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1969

SYMPOSIUM ON SMALLPOX

2 & 3 SEPTEMBER 1969

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INTRODUCTIOON
TO THE SYMPOSIUM
ON SMALLPOX

OPENING ADDRESS

F. Kogoj

Vice President, Yugoslav Academy of Sciences and Arts, Zagreb, Yugoslavia

Ladies and gentlemen,
Dear guests,
On behalf of the Yugoslav Academy of Sciences and Arts, the President of which is unfortunately absent, allow me to extend to you our heartfelt welcome. Our good wishes are not only an expression of formal courtesy but are sincere and cordial and I hope that you will feel this during your stay in Zagreb.

When after the Liberation our Academy again took up its regular activities in 1947, the natural sciences, among others, achieved the position that is theirs according to their importance for the well-being and progress of humanity. Thus in 1950 a special Department for Medical Sciences was formed. Since then the Academy has endeavoured to take a successful part in all up to date developments in the field of medicine. The Academy engages in studies which by their originality and concept can contribute towards our experiences in the so-called basic and clinical sciences – which I consider to be an inseparable whole. At the same time it does not neglect socio-medical aspects, and in its institutions deals with problems of industrial medicine and with questions concerning allergology and gerontology, studies the so-called national pathology and keeps close contact with institutions outside the Academy engaged in the scientific treatment of medical subjects. Therefore the Yugoslav Academy does not wish to play the role of a passive observer in your discussions on smallpox, but hopes to be an active participant at the Symposium. The international significance of this meeting at which along with Yugoslav scientists, there are guests from Austria, Australia, Denmark, France, Czechoslovakia, Hungary, the Federal Republic of Germany, the United States, the Soviet Union, Sweden, Switzerland,

Turkey and Great Britain, is not only a guarantee for a satisfactory solution of the problems this gathering will discuss, but follows precisely those trends which the Yugoslav Academy is emphasizing at all times and places: the close and equal cooperation of all peoples of the world in the field of culture and science.

Although for almost two hundered years (since 1798) it has been known how people can protect themselves from smallpox, which we still encounter as an endemic disease in some Asian countries (Burma, India, Indonesia, Pakistan) and in some other regions, nevertheless no remedy yet exists that would specifically cure smallpox at its manifest stage, which is the reason why lethality is outstandingly high and in some forms of variola reaches 80 and even more per cent. Therefore the practical implications of the subject you will be discussing here is beyond anv doubt. The Yugoslav Academy will have the duty and honour to make the results of your work and discussions available in book form to a wide circle of medical workers.

Wishing you all success in your work, I invite you, on behalf of the Academy, to visit its premises during your stay in Zagreb. We shall be very happy if you find the time to do so.

INTRODUCTORY REMARKS

I. ČUPAR

Secretary, Department of Medical Sciences, Yugoslav Academy of Sciences and Arts, Zagreb, Yugoslavia

It is my pleasant duty, on behalf of the Department of Medical Sciences of the Yugoslav Academy of Sciences and Arts, to address this scientific gathering in which, beside ours, eminent scholars from 13 European and other countries are taking part. It gives us great pleasure that a Symposium like this is taking place in our country.

The scientific problems on the agenda of this meeting are very topical. As far back as 1958 the 11th WH Assembly submitted the resolution for a global programme for smallpox eradication. In 1965 the 18th WH Assembly unanimously agreed that the eradication of smallpox throughout the world should be one of the main tasks of this organization. In 1967 the 20th WH Assembly drew up a resolution on a global programme of smallpox eradication, with the coordination of all international efforts and a more intensive development of research in this field.

In the past smallpox was a common world problem since it caused the death of millions of people every year. Nowadays the reservoir of smallpox is considerably reduced, but the disease has not been yet eradicated.

Smallpox is very suitable for eradication. The disease is spread directly from man to man, there are neither insects nor animals that could be the source of the disease. It appears rarely in sub-clinical form and can be easily and promptly recognized.

The efforts of experts to contribute – by solving the problems which mostly concern the field of epidemiology and of preparation of vaccine – to the success of the global programme of smallpox eradication make us hope that this goal will be achieved.

On the professional and scholarly aspects of the problems on the programme of this symposium you will hear from Prof. Ikić's report.

I wish you every success in you work.

Keynote Address

THE SIGNIFICANCE OF THE »SYMPOSIUM ON SMALLPOX« AND THE RESULTS EXPECTED BY SCIENCE

D. Ikić

*Corresponding Member of the Yugoslav Academy of Sciences and Arts,
Zagreb, Yugoslavia*

More than 170 years have passed since Jenner introduced artificial immunization in the control of disease (1798). When Jenner died (in 1823), his life work was only partly recognized. It was only in the second half of the 19th century that vaccination as method of controlling small-pox began to spread in Europe. At that time, institutes were established for large-scale production of smallpox vaccine in the scarified skin of calves. The laboratory which was built for this purpose in Zagreb 75 years ago (1894), and which is still working, marks the beginning of the development of our Institute of Immunology.

The generations that followed were for years vaccinated with the same type of vaccine. The problem of smallpox as a mass disease in developed countries was gradually disappearing and, with it, interest in this pro-blem. There was no significant progress in the preparation of the small-pox vaccine. Smallpox has remained a problem only in some parts of the world.

About ten years ago, the WHO started developing a programme for the eradication of smallpox. This was a historic decision. For the first time representatives of all countries, members of WHO decided, with their joint forces, to eradicate a mass disease from the earth.

Smallpox is a disease which is generally considered very suitable for eradication. The global eradication of smallpox is technically feasible and the unanimously accepted resolution of the 11th World Health As-sembly in 1958 was justified.

In the course of these ten years significant progress has been achieved. However, the problem of eradicating of smallpox must not be underesti-mated. Considerable efforts are still needed. The programme of eradica-

tion of smallpox has contributed towards the growing interest for a further development in the field of vaccine. The large-scale production of freeze-dried smallpox vaccine was started. This was the greatest achievement to date in the field of improving Jenner's vaccine.

The smallpox vaccine was the first vaccine to be developed. It is the oldest vaccine. It is, however, also the least developed field in active prophylaxis today.

A large number of important data on the preparation, testing and application of smallpox vaccine still need to be collected, together with information from the field of epidemiology and smallpox immunology.

The precise origin of the vaccinia strains used for the preparation of the vaccine in different institutes is unknown today. It is certain that each of the strains used in the different laboratories has been passed in different animals and tissue cultures. From available data on the severity of postvaccinal reactions, on the frequency of postvaccinal complications and on the laboratory properties of vaccinia virus, it is quite clear that individual strains differ in numerous characteristics. Additional laboratory and field studies are required in order to compare the relative efficacy and reactivity of individual strains and to relate these to their laboratory characteristics. Such studies would permit the choice of the most suitable strains to be used in the preparation of the vaccine.

While the main effort of the World Health Organization is directed towards the eradication of smallpox in endemic regions by using the freeze-dried vaccine, countries which for years have not had smallpox must increasingly question the need and the use of applying traditional and in such circumstances needlessly virulent virus strains for routine primary vaccination. It is indispensable to maintain at present a certain level of immunity of the population even in such epidemiological conditions, but there must be also an attempt to bring local and general reactions and complications nearer to the level of other vaccines of today.

The preparation of smallpox vaccine in animals is not a very good solution. At a time when tissue culture technique – and also virological technique – is so well developed it is natural that a new well defined substrate for the preparation of vaccine should be sought.

The challenge of skin resistance after vaccination is an important but at the same time unreliable and rough method for testing the acquired immunity and value of the vaccine. We do not, at present, have a laboratory method by means of which we could measure the strength of collective immunity or the prevaccinal and postvaccinal immunity status of the population. We do not have reasonably based requirements with regard to the protective qualities of the vaccine either. These are obstacles to quick progress in this field which are not easily removed.

This Symposium is being held at a time when the action of the World Health Organization on the eradication of smallpox throughout the world is in full swing, and this is one of the main tasks of the WHO.

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We hope that this Symposium will contribute to a more intense development of studies in this field, which will at the same time be the contribution of this Symposium to the WHO programme.

The programme of global smallpox eradication which the WHO is materializing must energetically be continued and all forces be employed for the creation of a focal immunity so that the smallpox virus should cease to circulate in the endemic parts of the world and thereby the final goal of global eradication of smallpox be achieved.

I am happy to be able to say that Yugoslavia has also joined the World Health Organization's programme to eradicate smallpox by putting at its disposal in 1967 1 million doses of smallpox vaccine.

GLOBAL EPIDEMIOLOGY AND PROBLEMS
OF SMALLPOX ERADICATION

THE STATUS OF THE GLOBAL SMALLPOX ERADICATION PROGRAMME IN SEPTEMBER 1969

A. HENDERSON

Smallpox Eradication Unit, World Health Organization, Geneva, Switzerland

Although presently confined to the developing countries of Africa, Asia and South America, smallpox continues to represent by far the most universal and serious threat of any infectious disease to countries throughout the world. Once introduced, smallpox can be readily transmitted in any country, in any climate – irrespective of the degree of economic development. Europe is quite as much at risk as Central America or Oceania. Case-fatality ratios among those infected with variola major are normally 35 to 40⁰/₀. No specific treatment is available.

Our only weapon for attack and virtually our only defence against smallpox is vaccination. As with no other disease, vaccination against smallpox is practiced today in every country throughout the world. As with no other disease, certificates of vaccination are universally required for international travel. Of all the immunizing agents available, far more smallpox vaccinations are performed annually than the total of all other immunizations combined.

Despite this extensive use of smallpox vaccine, despite the known frequency of complications which occur following its use, the vaccine itself and the techniques for producing it have really not changed substantially in a century or more. This symposium is, therefore, certainly most timely.

In dealing with smallpox, the principal emphasis of all countries until 1967 was based on a defensive posture – of keeping smallpox out of one's own country and, if introduced, of containing it quickly. The key principles were vaccination of one's own population, quarantine and surveillance. The simple axiom, »the best defense is a good offense«, was largely ignored.

In 1966, an intensive global programme of smallpox eradication was proposed and unanimously adopted by the World Health Assembly. Supported by a modest budget from the Organization and pledges of additional support from a number of countries, particularly the Soviet Union and the United States of America, the programme was initiated in January 1967. It is now in its third year. I should like to review with you the progress made to date and the present status of the programme for, in all respects, it is a unique venture in preventive medicine and could represent a milestone in man's efforts towards international co-operation.

During the first year of the eradication programme, reported cases of smallpox actually increased from 89000 cases to over 129000 cases (fig. 1). In part, this may be attributed to better reporting but, in part, longer term cyclical trends may have been responsible. During 1968, the incidence decreased by 40% to 79000 cases and, in 1969, a further decline of almost 40% has been observed to date. In 1967, 14 countries recorded 5000 more cases per 100000 population (fig. 2). Based on present trends, it is likely that only two countries, Indonesia and the Democratic Republic of the Congo, will record rates of 5.0 per 100000 or greater during 1969 (fig. 3). The decreasing incidence is also reflected in the number of countries which have been afflicted with the disease. At the beginning of the programme 42 countries recorded cases; last year, the number fell to 39; this year, only 30 countries have experienced smallpox. The benefits of the programme to Europe can be summarized succinctly by noting that it is now one year and two days since the last case was introduced.

Although programmes are now in effect in 26 of the 27 countries considered to be endemic at the beginning of this year, progress, not surprisingly has been greater in some areas than in others.

In the Americas in 1969, (fig. 4) cases have been recorded only in Brazil, the sole endemic country in this Region. The eradication programme has been intensified in Brazil during the past year. The number vaccinated in the systematic vaccination campaign is between 1.5 and 2 million persons per month; over 35 million have been vaccinated since the programme began. Smallpox incidence began declining approximately a year ago and, to date in 1969, 1803 cases have been recorded, 10% fewer cases than were recorded last year at this time.

The modest decline does not accurately reflect the situation, however. More than half of all cases recorded this year have been discovered by special surveillance teams which have been formed recently. In Brazil, as in many other countries now, such teams have been created specifically to investigate all suspect cases, to search for additional cases and to take appropriate containment measures. Through this approach, we

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feel that smallpox transmission may be interrupted much more quickly and the duration of the eradication effort shortened. Although reported incidence increases initially when surveillance teams commence their work, the incidence later falls at an accelerated rate. In the meantime in South America, those countries bordering Brazil have intensified their vaccination programmes and strengthened surveillance activities to prevent reintroduction of the disease.

By far the most dramatic progress to date has been in western and central Africa (fig. 5). Programmes in 20 countries embracing a population of 120 million persons were begun in January 1967, with bilateral assistance from the United States of America and additional help from the World Health Organization. By the end of this year, 100 million vaccinations will have been administered. Smallpox incidence has declined steadily. Except for a recent small outbreak of 31 cases in Dahomey, no cases have been detected in this extensive area since 25 June. Intensive efforts to detect cases continue, however, and will continue but we feel reasonably confident that this area will be smallpox-free by the end of this year. This is particularly remarkable when it is realized that five of the 10 countries which recorded the highest rates of smallpox in the world during 1968 were located in western and central Africa.

During 1969, recorded cases of smallpox in eastern and southern Africa (fig. 6) have declined more than 50% from the number reported in 1968. Smallpox incidence is presently at a record low level. No cases have been reported to date in Swaziland or Zambia and only four countries, the Democratic Republic of the Congo, Ethiopia, the Union of South Africa and Sudan, have reported more than 100 cases this year.

In the Democratic Republic of the Congo, 1050 cases have been recorded in 1969 compared with 2527 cases at this time last year. In this country of 17 million persons, vaccination activities have been sharply increased during the past two years as indicated below:

	No. of Vaccinations			
	Eradication Programme		Other health services	Total
1967		302 000		302 000
1968	2 275 000		574 000	2 849 000
1969 (7 months only)	2 914 000		2 187 000	4 101 000

Since the beginning of 1969, special efforts have been made to improve the completeness of routine case notification and plans are being developed to undertake intensified investigation and containment activities later in the year. A successful programme in the Congo is particularly important as this country occupies a strategic position in Africa, having common borders with nine other countries.

The two other countries of major concern, Ethiopia and the Sudan have this year recorded a total of 283 cases, an increase of 20% over the number of cases recorded at this time last year. In the Sudan, smallpox outbreaks commenced in mid-December in the southern part of the country and continued into May. A total of 125 cases were detected in 34 towns and 4 provinces. The outbreaks coincided with a very large seasonal migration of agricultural workers into east central Sudan from the southern part of the country and from Ethiopia. While an eradication programme has begun in the Sudan, little information is available regarding smallpox activities in Ethiopia. No formal programme of control or eradication is planned. Reporting is recognized to be very incomplete and it is reasonable to assume that the actual incidence of disease is many times that which is presently recorded. Ethiopia, at present, represents the most serious threat to the eventual success of the eradication effort.

Smallpox incidence in Asia (fig. 7) declined by 40% in 1968 and appears to be declining at a comparable rate in 1969. However, from one country to the next, progress in the smallpox programmes differs widely as do the trends in incidence and factors influencing these trends.

The most active programme in Asia is in Indonesia. The programme commenced in July 1968 and has progressively been extended throughout the country. Paradoxically, in 1969, the reported incidence of smallpox is little different from that in 1968. Reporting, however, has been greatly intensified and containment teams, initiated in January of this year, have steadily broadened their extent of activity. The impact of the vaccination programme in reducing reported incidence has thus been nullified by the improvement in notification. Comparatively few foci of smallpox are present outside of the island of Java, on which resides 65% of the population and on Sumatra. East Java and Bali with a population of 30 million are now smallpox-free. Intensive containment operations in parallel with the systematic vaccination programme have sharply curtailed smallpox in Central Java.

Increased notifications were received during 1968 from both Afghanistan and Nepal, and a further increase in 1969 from Nepal. In both countries, eradication programmes are steadily being intensified and more complete reporting is apparent.

A marked decline in smallpox occurred this year in East Pakistan which, in 1968, recorded its highest incidence in a decade. However, the fall in incidence must be attributed to expected cyclical variation as little progress has yet been made in the eradication programme. The opposite pattern has occurred in West Pakistan which is one of the few reporting areas which has recorded an increase in smallpox in 1969. An eradication programme in West Pakistan is just beginning; surveillance activities have not yet been organized.

In India, an increased emphasis has been placed on vaccination of those never previously vaccinated, particularly pre-school children; the use of liquid vaccine has been totally abolished; vaccine storage has been improved; and the bifurcated needle is being substituted for the rotary lancet in the vaccination programme. Although reporting is still very incomplete and surveillance activities are still very limited, there appears to be a continuing decline in incidence from 1968 and 1967.

General Programme Activities

In the development of the eradication programme, initial efforts were directed toward the development of the technical and operational strategy. These were fully discussed by a Scientific Group on Smallpox Eradication which met in October 1967 and presented in a report *(Technical Report Series* No. 393). A »Handbook for Smallpox Eradication« was also written, which is being revised this year to take into account the experience of the past two years. Additionally, a special manual which discusses the theory and practice of surveillance-containment operations has been prepared.

Special seminars dealing with programme execution have been conducted in 1967 for countries in Asia and in 1968 and 1969 for countries in Africa.

Because of the critical need for adequate supplies of freeze-dried vaccine which meet standards established by WHO, major efforts have been devoted to this problem. Assistance in the form of consultation, vaccine testing, equipment (in conjunction with UNICEF) and antigens for testing have been provided to laboratories throughout the world. To date, WHO consultants have visited 24 production laboratories; equipment, special reagents and testing materials have been provided to 30 laboratories. All countries have been urged to submit vaccine regularly for testing purposes. These are tested at one of two WHO Reference Centres, at the Rijks Institute, Netherlands, or the Connaught Medical Research Laboratory, University of Toronto, Canada. This service has been increasingly used as shown in the table below:

	1965	1966	1967	1968
No of samples tested	12	43	83	167

It is satisfying to note that almost all vaccinations now performed in endemic countries are performed with freeze-dried vaccine which conforms to the potency standards recommended by WHO. At the inception of the programme two years ago, it is probable that not more than 10 to 20% of vaccinations in endemic countries were performed with satisfactory vaccine.

Recommended vaccination techniques have been altered substantially to provide simpler methods which assure higher take rates and use smaller quantities of vaccine. In 1967, after several years of testing and evaluation, the foot-operated jet injector was first employed for routine field operations. It is now in widespread use in Brazil, in the Democratic Republic of the Congo, as well as in the countries of western and central Africa. It is being employed also in several other countries for special programmes of epidemic containment and for vaccination of large groups. Early in 1968, after a number of special field studies, the bifurcated needle, developed by Wyeth Laboratories, USA, was introduced for field use and has now been adopted in essentially all programmes. Employing bifurcated needles, vaccinators in western Africa have been able to vaccinate 400 to 700 persons daily, while realizing savings of several fold in vaccine. By the end of 1969, virtually all vaccinations in endemic countries will be performed either with the jet injector or the bifurcated needle, techniques unknown to routine vaccination programmes prior to the beginning of the global eradication effort.

Since the inception of the global programme, the importance of more complete reporting of cases of smallpox has been stressed and the majority of countries have made special efforts to strengthen their reporting and surveillance activities. In addition to various administrative measures to assure the regular notification of cases from health facilities throughout their countries, several have initiated the telegraphic reporting of cases; special case investigation teams have been established in many areas; and smallpox surveillance reports are now published regularly by seven of the endemic countries.

To facilitate the more rapid exchange of current information regarding the global status of smallpox and eradication activities throughout the world, WHO has, since June 1968, prepared a special surveillance report on smallpox which is published every two to three weeks in the Weekly Epidemiological Record. To permit more rapid and detailed analysis of disease trends, smallpox morbidity data is now being recorded and tabulated by computer.

Reliable reporting rests in large measure upon the accurate clinical diagnosis of the disease. To assist health personnel and others responsible for the reporting of smallpox, WHO has produced for the African countries a series of teaching aids, including brochures, posters and slides, which show cases of smallpox and varicella at different stages of the evolution of the rash. It is anticipated that a similar series of teaching aids which show smallpox in Asian patients will be prepared next year

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A network of diagnostic laboratories to provide geographically convenient diagnostic services to every country is also being developed by WHO. It is planned for each participating laboratory to be able to conduct at least three basic examinations for the identification of variola virus – a microscopic smear examination, a precipitation-in-gel test and virus isolation on the choriollantoic membrane of chick embryos. A 48-page manual has been prepared entitled »Guide to the Laboratory Diagnosis of Smallpox« which describes in detail and pictorially each of the tests noted. Arrangements have been made with collaborating laboratories to produce requisite antisera and antigens and additional materials for each of the tests have been procured.

Training courses have already been conducted in the Americas and a network of 12 diagnostic centres enstablished. During 1969 and 1970. it is planned for additional courses to be conducted in other regions. Following the training course and the designation of laboratories as diagnostic centres, arrangements are being made to distribute twice each year to each of the laboratories specimens as »unknown« to ensure that each of the laboratories has retained its competence or, if not, to assist in retraining the technicians concerned.

Fig. 1

Reported Cases of Smallpox in the World – 1955 1969
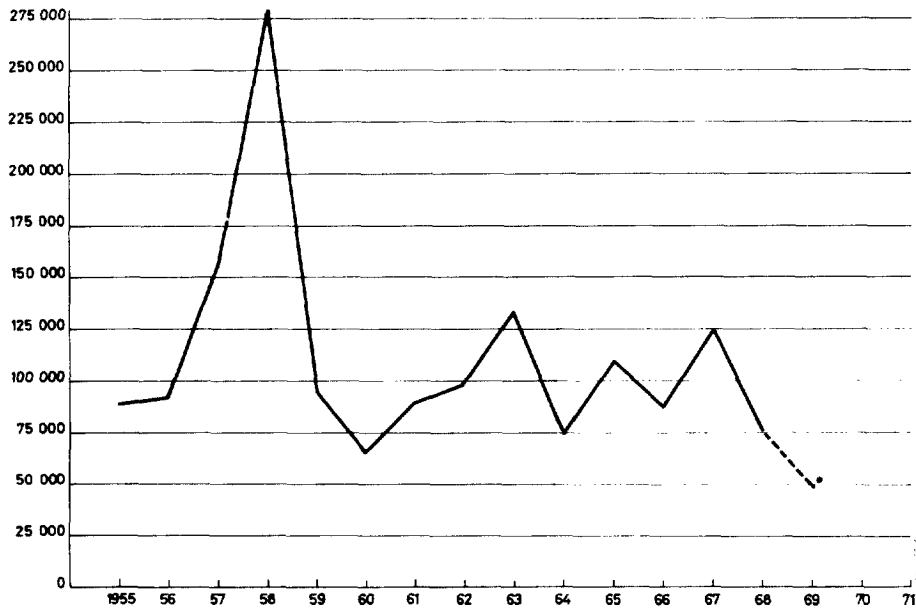
* Estimated en present trends

Fig. 2

1968 – Smallpox Cases per 100 000 Population
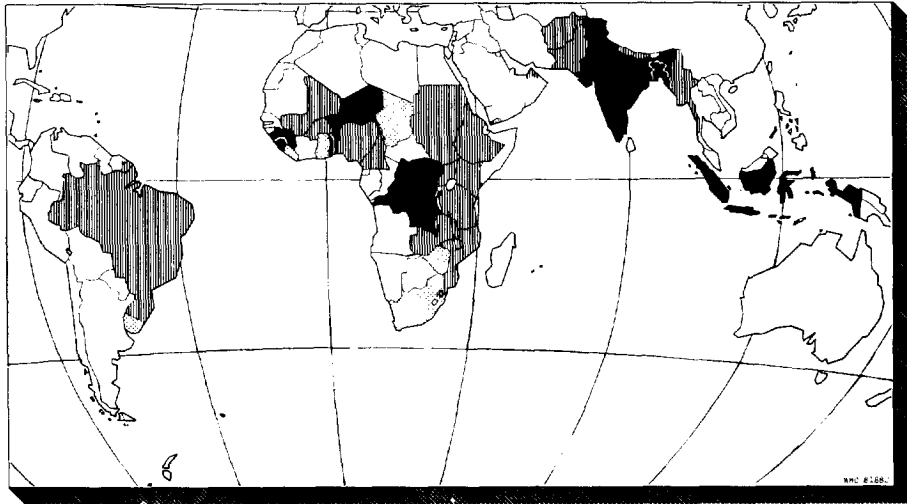
> 5.0 ■   0.5–5.0 ▥   > 0.5 ▢

Fig. 3

1969 – Estimated Smallpox Cases per 100 000 Population Based on Present Trends
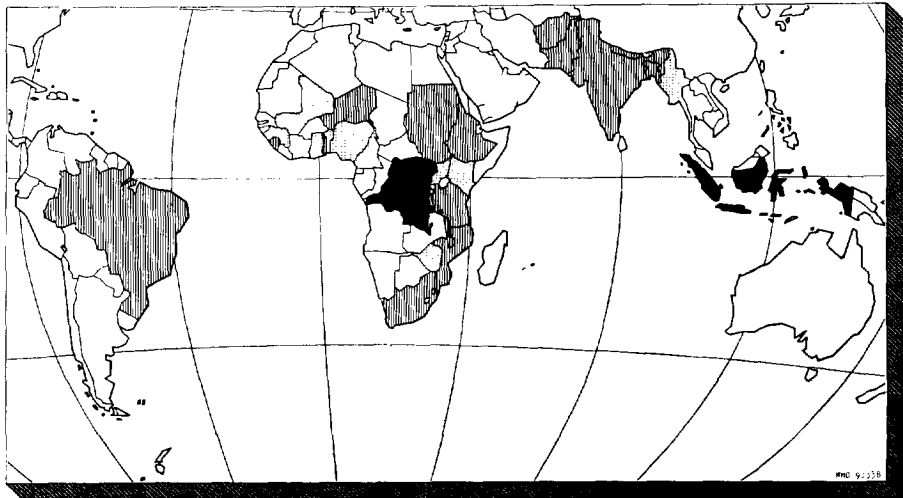
> 5.0 ■   0.5–5.0 ▥   < 0.5 ▢

Fig. 4

Smallpox Incidence: South America, 1967–1969

Cases
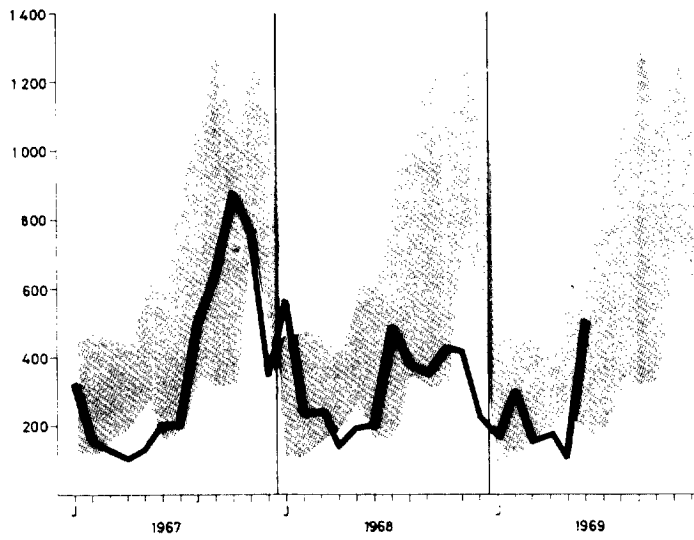
Note – The grey area represents the range between the highest and
lowest incidence reported during the 5-year period 1962–1966

Fig. 5

Smallpox Incidence: Africa, West and Central, 1967–1969

Cases

Note – The grey area represents the range between the highest and lowest incidence reported during the 5-year period 1962–1966

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Fig. 6

Smallpox Incidence: Africa, East and South, 1967–1969

Cases

Note – The grey area represents the range between the highest and lowest incidence reported during the 5-year period 1962–1966
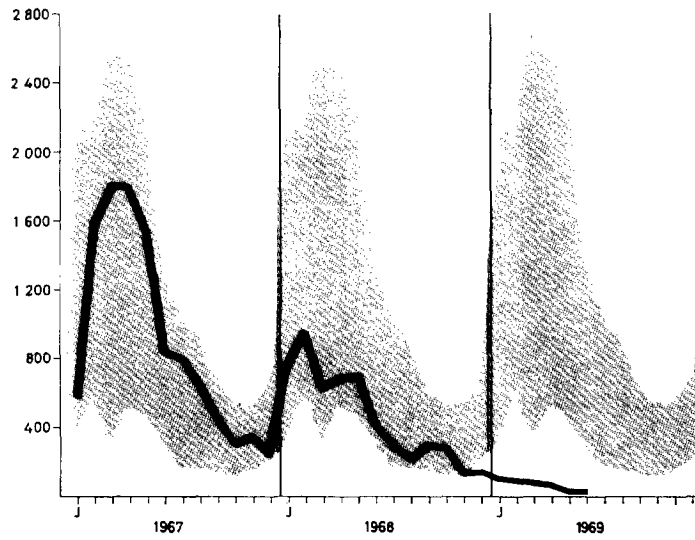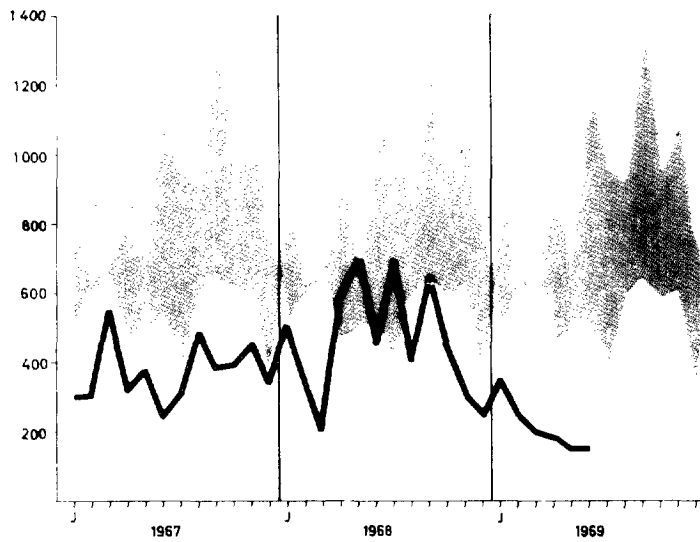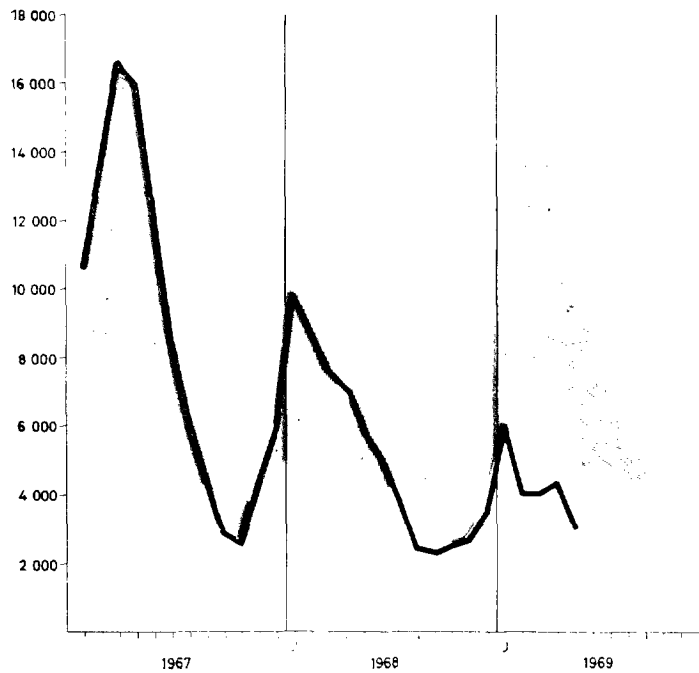
Fig. 7

Smallpox Incidence: Asia. 1967–1969

Cases

Note – The grey area represents the range between the highest and lowest incidence reported during the 5-year period 1962–1966

SUMMARY

Since the inception of the global programme of smallpox eradication
in 1967, recorded cases have fallen at a rate of 40% per year, despite
steadily improved reporting. While 10 countries recorded rates of more
than 5.0 per 100,000 in 1968, present trends indicate that only two will
exceed this rate in 1969.

Eradication programmes are now in progress in all but one of the
countries originally considered to have endemic disease. Most program-
mes are making excellent progress. The most dramatic progress to date
has been in Western and Central Africa where almost 100 of the 120
million inhabitants have been vaccinated and smallpox has now decre-
ased to the point that this region could become smallpox-free by the end
of the year.

As a result of substantial assistance provided by WHO to vaccine pro-
duction laboratories and because of contributions of vaccine by many
countries, particularly the Soviet Union and the USA, virtually all vac-
cine now in use in endemic regions is freeze-dried and fully meets WHO
standards. The jet injector and the bifurcated needle have replaced
older techniques of vaccination. With these instruments higher take ra-
tes are consistently observed while smaller quantities of vaccine are
employed.

The principal focus of the eradication programme is now on improved
reporting, investigation and containment of cases and outbreaks. These
activities, termed »surveillance« have been increasingly widely used and
are serving to accelerate significantly the pace of the programme.

Although the disease trends virtually everywhere are highly encourag-
ing, no major epidemiological region has yet become smallpox-free. This
is the important next objective.

FREEZE – DRIED
SMALLPOX VACCINE

FREEZE-DRIED VACCINE FOR THE SMALLPOX ERADICATION PROGRAMME

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The use of freeze-dried smallpox vaccine of suitable quality is pre-requisite to the success of the smallpox eradication programme. There-fore, at the beginning of the eradication programme in 1967, particular efforts were made to improve the quality and to increase the quantity of vaccine avaliable. This paper describes WHO activities dealing with vaccine quality and summarizes certain aspects of the present status of vaccine production.

WHO Survey on Vaccine Production Status

In 1967, when WHO, in cooperation with its member countries, start-ed the eradication programme on a world-wide basis, our first concern was to ensure an adequate supply of potent, heat stable vaccine to the programme. At that time, only limited information on the status of vaccine production in individual laboratories was available. Accordingly, early in 1967, questionnaires were sent to all member states where the production of freeze-dried vaccine was believed to be under way. The information provided by the laboratories was summarized early in 1968 and since then the data have been revised whenever additional informa-tion has been received. The data presented in Tables 1, 2 and 3 are based on this information and are believed to reflect fairly well the present status of vaccine production.

At present, 58 countries are participating in the production of freeze-dried vaccine (Table 1). In these countries, 81 laboratories are either producing or preparing to produce vaccine. Sixty-four laboratories are now in routine production.

Table 2 summarizes the types of strains being used in the 64 laboratories currently in routine production. The designation of the strains is provisional and represents simply the names of strains as reported to us. The Lister strain is most frequently used. Of 64 laboratories, 20 use the Lister strain the New York Board of Health strain is used in 5 laboratories in the Americas. In Europe, more than 10 types of strains are used in various laboratories, reflecting the very long history of vaccine development in this region. The Patwadangar strain is used in three laboratories in India; the EM63 strain, derived from the Ecuador strain, was selected for use in the Soviet Union after careful comparative studies of various vaccinia strains. It should be noted that there are several laboratories where the origin of the strain or the type of strain is unknown.

Among the animals and media available for production of vaccine, calves are most frequently employed (Table 3). Only three laboratories (one in the Americas and two in Europe) are producing vaccine with eggs or tissue cultures – other than animal skin.

The WHO questionnaires in 1967 requested information regarding potency and stability of vaccine as tested in the individual laboratories. Only 16 of 45 laboratories recorded satisfactory results for both the potency and heat stability of their vaccines. During 1967, 16 laboratories in 16 countries submitted samples of vaccine to WHO for independent testing. Of these, vaccina from only 7 laboratories (43%) consistently met WHO requirements. Thus, the quality of the vaccine was generally unsatisfactory at that time and it was felt that if the eradication programme were to be successful, urgent measures were required to improve vaccine quality. Two principal approaches were taken. First, a Seminar on Vaccine Production was convened in March 1968 and, second, steps were taken to provide consultation, fellowship training, and independent testing of batches of vaccine.

Seminar on Vaccine Production

The purpose of this Seminar is best expressed in the introductory paragraph of its final report,[1] as quoted below:

»The various vaccine laboratories employ a wide variety of production methods evolved over many years through trial and error, experimentation, adaptation and arbitrary decision. Although the basic principles of smallpox vaccine production and testing have been elaborated, a detailed methodology has never been published. Accordingly, WHO convened a working group, composed of those with expertise in vaccine production and a broad knowledge of production problems in the developing countries, to consider alternative methods for the production and testing of freeze-dried smallpox vaccine and to recommend the simplest, most practicable methods«.

Staff from five laboratories participated in the Seminar: Rijks Institute voor de Volksgezondheid, Netherlands; Wyeth Laboratories, Inc.,

USA; Research Institute of Virus Preparations, Moscow, USSR; Connaught Medical Research Laboratories, Canada; and Research Institute of Immunology, Prague, Czechoslovakia. The group, after discussions in Geneva, visited two laboratories, the Research Institute of Virus Preparations in Moscow and the Wyeth Laboratories, USA, to observe actual production processes and to discuss further the proposed methods. A manual termed »Methodology of Freeze-dried Smallpox Vaccine Production« was prepared during the Seminar and this has been made available to producers on request. It might be useful to mention briefly several important points noted in this manual. First, the group recommended the establishment of a seed virus system with a high potency seed lot of more than $10^{8.7}$ p.f.u./ml. to ensure consistency in the vaccine strain and a high concentration of virus in the pulp. It was noted that with good technique, the pulp should contain 10^{10} p.f.u./ml. of virus. Copious cleansing of the animals and subsequent appropriate use of phenol, in addition to the usual precautions to avoid contamination, should result in a very low or nil bacterial count in the final bulk material. The choice of freeze-driers, problems of sealing, types of final containers such as ampoules, vials, and vampoules are discussed and illustrated in the manual. A vaccine fill of 0.25 ml. was recommended in view of the universal use of the bifurcated needle in the eradication programme. For diluent, 25% glycerol was suggested since it is less virocidal than the customarily used higher concentrations. For egg testing, the use of a 0.1 ml. inoculum on CAM was recommended as being more sensitive than the use of 0.2 ml. It was noted that some producers are not certain about the history of their seed virus and some normally observe different morphological types of pocks from seed virus inoculated on CAM, indicating that their seed virus may not be homogenous. While it is difficult to translate the significance of such observations into practical terms of reactogenicity and immunogenicity, the group was of the opinion that it would be more practical and realistic to replace questionable strains with strains such as the Lister or EM63, which have proved to be satisfactory both with respect to immunogenicity and reactogenicity.

Provision of Consultant Services, Fellowship Training and Vaccine Testing by WHO

It was felt most desirable to establish the closest possible contact between producers and consultant laboratories. In the Americas, since 1967, the Connaught Medical Research Laboratories, Toronto, Canada, have assumed responsibility, under contract with WHO, for the provision of consultation, fellowship training and vaccine testing to vaccine producers in South and Central America. Thirteen South and Central American laboratories have now established close communication with the Connaught laboratories. For laboratories in other parts of the world,

the National Institute of Public Health, Utrecht, Netherlands, has undertaken to provide a vaccine testing service as well as consultative assistance; additional help is provided also by special consultants from the United Kingdom, Czechoslovakia, the Soviet Union, Austria, France and Sweden. Vaccine testing includes determination of initial potency, heat stability (both at 100°C for 1 hour and at 37°C for 4 weeks), bacteriological testing, phenol content, moisture content and degree of vacuum in the final containers.

These laboratories are also prepared to provide seed lot virus, ready for inoculation, to producers who wish to replace their current strain. During the Seminar on Vaccine Production, the availability of reference vaccine was of concern since some laboratories in developing countries are not able initially to produce a national reference vaccine for purposes of routine vaccine testing. For such laboratories, the two testing laboratories agreed to provide their own national reference preparation until producers could develop their own reference standard. Developmental studies, related to vaccine production problems have also been carried out in these two laboratories. In the Utrecht laboratory, a bank of vaccinia strains has also been established. In 1969, these two laboratories were officially designated as the WHO Regional Reference Centre for Smallpox Vaccine (Connaught Medical Research Laboratories) and the WHO International Reference Centre for Smallpox Vaccine (Rijks Institute).

Potency and Heat Stability of Vaccines Presently in Use

With the assistance particularly of the two WHO Reference Laboratories, closer contact with producers throughout the world has been established. Recent results of vaccine testing, as performed by these Reference Laboratories suggest that substantial improvements in vaccine quality have been made although further improvements would be desirable.

Table 4 presents the results of 201 lots tested during 1968 and 1969 to date. Twenty-two producers submitted samples. Of the 201 lots tested, 150 lots met WHO recommended standards for potency, heat stability and purity. Of 51 lots which showed unsatisfactory results, 45 lots were unsatisfactory with respect to heat stability. Only four lots revealed unsatisfactory bacterial counts. Of the lots with satisfactory results, about 40% had nil bacteria by plate count assay.

Since the heat stability of vaccine remains the most significant problem, a further analysis was made of the results of heat stability testing obtained in tests of up to five successive lots from 20 different producers (Table 5). With the exception of two producers (one in Africa and one in Australasia), the average loss of titre after incubation of the vaccine for 4 weeks at 37°C was less than 0.67 log. In 10 laboratories the average loss of titre was less than 0.3 log.

In some endemic countries, the pressing demands for vaccine supply and the lack of storage space recommended the evaluation of a test for vaccine stability which would take less time than the conventional 4 week stability test. During the Seminar on Vaccine Production, further evaluation of the test requiring incubation at 100°C for 1 hour was proposed. Since 1968, the WHO Vaccine Reference Laboratory in Utrecht, has conducted in parallel this rapid heat stability test and the conventional heat stability test.

Table 6 shows a comparison of the average loss of titre between the conventional and rapid heat stability tests for several successive sample lots produced in 12 laboratories. With the rapid heat stability test, the loss in titre is greater; vaccine produced by 6 of 12 producers showed a titre reduction of more than 1.0 log after incubation. The correlation in results obtained with these two tests is at best approximate; results obtained with vaccine provided by producer 5 are particularly at variance with the others.

A further examination of the data was made to determine the possible application of the rapid heat stability test as a screening test for lots of vaccine – in other words, to ascertain if vaccine which contained a defined minimum titre of virus and lost nor more than a defined maximum amount of virus after incubation at 100°C for 1 hour, could be assured of passing the conventional stability test involving incubation for 4 weeks at 37°C. A comparison of results obtained for 139 lots from 24 producers tested during 1968 and 1969 is shown in Table 7.[2] Of the 139 lots examined, 106 passed the usual heat stability test. As can be seen in the figure, all lots except one, which passed the conventional heat stability test had an initial titre of $10^{8.5}$ p.f.u./ml. or more and retained a titre of $10^{7.5}$ or more after incubation at 100°C for one hour.

With regard to the exceptional lot, it should be noted that there was a substantial difference between titres obtained before incubation when this lot was tested on two occasions, suggesting that the lot of vaccine itself may not have been homogenous.

On the basis of these observations, it is proposed to accept as satisfactorily stable, without further testing, lots of vaccine which have an initial titre of $10^{8.5}$ p.f.u./ml. or more and which retain a potency of $10^{7.5}$ p.f.u./ml. or more after incubation for 1 hour at 100°C. Of the 106 lots which were satisfactorily stable by the conventional stability test, 68 (64%) conformed with these criteria, and could be immediately released for use without further stability testing.

Table 1

Geographical Distribution of Producers of Freeze-dried Smallpox Vaccine

Africa	Routine Production	Developing	Americas	Routine Production	Developing
Angola	1	—	Argentina	1	—
Algeria	1	—	Bolivia	—	1
Congo (D. R.)	—	1	Brazil	2	2
Ethiopia	1	—	Canada	1	1
Guinea	—	1	Chile	1	—
Kenya	1	—	Colombia	1	—
Mozambique	1	—	Cuba	—	1
Nigeria	1	—	Ecuador	—	1
Rwanda	—	1	Peru	1	—
Senegal	—	1	USA	2	—
Tunisia	1	—	Venezuela	—	1
Union of South Africa	1	—			
United Arab Republic	1	—			
Total (13)	9	4	Total: (11)	9	7

Australiasia	Routine Production	Developing	Europe	Routine Production	Developing
Australia	—	1	Austria	1	—
Burma	1	—	Belgium	1	—
Cambodia	1	—	Bulgaria	1	—
Ceylon	—	1	Czechoslovakia	1	—
India	4	—	France	3	—
Indonesia	1	—	Germany	3	—
Iran	1	—	Hungary	1	—
Iraq	—	—	Italy	3	—
Japan	6	—	Netherlands	1	—
Jordan	—	1	Portugal	1	—
New Zealand	1	—	Spain	1	—
Pakistan	1	1	Sweden	—	1
Philippines	1	—	Switzerland	1	—
Taiwan	1	—	Turkey	1	—
Syria	—	—	United Kingdom	1	—
Thailand	1	—	USSR	6	—
Vietnam	—	1	Yugoslavia	1	—
Total: (17)	19	5	Total: (27)	27	1
			Grand total: (58)	64	17

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Table 2

Vaccinia Strains Used in Laboratories Presently in Routine Production

	No. of Laboratories	Lister	New York Board of Health	Institut Pasteur	Berne	Patwadangar	EM 63	Other*	Unknown
Africa	9	3	0	1	0	0	0	2	3
Americas	9	2	5	0	0	0	0	1	1
Australasia	19	7	0	1	0	3	0	7	1
Europe	27	8	0	1	2	0	4	10	2
Total	64	20	5	3	2	3	4	20	7

* Includes Institut Chambon (Paris), Ikeda, Budapest, Bohemia, Hamburg, Bordeaux, Aosta, Minsk.

Table 3

Medium Used for Production of Vaccinia Virus in Laboratories Presently in Routine Production of Freeze-Dried Vaccine

	No. of Laboratories	Calves	Sheep	Eggs	Tissue Culture	Unknown
Africa	9	4	5	0	0	0
Americas	9	8	0	1	0	1
Australasia	19	13	5	0	0	1
Europe	27	21	6*	0	2**	1
Total	64	46	16	1	2	3

* One laboratory uses both calves and sheep
** Two laboratories use bovine embryo muscle tissue cell cultures, in addition to animals

Table 4

*Testing Results of Vaccines from 22 Producers by WHO Reference Laboratories –
1968 and 1969 (to June)*

		No. of Lots Tested	No. of Lots Satisfactory*	No. of Lots Unsatisfactory	No. of Lots Unsatisfactory when Tested		
					Initial Potency	Heat Stability	Bacterial Count
Africa Producer	No. 1	23	23	—	—	—	—
	2	5	—	5	5	5	—
	3	2	2	—	—	—	—
	4	4	—	4	4	4	—
	5	3	3	—	—	—	—
America Producer	No. 1	2	2	—	—	—	—
	2	5	5	—	—	—	—
	3	3	—	3	1	3	—
	4	10	—	10	—	10	—
	5	2	—	2	2	2	—
	6	1	1	—	—	—	—
Australasia Producer	No. 1	12	3	9	—	9	—
	2	3	3	—	—	—	—
	3	60	54	6	2	2	3
	4	5	5	—	—	—	—
	5	6	3	3	—	3	—
	6	11	10	1	—	—	1
	7	2	2	—	—	—	—
	8	16	16	—	—	—	—
Europe Producer	No. 1	2	2	—	—	—	—
	2	2	2	—	—	—	—
	3	22	14	8	1	7	—
Total		201	150	51	15	45	4

* Vaccine with potency of over 1×10^8 p. f. u./ml., with heat stability maintaining over 1×10^8 p. f. u./ml after 4 weeks at 37° C and which bacterial count less than 500/ml.

Table 5

Heat stability after 4 weeks at 37° C of most recent successive five lots produced by 20 laboratories and tested by WHO reference laboratories
Titre of p. f. u./ml. expressed as Log.

	Producers	Initial Titre		After 4 weeks at 37° C		Average Loss of Titres (A)–(B)
		(A) Average	(Range)	(B) Average	(Range)	
Africa	A	8.78	(9.04–8.57)	8.57	(8.84–8.25)	0.21
	B	8.26	(8.55–8.04)	7.73	8.38–7.36)	0.53
	C****	7.83	(8.51–7.17)	6.80	(7.47–⟨6.04)	⟩1.03
Americas	A	8.40	(8.63–8.25)	8.19	(8.30–8.08)	0.21
	B	8.05	(8.17–7.90)	7.45	(7.60–7.30)	0.60
Australasia	A	9.43	9.51–9.34)	9.28	(9.39–9.20)	0.15
	B	8.63	(8.75–8.50)	8.42	(8.61–8.27)	0.21
	C	7.97	(8.20–7.74)	7.72	(7.91–7.56)	0.25
	D	8.89	(9.11–8.80)	8.63	(8.95–8.27)	0.26
	E	8.59	(8.76–8.46)	8.33	(8.41–8.17)	0.26
	F**	8.82	(8.88–8.77)	8.48	(8.53–8.43)	0.34
	G***	8.78	(8.95–8.51)	8.39	(8.60–8.00)	0.39
	H	9.00	(9.60–8.51)	7.79	(8.75–6.98)	1.21
Europe	A	9.02	(9.11–8.82)	8.81	(9.14–8.55)	0.21
	B	8.43	(8.63–8.25)	8.21	(8.36–8.14)	0.22
	C*	8.07	8.07	7.80	7.80	0.27
	D	8.92	(9.11–8.65)	8.56	(8.81–8.23)	0.36
	E**	8.60	(8.75–8.46)	8.22	(8.30–8.14)	0.38
	F	8.95	(9.39–8.79)	8.56	(8.79–8.32)	0.39
	G*	8.74	8.74	8.07	8.07	0.67

* Only one test lot
** Two test lots
*** Three test lots
**** Four test lots

Table 6

Comparison of Loss of Titres between Heat Stability Tests after 1 hour at 100° and after 4 weeks at 37° C

Serial No. of Producers	No. of Test Lots	Average loss of titre after 4 weeks at 37°C	Average loss of titre after 1 hour at 100°C
1	5	0.15	0.97
2	5	0.21	0.61
3	5	0.21	0.46
4	—	0.21	0.41
5	5	0.25	⟩3.56
6	5	0.26	0.68
7	5	0.26	1.39
8	1	0.27	1.04
9	2	0.34	1.63
10	5	0.39	0.89
11	3	0.39	1.19
12	5	1.21	⟩4.36

Table 7

Results of Testing of 189 lots of Vaccine for Potency and Stability by Conventional and Rapid Heat Stability Tests
(Titre of p. f. u./ml. expressed as Log)

o: Lot passed conventional test (4 weeks at 37º C).
x: Lot failed conventional test (4 weeks at 37º C).

Titre after 1 hour at 100° C

Initial Titre	9.00 or greater	8.50 – 8.99	8.00 – 8.49	7.50 – 7.99	7.00 – 7.49	6.50 – 6.99	6.49 or less
9.00 or greater		ox¹0000 00	0000000 0000000	00000	0		ooxo
8.90 – 8.99		0	0000000	0			
8.80 – 8.89			0000000 000	0	0	00	0
8.70 – 8.79			000000	0	0	0	000
8.60 – 8.69			000000 00	000	xo		xo
8.50 – 8.59			00	0	xo	0	x
8.40 – 8.49			0	0000x		0	
8.30 – 8.39			0	ooxxox	xoxo		x
8.20 – 8.29				ox	xx		xox
8.10 – 8.19			0	ooxx	xoo		xxxox
8.00 – 8.09				xxx	xox	xx	xx

¹The titre of this vaccine is:

	Before	After	Loss
100ºC ½ hour	9.25	8.53	0.72
100ºC 1 hour	9.25	8.53	0.72
37ºC 4 weeks	8.72	7.65	1.07

SUMMARY

In 1967, when the intensified smallpox eradication programme started, particular efforts were made to improve the vaccine quality and to increase the quantities of vaccine available in 1967, a WHO survey was conducted on the production status of freeze-dried smallpox vaccine; early in 1968, a WHO Seminar on Vaccine Production was held to recommend the simplest and most practical method of production; a WHO testing and advisory service was established.

At present, 64 laboratories are producing freeze-dried smallpox vaccine on a routine basis. Virous types of vaccinia strains are employed by different laboratories but the Lister strain is most frequently used. During the past two years, considerable progress has been made in the production of vaccine which is satisfactory with respect to potency, stability and purity. However, of 201 lots from 22 producers, tested by WHO during 1968 and 1969 (through July), 51 lots did not yet conform with WHO requirements, mainly due to unsatisfactory heat stability. Observations have been made on the heat stability of vaccines from different producers, with regard to the results of both the rapid heat stability test (100°C for 1 hour) and the conventional test (37°C for 4 weeks). Based on these observations, criteria are proposed for the acceptance of vaccine based on the rapid heat stability test as a screening procedure.

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REVIEW AND CHARACTERISTICS
OF VACCINAL STRAINS

SELECTION OF A STRAIN OF VACCINIA VIRUS FOR PRODUCTION OF SMALLPOX VACCINE

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It is recommended in WHO Requirements for Smallpox Vaccine (1966) that the vaccinia strains used should have a low pathogenicity for men, provided they give satisfactory immunity. It is also pointed out that there is no evidence that a strain which produces severe local lesions and marked systemic disturbance confers better protection than one that gives milder clinical reaction.

A thorough clinical comparison of four smallpox vaccines was carried out by Polak et al. (1963) in Holland, using the Danish, Lister and Equador vaccines, together with a vaccine from Berne, for primary vaccination of young adults. The four vaccines could be classified into three significantly different groups on the basis of the clinical reactions.

A comparison of the virulence of a large number of smallpox vaccines performed at Statens Seruminstitut revealed that one vaccine had an unusually low virulence for suckling mice infected intranasally, viz. the vaccine obtained from Equador. This was included in Polak's investigations and also in the International Colleborative Assay carried out by WHO with the aim of establishing the International Reference Preparation of Smallpox Vaccine (Krag et al., 1963).

It would be highly desirable to have a laboratory method which would give positive correlation with the pathogenicity in man.

This paper gives information concerning the properties of three vaccinia strains, based on own animal experiments supplemented by results of vaccination of man performed by others.

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Danish strain

The strain of vaccinia virus in use at Statens Seruminstitut for the production of smallpox vaccine was obtained some 50 years ago from Germany. The origin is unknown.

The strain has since been maintained by cutaneous passages in rabbit and calf. The vaccine used for man is the third passage on calf from seed virus obtained from the third passage on rabbit.

Lister strain

The International Reference Preparation of Smallpox Vaccine was used. This was prepared from a strain of vaccinia virus used in the United Kingdom for more than 60 years. Before 1946 it was passed in calves in the old Government Lymph Laboratory and since then has been passed alternately in rabbit and sheep at the Lister Institute of Preventive Medicine, Elstree.

Equador strain

This was obtained from a batch of smallpox vaccine prepared in 1956 at the Institute Nacional de Higiene, Guayaquil, Equador. The strain of vaccinia virus used was originally obtained from the laboratories of the State Health Department, Massachusetts. This information was obtained in 1963 through WHO, and its reliability was made probable in 1968 when a smallpox vaccine received from the State Health Department in Massachusetts showed the same low virulence for suckling mice as the Equador strain. The origin of the Massachusetts strain of vaccinia virus is not certain, but it was probably obtained in 1905 from the New York City Laboratories (Edsall, personal communication 1969).

At Statens Seruminstitut the strain has been through two consecutive series of cutaneous passages, the first consisting of three rabbit and two calf passages, the second of three rabbit and three calf passages.

ANIMAL EXPERIMENTS

Pathogenic properties

1. Suckling mice

The result of virulence testing of three vaccinia strains in suckling mice is shown on Fig. 1 (Ørskov et al., 1948). The mice were infected intranasally and the same infecting dose, as estimated by pock count, was used for each vaccine. The graphs are based on the pooled results of several experiments in which vaccines from cutaneous passages in

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calves and rabbits, passages in chick embryo tissue culture, and on the chorioallantoic membrane of chick embryos, were used. As there was no convincing difference in virulence due to the animal species or tissue used for preparation of the vaccines, the percentages of deaths were calculated from the total number of mice in each dose group and plotted against log pock-forming units/ml. The number of mice in each group varied from 20 to 46, with the largest number in the groups giving only partial percentages of deaths.

The Danish strain was found to be the most virulent, with an LD/50 of about 25 pock-forming units and time of death between 4 and 8 days. The Lister strain had an LD/50 of 500 and a time of death of 8–14 days. The virulence of the Equador strain was too low to be expressed as number of LD/50 and the few deaths occurred from 9 to 16 days after infection.

Fig. 2 shows the result of a virulence test performed with the same four vaccines as used by Polak et al. (1963) in the clinical trial, only 4–8 young were used per dose. The result agrees fairly well with the pooled result shown on Fig. 1, except that the virulence of the Equador vaccine was found to be a little higher.

According to the clinical reactions in adults, the four vaccines were classified as Lister < Equador < Danish = Berne. According to the death rate in suckling mice, the classification was: Equador < Lister < Danish = Berne.

Table 1 shows the cause of infection in suckling mice infected intranasally with either the Equador or the Danish strain of vaccinia virus. The same number of pock-forming units was used for each strain. One mouse was killed from each group at increasing intervals after infection and the organs titrated on the chorioallantoic membrane of embryonated eggs. Both strains caused a general infection, with a high virus content in the different organs. The mice infected with the Equador strain recovered quickly, while the mice infected with the Danish strain died 4–8 days after infection.

2. Suckling rabbits

Two-day-old rabbits were infected intracutaneously with 4×0.1 ml vaccine containing 10^5 pock-forming units (Ørskov et al., 1938). The young infected with the Equador strain all survived, while those infected with the Danish strain died 5 days after infection. The photographs in Fig. 3 show the difference in local reactions to the two strains 4 days after infection.

Immunogenic properties

The only way of ensuring that a vaccine is able to protect against smallpox is either to carry out time-consuming and expensive field trials in countries where smallpox occurs endemically or to perform experimental studies with variola virus.

An attempt has been made to demonstrate the immunogenic properties of different vaccinia strains by the transfer of antibodies from vaccinated female mice to their offspring. In Denmark, where we have had no case of smallpox for many years, it is not permitted to work experimentally with variola strains. A monkey-pox strain was therefore used for challenge.

The monkey-pox virus was isolated in 1958 at Statens Seruminstitut from outbreaks of a pox-like disease in Cynomolgus monkeys. It resembles variola virus in the lesions produced on the chorioallantoic membrane and on the scarified cornea of rabbits, but unlike variola virus it could be maintained in serial passages both in mice infected intracerebrally and in the rabbit skin (von Magnus et al., 1959).

Technique

Groups of female mice were vaccinated subcutaneously with one of the three vaccinia strains and the monkey virus strain. For the vaccinia virus strains, the first egg passage from calf or sheep vaccine was used and for monkey-pox virus the third egg passage from the original pustular material. The mice received four doses of 0.5 ml containing approximately 5×10^6 pock-forming units at intervals of one week. Four days after the last vaccination the mice were mated, and the 2-day-old babies were infected intranasally with the monkey-pox virus (approximately $50 \times LD/50$) (Ørskov et al., 1948).

The results are shown in Table 2. The homologous vaccination with the monkey-pox virus resulted in 75 per cent survivors. Vaccination with the vaccinia strains gave a lower degree of protection with great variations from one litter to another. Obviously the material is insufficient to demonstrate differences between the protective effect of vaccinia strains against infection with monkey-pox virus.

The relatively low degree of protection from vaccination with vaccinia strains against infection with monkey-pox virus seems surprising considering the close antigenic relationship reported previously.

Active protection test in rabbits

Rabbits which had recovered from infection by scarification with either the Lister, Equador or Danish vaccinia strains showed the same degree of immunity to subsequent infection by the same route by each vaccine diluted 1 : 10, 1 : 100 and 1 : 1000. There was no indication of better protection against the homologous than against the heterologous strains.

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Primary vaccination

The thorough clinical comparison carried out by Polak et al. (1963) concerning reactivity of four smallpox vaccines after primary vaccination of young adults provides valuable information. The vaccines were classified according to the severity of clinical reactions as Lister < Equador < Danish = Berne.

The more virulent Danish strain has been used with quite satisfactory result for more than 50 years in Denmark, both for primary vaccination of children and for revaccination. In fact, conscientious vaccinators in charge of the public vaccination did not wish to change to a less virulent strain because they obtained satisfactorily mild reactions. However, less skilled vaccinators might obtain rather severe local reactions.

In Denmark the Equador vaccine, with a potency of 2×10^8 pock-forming units/ml, has been used for primary vaccination of 252 children. The take rate was good, and the general impression was that the Equador vaccine gave slightly milder reactions, with less swelling of the arm, than the Danish vaccine.

Revaccination

The Equador vaccine has not been used for revaccination in Denmark, but valuable information can be drawn from the International Collaborative Assay carried out by WHO in order to establish the International Reference Preparation of Smallpox Vaccine (Krag et al., 1963). The Equador vaccine was one of the test vaccines in that assay and the Lister vaccine was represented both as the reference and as a test vaccine. Five laboratories collaborated in the revaccination studies in adults. A comparison of the effectiveness of the vaccines was made on the basis of index figures based on the degree of reactions.

The results obtained from two of the five collaborating laboratories showed well-defined index differences because of the relatively high take rate. The average intervals between the last vaccination and the test vaccination from these two laboratories were 13 and 15 years (unpublished data). The ability of the Lister and Equador vaccines to give positive takes after revaccination was found to be almost equal, in spite of the fact that the Equador vaccine was slightly less potent than the Lister vaccine. The average relative potency of the Equador vaccine was found to be —0.34.

In the two laboratories mentioned the local vaccines showed a similar take rate to that of the Lister and the Equador vaccines. The results from other three laboratories showed similar distributions, but these comparisons were less acurate due to the low percentage of takes.

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Other properties

The rate of deterioration of the Danish and Equador vaccines is shown on Fig. 4. The glycerinated vaccines were kept at 25⁰C in small nylon capsules. At intervals of one week one capsule was removed and placed at —18⁰C. The samples, together with a control kept at —18⁰C, were titrated simultaneously, on one day by pock count and on another by plaque count on chick embryo tissue culture. No significant difference in the deterioration rate could be demonstrated.

All three strains developed white, easily countable, pocks on the chorioallantoic membrane, but the percentage of eggs giving unclear pocks seemed to be higher for the Danish strain than for the Lister and Equador strains.

The Danish and Equador strains developed round and easily countable plaques on chick embryo tissue culture, while the Lister strain had a tendency to give smaller and irregular plaques.

The three strains of vaccinia virus were all dermal strains obtained from production laboratories. The Danish and Equador vaccines were produced on calves, while the Lister vaccine was produced on sheep. At Statens Seruminstitut we have had difficulty in getting sufficient takes on our calves with the Lister vaccine, even when using a calf-adapted strain obtained from Holland, while in Holland no such difficulties were encountered.

We will doubtless be able to produce the Lister vaccine on calf when we change from the Red Danish breed to the white-spotted Holstein-Friesian breed and use the whole-area scarification method instead of the single scratch method.

DISCUSSION

It seems possible by means of the virulence test in suckling mice to distinguish between more or less virulent vaccinia strains but the correlation between the classification as estimated by clinical reactions in primarily vaccinated adults and by the death rate in suckling mice was not absolute. Classification of the two less virulent strains was in the opposite order, while there was full agreement concerning the two virulent strains.

The experiment on passive transfer of protective antibodies from mother mice to their offspring against challenge with monkey-pox virus seems to indicate a lower degree of immunity after vaccination with vaccinia strains than when using the homologous strain. A similar observation was made by Stickl et al. (1965) using an immunocytolysis test for detection of tissue immunity. Three years after a probable attack of smallpox, the convalescent showed a high haemagglutinin inhibition titre but a low degree of cytolysis, when the leucocytes were tested against

vaccinia virus. Leucocytes from vaccinated persons showed a low degree of lysis when tested against variola virus, while the lysis was strong when tested against vaccinia virus.

These observations stress further the importance of adequate vaccination. During the last five years, all batches of gamma-globulin prepared at Statens Seruminstitut have been tested for neutralizing antibodies against vaccinia virus and were found to contain between 100 and 200 units/ml, while human anti-vaccinia gamma-globulin contains only 3 times as much neutralizing antibody. This can be taken as an indication of the high degree of immunity in the Danish population.

Our knowledge concerning the duration of immunity following vaccination with different vaccinia strains is insufficient. It would be desirable if an investigation into the rate of disappearance of neutralizing antibodies and tissue immunity could be carried out before a single strain of vaccinia virus is selected for vaccine production throughout the world.
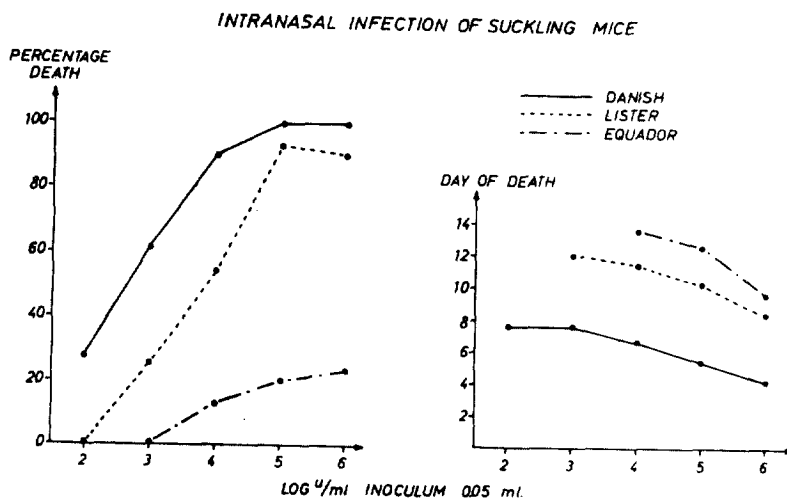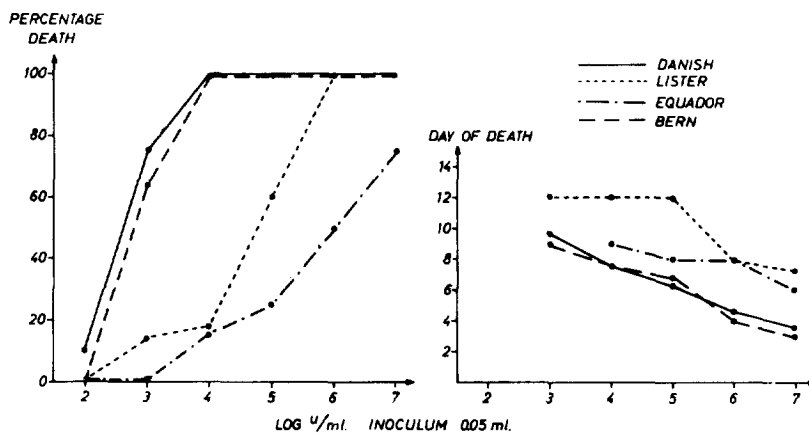
Fig. 1

INTRANASAL INFECTION OF SUCKLING MICE

Fig. 2

INTRANASAL INFECTION OF SUCKLING MICE

PERCENTAGE DEATH

DAY OF DEATH

—— DANISH
········ LISTER
—·—· EQUADOR
— — — BERN

LOG $^U/ml.$ INOCULUM 0.05 ml.

Table 1

COURSE OF INFECTION IN 2-DAY-OLD MICE
INTRANASALLY INFECTED WITH VACCINIA VIRUS

DAY AFTER INF.	BLOOD	LUNG	LIVER	SPLEEN	BRAIN
EQUADOR: WHO A. 1' EGG PASSAGE					
1	0	4.4	0	0	0
3	· 2.3	7.3	4.3	4.3	2
6	2.18	7.45	3.3	<3.0	3.6
9	0	7.34	0	0	3.5
14	–	0	–	–	0
DANISH: CALF VACCINE 1/50 III					
1	0	3.66	0	0	0
3	3.04	7.62	4.64	>5.2	2.54
6	5.0	8.42	5.67	8.08	6.08
9	ALL MICE DIED 4-7 DAYS AFTER INFECTION				

FIGURES INDICATE LOG TITRE ESTIMATED BY POCK COUNT ON
CHORIOALLANTOIC MEMBRANE.
INFECTING DOSE ABOUT 0.05 ml CONTAINING 5000 POCK-FORMING U.

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Table 2

Passive transfer of protective antibodies in mice

Virus Strain	Litter No.	†/T	Total †/T	Per cent Survivors	Ave, Day of Death
Equador	1	5/6			8.6
	3	6/6			5.2
	4	6/6			4.8
	5	1/6			7
	6	4/30	22/30	27	6
Lister	7	4/6			12
	8	3/3			5
	9	2/4			8
	12	6/6	15/19	21	5.3
Danish	15	6/6			4.3
	17	2/5			9
	18	6/6	14/17	18	6.2
Monkey Pox	19	1/6			9
	21	2/6			10
	23	0/6			
	24	3/6	6/24	75	8
Control	26	1/1			9
	27	3/3			5
	28	6/6			5.2
	30	6/6	16/16	0	5

Challenge strain: Monkey Pox
Infect. Dose: 30 LD/50. †/T: No of deaths of total no.

Fig. 3
Intracutaneous infection of 2-day-old rabbits

	Dose	†/T	Day of death
Equador	5	0/5	
Danish	5	5/5	5.2

Dose: Total log pock-forming units in 4×0.1 ml.
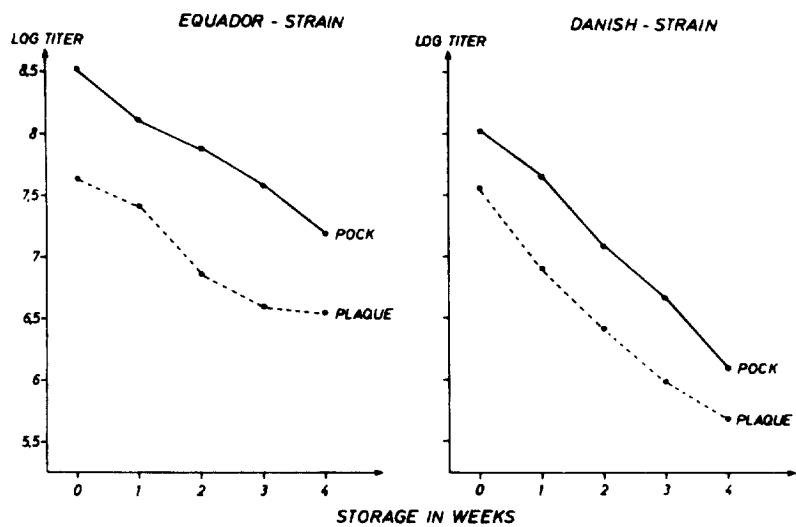†/T: Number of deaths of total number of rabbits.

Equador   Danish

Reactions in suckling rabbits 4 days after intracutaneous injection.

Fig. 4

DETERIORATION AT 25°C.

EQUADOR - STRAIN   DANISH - STRAIN

STORAGE IN WEEKS

SUMMARY

A virulence test for vaccinia strains is suggested.

The mortality rate and the time of death in suckling mice infected intranasally with serial dilutions of smallpox vaccines enable distinction to be made between more or less virulent vaccinia strains. Such a rough distinction seems to agree with the degree of reactions in human beings, but a close correlation could not be established between the morbidity rate in human adults and the mortality rate in mice.

Transfer of protective antibodies from vaccinated mother mice to their offspring against challenge with monkey-pox virus indicates a lower degree of protection in mice vaccinated with vaccinia virus than in mice vaccinated with the homologous monkey-pox virus.

If such a difference in protection within the pox group is valid also for smallpox in human beings, it would further stress the importance of adequate vaccination.

It is suggested that further investigations regarding the duration of humoral and tissue immunity in man after vaccination with smallpox vaccines of low virulence should be made before a single vaccinia strain is selected as the ideal strain for vaccine production.

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CHARACTERISTICS OF VIRUS STRAINS FOR PRODUCTION OF SMALLPOX VACCINE

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Since the time of E. Jenner's discovery until now vaccination against smallpox has remained the main means of control of this infection. It is evident, therefore, that until complete global eradication of smallpox has been achieved, the complex of problems connected both with vaccination and the quality of the vaccine will be in the spotlight.

Investigations of recent years showed that the quility of smallpox vaccine was determined to a considerable degree by characteristics of strains of which the vaccine was made (Marennikova et al., 1962; Polak, 1963; Krag et al., 1963; Marennikova and Tashpulatov, 1966; and others).

The fact of differences between smallpox vaccines was observed by old authors (N. F. Gamaleya, 1913; and others). These differences, however, could not be conclusively associated with the quality of vaccine strains since at that time there was no standard technology for production of a stable preparation and no accurate methods for the quantitation of the virus in it were available.

Investigations along these lines are particularly important because at the present time such a variety of production strains is used for smallpox vaccine as for no other virus vaccine. As the experience of investigation of the history of these strains shows, in addition to indefinite origin, they are maintained in extremely diverse ways both with respect to animal species used for their cultivation and the scheme of passages used. It is no mere chance, therefore, that the WHO included this problem among the subjects to be specially studied (WHO, Smallpox Eradication, 1968).

This paper presents materials characterizing the properties of strains used for smallpox vaccine production and evaluates them in connection with the quality of the final product.

The general evaluation of the materials obtained in laboratory studies under similar conditions of over 20 vaccine strains permits to divide the strains into certain groups according to different genetic properties.

Thus, according to the composition and homogeneity of the virus population as well as to the range and degree of pathogenicity for laboratory animals, the strains under study were divided into several groups (Table 1).

The composition and homogeneity of the virus population was studied by determination of morphology of pock lesions on the chorio-allantoic membrane (CAM) of chick embryos and in some cases of morphology of plaques in tissue cultures under the agar overlay.

The pathogenicity of strains in the above classification was evaluated by the reaction to inoculation of constant doses of virus:

1. intracerebrally and intracutaneously to rabbits (10^6 and 10^7 PFU/0.1 ml);
2. Intracerebrally to white mice (10^5 and 10^6 PFU/0.03 ml);
3. Intranasally to suckling white mice (10^5 and 10^6 PFU/0.01 ml);
4. Intravenously to white mice (1.5×10^7 PFU/0.5 ml) and to white rats (10^6, 10^7 and 10^8 PFU/1 ml) which had previously received total irradiation (Co^{60}, 400 r).

According to the composition and homogeneity of the virus population, we distinguish 3 groups of strains:

1. with homogeneous virus population;
2. with inhomogeneous population in which predominates the virus causing white pocks on the CAM;
3. with inhomogeneous population in which predominates the virus producing superficial pocks on the CAM.

Practically, all the above groups of strains consisted in their turn of 3 subgroups:

1. strains of marked pathogenicity,
2. strains of moderate pathogenicity,
3. strains of low pathogenicity.

The group of markedly pathogenic strains includes those producing death of intracerebrally inoculated rabbits and white mice, death of irradiated white mice and white rats inoculated intravenously, necroses after intracutaneous inoculation, marked orchitis (sometimes with generalization of the process) after inoculation of rabbits into the testis, etc.

Strains of moderate pathogenicity were partially pathogenic for rabbits by the intracerebral route and for irradiated animals by the intravenous route, but produced necroses (sometimes in a portion of inoculated animals) after intracutaneous inoculation.

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The third group of strains of low pathogenicity included strains which produced no death in intracerebrally inoculated rabbits, no necroses by intracutaneous inoculation, and were apathogenic or of low pathogenicity for irradiated animals.

Analysis of the importance of the genetic homogeneity and composition of the virus population was carried out by cloning of several inhomogeneous strains followed by investigation of the properties of each clone. It was established that properties of clones with different morphology of pocks and of vaccines prepared from them differed markedly; properties of clones with similar morphology of pocks but derived from different strains also varied. It was also demonstrated that the properties of the original strain were determined mainly by the properties of the dominating virus population (Marennikova, Maltseva, 1965; Marennikova, 1967).

Despite the fact that the composition of population (for inhomogeneous strains) or homogeneity (for homogeneous ones) are sufficiently stable properties, some of our observations indicate the possibility of practically unpredictable changes. Thus, in particular, we observed an appearance and gradual increase with passages of a population of virus producing superficial pocks in the strain which had before produced only white pocks. It is important to emphasize that in parallel the reactogenicity of the strain increased slightly.

Thus, the structure of a strain requires periodic control, since its changes and breaks may be accompanied by changes in the quality of the vaccine.

For the evaluation of the practical importance of the degree of strain pathogencity for laboratory animals, we studied under comparable conditions the pattern and severity of vaccination process in primary vaccination of children using vaccines differing in these properties.

The study of the febrile reaction and some other signs caused in the children by vaccination revealed significant differences between vaccines depending on the strain used for vaccine preparation. It was found, for example, that the vaccines prepared from the strains highly pathogenic for animals (Tashkent) produced fever above 39⁰C in 11–16⁰/o of vaccines, whereas the vaccine made of the strain of low pathogenicity (EM–63) with an equal or even 1 lg higher infectious titer produced the same fever in 0–2.5⁰/o of vaccinees.

Vaccinal reaction with fever not exceeding 37⁰C was observed in 39–42⁰/o of vaccinees using the latter vaccine, whereas vaccines made of pathogenic strains caused vaccination process with normal temperature in only 9–21⁰/o of vaccinees.

Differences of the same kind were found in consideration of other characteristics of severity of vaccination reaction: incidence of lymphadenites, additional pustules, confluent areas, etc. It should be mentioned that vaccines made of some highly pathogenic strains (Tashkent) were so highly reactogenic that it was impossible to prepare from them the

vaccine with the infectious potency required by the WHO instructions (1966). These vaccines, even with titers 10 times lower than the minimum acceptable by the WHO requirements, produced a high per cent of severe reactions with many additional pustules, severe lymphadenites and marked general intoxication.

Because of the foregoing data, it would be expedient to revert to consideration of requirements for production strains of vaccine virus. As is known, requirements for the strains, as determined by the WHO, have only one definite quantitative criterion – the infectious titer. This criterion, even though very important, cannot characterize the preparation completely. Indeed, with similar titer one vaccine may give moderate reactions and the other severe.

Proceeding from this, it would be expedient to supplement the current requirements of the WHO with more concrete determination of properties of the production strains as well as to introduce an additional criterion characterizing the reactogenicity of the vaccine, as has already been done for some other preparations.

Thus, the available evidence indicates that it is possible to make a preliminary laboratory evaluation of reactogenicity of smallpox vaccines on the basis of tests for determination the degree and range of pathogenicity for laboratory animals. It should be kept im mind that the most complete characterization of a strain may be possible only by using all the above-described tests.

Considering the preference given by the WHO (WHO, Smallpox Eradication, 1968) to smallpox vaccine producing no severe reactions, we studied in greater detail some strains selected on the basis of the above classification and belonging to the group of strains with moderate and low pathogenicity – a strain of Lister Institute,[1] Wyeth (USA),

A controlled trial of this group of vaccines in limited contingents of primarily vaccinated subjects (vaccination by 2 scarifications) demonstrated for all the preparations under study a high per cent of vaccination reactions with normal temperature (up to 37°C). The Lister Institute strain differed slightly from the others, producing a low per cent of high fever (above 39°C). Inoculation of the same vaccines by jet injector produced milder vaccination reaction than by scarification. This difference appears to be due to the fact that in the former case the virus is inoculated and multiplies at one site, whereas in scarification method in two sites.

Subsequent observations were connected with the evaluation of the antigenic and immunogenic activity of these strains. These values were determined experimentally in different animals – rabbits, white rats irradiated with γ-rays and mice.

The antigenic activity was evaluated by accumulation of virus-neutralizing antibody and antihemagglutinins after immunization with equal

doses of the vaccine. The immunogenicity was evaluated by determination of the minimal immunizing dose of vaccines in experiments in irradiated white rats (Chimishkyan, in press), of resistance of rabbits to intracerebral inoculation of different doses of neurovaccine and resistance of irradiated white rats to intravenous inoculation of large doses of »Tashkent« strain.

The results obtained showed that the strains under study differed in the above tests. The highest antigenic activity among the three strains used in the tests was found in the Lister Institute strain (Fig. 1). Similar data were obtained with regard to the immunogenic activity. Thus, the minimal immunizing dose in experiments in irradiated white rats and mice (Table 2) was found to be slightly higher for the EM–63 strain than for Byelorussian and Lister strains (the minimum immunizing dose – MID_{50} – was considered to be the highest dilution of virus-containing suspension which protected against »vaccination disease« 50% of irradiated animals, as calculated by Reed and Muench formula, 1938). At the same time, when resistance in rabbits to 100,000 LD_{50} of neurovaccine virus was tested (the immunization dose $6.0–8.0 \times 10^6$ PFU/0.2 ml), all the 3 strains produced similar protection.

In subsequent observations carried out in limited groups of primovaccinees and revaccinees, the dynamics of accumulation of virus-neutralizing antibody and antihemagglutinins was determined after vaccination with vaccines made of the above strains (Fig. 2, Table 3 and Fig. 3). These studies showed that antibody response in vaccinees varied, as had been demonstrated in animal experiments. In primovaccinees, higher titers of virus-neutralizing antibody were obtained with Lister Institute and Wyeth vaccines (Fig. 2). These results on the whole were observed also in children vaccinated by jet injector with vaccines under study. It should be mentioned, however, that the latter method of vaccination gave lower antibody levels for all the strains, which, as well as the severity of reaction in vaccination by this method, may be explained by different conditions of virus multiplication.

In analysing the data obtained for the group of revaccinees, consideration should be given to the influence on the results of the immune background preceeding revaccination: the lower the antibody level, the higher its increase after revaccination (Svet-Moldavskaya et al., 1969). The results obtained in these experiments indicated (Table 3) that all the vaccines provided high (100 or close to 100) per cent of seroconversions. Determination of virus-neutralizing antibody titers one year after revaccination demonstrated that despite their decline in all the groups antibody levels remained much higher than before revaccination. Even though there are certain difficulties in more detailed comparative evaluation of the antigenic activity of different vaccines associated with differences of the initial antibody levels in some groups of revaccinated subjects (by geometric mean titers), it is evident that the general regularity revealed in primary vaccinations holds true in revaccinations: the vaccine of the Lister Institute strain is more antigenically active. At the

same time, in contrast to what had been found in primary vaccinations, it was shown that in revaccination the method of vaccine administration had no significant effect on the intensity of humoral response.

Additional studies were carried out in order to explain the mechanism of revealed differences. Thus, preliminary experiments demonstrated that virus reproduction in rabbit skin was more active with strains of high antigenic activity. On the other hand, when the antigenic activity was studied under conditions excluding virus multiplication in the organism (immunization of animals with vaccines from the same strains inactivated with γ-rays), no differences established in animals experiments and observations in people were found.

No differences in the antigenic structure of the Lister, Byelorussian, Hyeth and EM–63 strains were found when tested by the agar percipitation test (by Auchterlony) with sera prepared both for the newly isolated strain of variola virus and for the vaccine strains (Fig. 4).

Thus, the available evidence suggested that differences in the antigenic and immunogenic activity of the strains might be due to a certain extent to intensity of their multiplication in the organism. In this connection it seemed of interest to study the capacity of the strains to induce interferon production and their sensitivity to its effect. Preliminary data of these experiments (carried out in collaboration with T. A. Bektemirov and L. S. Shenkman) revealed no differences between EM–63 and Lister Institute strains which proved to be poor interferohogens in the models tested (intravenous inoculation of white mice). No significant differences between these strains were found with regard to sensitivity to interferon: both were more sensitive than Tashkent strain (highly pathogenic for animals). Studies of this kind are in progress now.

For all countries where vaccination prophylaxis of smallpox is carried out, and particularly for those countries where smallpox has been eradicated as an endemic disease, postvaccination complications are not less, if not more, important than reactogenicity. There exists a number of factors which may influence the incidence of the majority of complications (consideration of contraindications, age for primary vaccination, etc.). As for the influence on the incidence of complications of the quality of vaccine as such, and, in particular, of the strain from which it is prepared, the majority of authors find no correlations between them (Wilson, 1967).

The preliminary analysis of the number of postvaccination encephalities due to application of vaccines prepared from highly pathogenic (reactogenic) strains and strains with lower reactogenicity shows, however, that use of the latter is accompanied by smaller number of postvaccination encephalites. Proceeding from the foregoing, there are certain reasons for review of unconditional denial of association between the quality of the vaccine and incidence of postvaccination encephalitis.

In this paper we did not touch upon a number of problems relevant to this subject (intensity of strain accumulation in the organism of pro-

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ducent, the effect of cultivation conditions on the properties of strain, methods for stabilization of the properties of strain, etc.). At the same time, it seems to us that the material presented testifies conclusively to the fact that the properties of vaccine strains are very important both for the quality of the preparation produced from them and for the practice of smallpox vaccination on the whole.

Table 1

Classification of strains by some genetic properties

Composition of population	Degree of pathogenicity	Country where the strain is or was used
Homogeneous (99.5–98.8%)[a]	Pathogenic	Denmark, Hungary, USSR (T. B. K.)[e]
	Moderately pathogenic	England (Lister Institute strain)[d], India (Patvadangar), Bulgaria, Thailand
	Low-pathogenic	USSR (EM-63)
Inhomogeneous, with prevalence of white pocks (68–88.4%)[b]	Pathogenic	USSR (Tashkent, Per, Tom),[e] France
	Moderately pathogenic	USSR (BIEM), Poland, Germany (Bern)
	Low-pathogenic	USA (Wyeth)
Inhomogeneous with prevalence of superficial pocks (48–94%)[c]	Pathogenic	Japan (Ikeda, Dairen), China, USSR (Gam., MRIVP-as)[e]
	Moderately pathogenic	Indonesia
		—

a, b – content of white pocks
c   – content of superficial pocks
d   – according to data of studies of 1968, 1969
e   – previously used in the USSR

Table 2

Data of comparative study of immunogenicity of different smallpox vaccines in irradiated white rats and mice
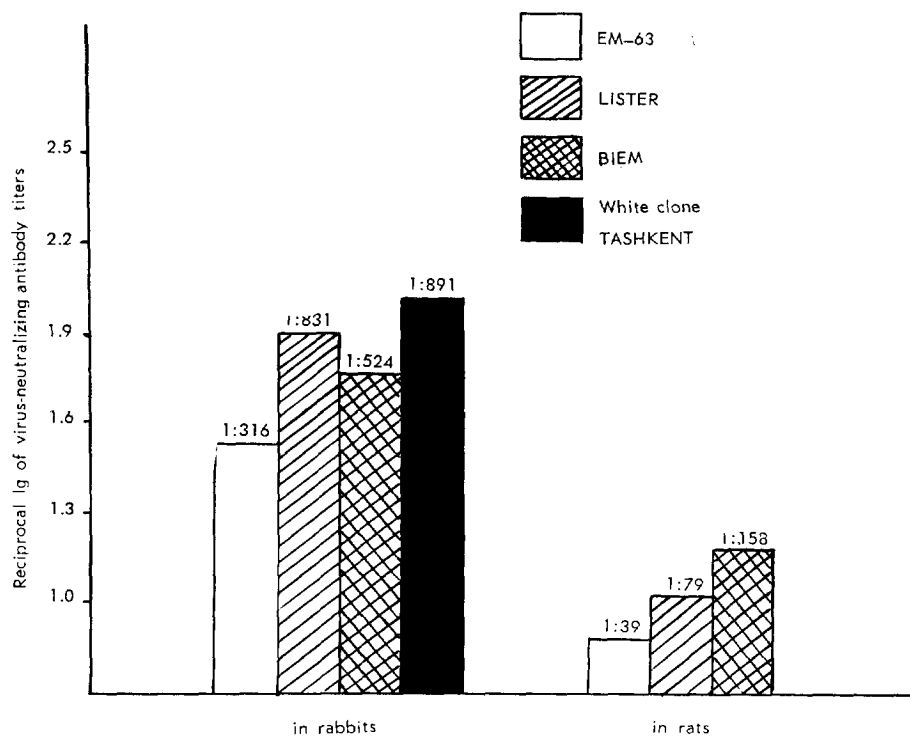
Strain	Animal species	Immunising dose (PFU/ml)	Resistance to the lethal dose of Tashkent strain	lg MID_{50}
EM-63	Rats	$6.0 \times 10^3/0.2$ $6.0 \times 10^2/0.2$ $6.0 \times 10^1/0.2$	4/10 9/9 10/10	2.83
	Mice	$2.0 \times 10^5/0.1$ $2.0 \times 10^4/0.1$ $2.0 \times 10^3/0.1$ $2.0 \times 10^2/0.1$	0/5 0/5 3/5 5/5	3.16
LISTER	Rats	$6.0 \times 10^3/0.2$ $6.0 \times 10^2/0.2$ $6.0 \times 10^1/0.2$	4/7 4/8 7/10	1.72
	Mice	$2.0 \times 10^5/0.1$ $2.0 \times 10^4/0.1$ $2.0 \times 10^3/0.1$ $2.0 \times 10^2/0.1$	0/5 0/5 2/5 5/5	2.83
BIEM	Rats	$6.0 \times 10^3/0.2$ $6.0 \times 10^2/0.2$ $6.0 \times 10^1/0.2$	3/10 3/8 8/10	1.92
	Mice	$2.1 \times 10^5/0.1$ $2.1 \times 10^4/0.1$ $2.1 \times 10^3/0.1$ $2.1 \times 10^2/0.1$	0/5 0/5 0/5 3/5	2.16
White clone TASHKENT	Mice	$2.2 \times 10^5/0.1$ $2.2 \times 10^4/0.1$ $2.2 \times 10^3/0.1$ $2.2 \times 10^2/0.1$	0/5 0/5 1/5 5/5	2.62

x-numerator-number of dead animals; denominator-number of animals in the group

Table 3

Antigenic activity of smallpox vaccines in revaccination

Exp. No.	Method of vaccination	Contingent — previous vaccination	age (years)	No. of subjects in the group	Vaccine strain	Lot No.	Infectious activity (PFU/ml)	Prevaccination Range	Prevaccination Geometr. mean titer	Postvaccination (at 4 weeks / at 2 weeks) Range	Geometr. mean titer	fold increase	Postvaccination (one year / at 4 weeks) Range	Geometr. mean titer	fold increase	Number of positive seroconversio
1	Scarification	Vaccination and several revacci-	8	9–14	EM-63	0182	1.8×10^8	<10–160	15.4	20–2560	331.1	22	10–160	68.1		13/14
					BIEM	499a	1.6×10^8	<10–160	14.7	20–2560	575.4	38.3	10–160	67.6		100%
					Lister	4079	3.8×10^8	<10–80	14.1	160–1280	512.9	36.6	20–640	104.7		100%
2	Jet injection	same as above	18	12–14	EM-63	0182	1.8×10^8	<10–160	16.6	20–2560	416.9	24.5	80–2560	446.7	26.3	100%
					BIEM	499a	1.6×10^8	<10–320	36.3	80–2560	489.8	13.6	80–1280	446.7	12.4	13/14
						4079	3.8×10^8	<10–320	16.2	160–2560	562.3	35	160–2560	588.8	36.8	100%
					Lister	244902	9.0×10^7	<10–160	32.2	80–2560	446.7	13.8	40–2560	416.9	13	100%
3	Jet injection	same as above	18	8	EM-63	0182	1.8×10^8	<10–160	13.2				40–2560	190.5	14.3	100%
					BIEM	499a	1.6×10^8	<10–160	7.4				80–1280	141.3	19.0	100%

Titres of virus-neutralizing antibody — Postvaccination groups: for Exp. 1 "at 4 weeks" and "one year"; for Exp. 2 and 3 "at 2 weeks" and "at 4 weeks".

Fig. 1

Antigenic activity of smallpox vaccines in primary vaccination
Titers of virus-neutralizing antibody of vaccines at 28–30 days after vaccination (per cent). The geometric mean titer in subjects vaccinated by scarification with EM-63 strain vaccine is taken for 100%.
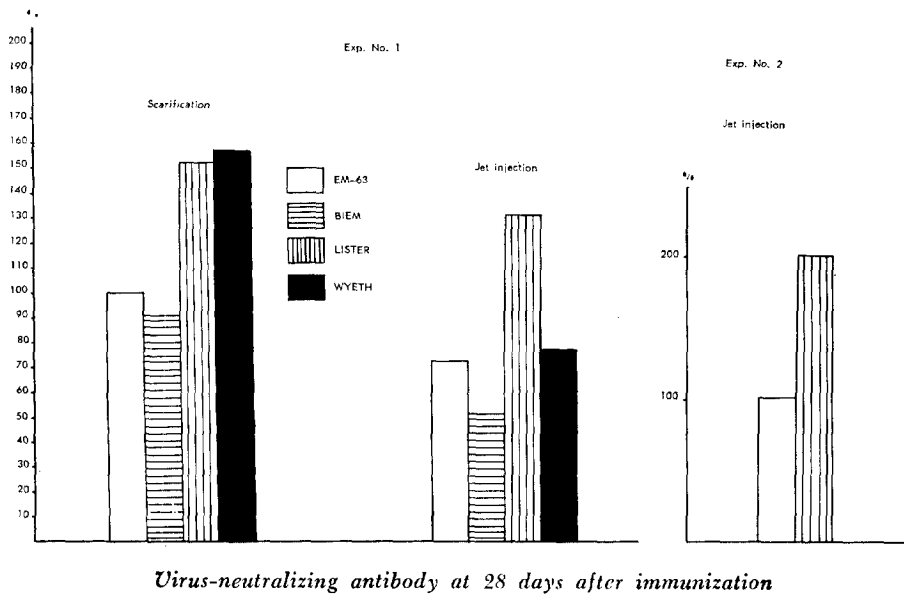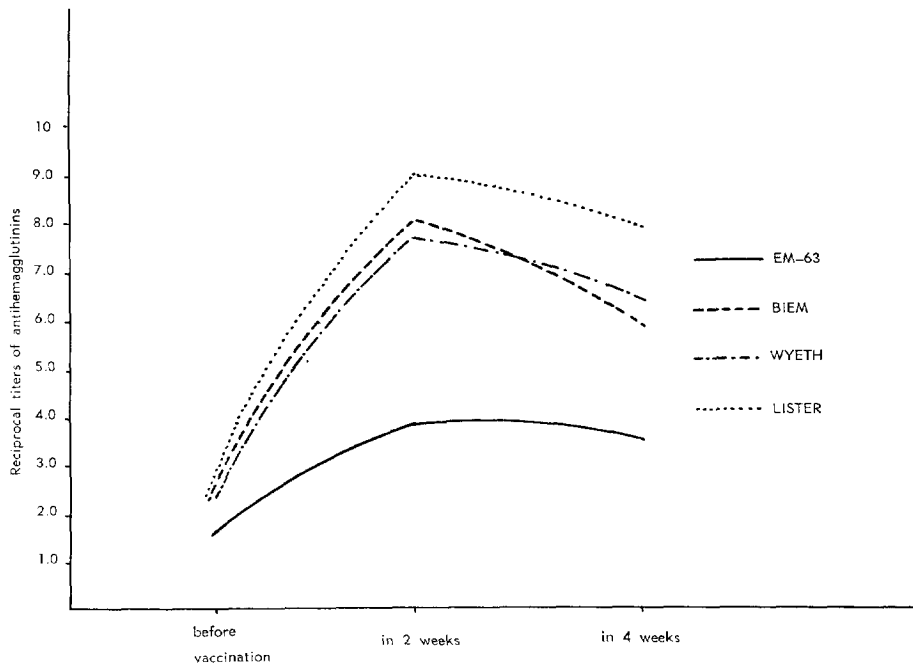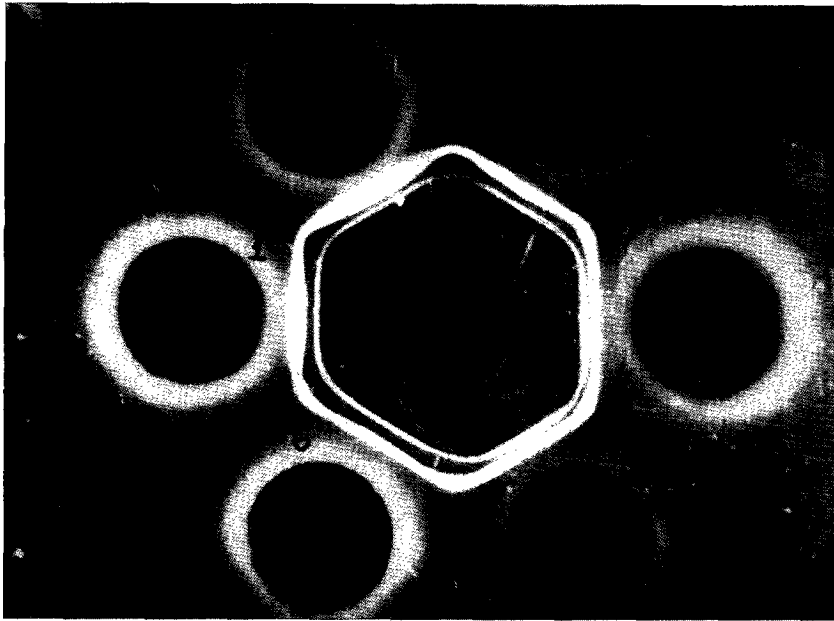
Fig. 2

Virus-neutralizing antibody at 28 days after immunization

Fig. 3

The dynamics of antihemagglutinins of the vaccines under study in revaccination by jet-injection (Experiment No. 2)
Reciprocal titres of antihemagglutinins

Fig. 4

Investigation of the antigenic structure of strains by the agar gel diffusion test
In the central well-rabbit immune serum for a freshly isolated strain of variola
virus (India – 1967)
In wells along the circumstance-chorionallantoic cultures of strains: No. I-EM-63

    No. 1 – EM-63
    No. 2 – Lister
    No. 3– Wyeth laboratory
    No. 4 – BIEM
    No. 5 – variola virus (Nepal – 1967)
    No. 6 – variola virus (India – 1967)

SUMMARY

As a result of studies of virus strains used for production of smallpox vaccine, their classification is presented according to a number of genetic properties: homogeneity of virus population and degree and range of pathogenicity for laboratory animals. The importance of these properties of strains for the quality of the vaccine is discussed on the basis of the available evidence.

Correlation between some genetic markers determining the pathogenicity of the strain and its reactogenicity in primary vaccinations of children has been demonstrated. A complex of laboratory markers of strain reactogenicity is suggested, including pathogenicity for rabbits by the intracutaneous route, for rabbits and white mice by the intracerebral route, as well as pathogenicity for white rats irradiated with γ-rays by the intravenous route.

In comparative experiments in laboratory animals as well as in trials in primarily vaccinated and revaccinated children, different antigenic activities of the strains classified as moderately pathogenic and low-pathogenic was established.

The method of vaccination (two scarifications or jet injection) has been shown to determine to a certain extent the severity of the vaccination process and the degree of primary antibody response, and to exert no significant influence on the intensity of humoral response in revaccination.

The paper discusses the problem of association between the incidence of postvaccination encephalitis in smallpox vaccinations and the properties of the strain from which the vaccine is prepared.

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CHARACTERISTICS OF BERNE-ZAGREB VACCINAL STRAIN

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The vaccination against smallpox being compulsory in this country and the vaccine being produced in one institute – the Institute of Immunology – we have been given the opportunity to well characterize our vaccinal virus strain, having at our disposal a substantial body of data on the reactivity of this vaccine.

An impressive amount of 30,000,000 doses of smallpox vaccine prepared at the Institute of Immunology in Zagreb has been used in Yugoslavia since 1945 for primary vaccination and and revaccination of children.

According to our regulations[*] primary vaccination against smallpox is compulsory for all children from three months to three years of age. Children older than three years are vaccinated only in special epidemiological circumstances.

Children that have been successfully vaccinated before their third year of life are revaccinated for the first time when about seven years old (the first form of elementary school) and for the second time when about fourteen (the eighth form of elementary school). The third vaccination covers Army recruits on enlistment.

Institute of Immunology in Zagreb is the only manufacturer of smallpox vaccine in Yugoslavia. Until 1964 liquid vaccine was prepared (lymph) and since then all smallpox vaccine have been freeze-dried.

[*] Article 15, para. 1. Basic Decree on the Prevention and Control of contagious diseases. Službeni list SFRJ, No. 17/1964. Regulations covering the conditions and method of implementing obligatory immunization against contagious diseases. Službeni list SFRJ, No. 2/1965.

Before release each vaccine lot is tested in children (after having passed all the obligatory laboratory tests) and the clinical course of reactions is followed up.

MATERIAL AND METHODS

Vaccine

Seed virus. Our seed virus is prepared from the Berne strain which was received from the Berne Institute in 1936. The 1 : 3 solution of seed virus and glycerole, homogenised, is stored at —20⁰C. The pulp for the vaccine production is obtained only by passage through calves without changing the host.

Preparation of the virus infected pulp. For the production of pulp one year old heifers of Simenthal breed, weighing between 300 and 400 kilograms, are used.* The animals are kept in quarantine and under veterinary supervision for 14 days. During this time the tuberculin test and the examination of stool for bacteria and parasites are made. The animals are placed on an operation table and after being shaved and washed with water and desinfectants, the abdomen, the chest and the inner side of legs are infected with seed virus having the titre of $10^{8.5}$ PFU/ml (WHO–SE/68,3. Rev. 1).

The site of infection is covered with a thin layer of sulphonamide ointment and sterile gauze and then with a sterile protective «apron«, to minimize the risk of infection of the scarified area. Special attention is paid to the cleaning and washing of the cattle shed. There is a day-and night watch-keeping from the time of infection until harvest, which is done at the end of four or five days, depending on the local reaction.

The pulp of each animal separately is collected in bottles, without preservative, and left at —20⁰C until use. Each animal used for obtaining the pulp undergoes autopsy.

Preparation of vaccine. A certain amount of tissue is dissolved and homogenized for 10 minutes at 45.000 r.p.m. in a homogenizer (Equipments Industriels, Paris), filtered and diluted in McIllvain's solution and 5 per cent peptone (Difco) aá to the desired concentration. The virus suspension is filled in ampoules, 10 or 50 doses in each, and freeze-dried in nitrogen atmosphere (freeze-dryer Usifroid).

No antibiotics are added in any production stage.

If purified vaccine is required, purification is done only by centrifugation (mechanically) at 3000 r.p.m. to remove skin particles and a great deal of bacteria.

Titration and control of vaccine. The titration was done by inoculating chorio-allantoic membrane (CAM) of 12 days old chick embryos (Leg-

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* Production protocols of the Institute of Immunology 1961, rev. 1966.

horn breed) (Burnet and Faris 1942). The inoculum was 0.1 ml of virus suspension per egg. Not less than 10 eggs for each of two tenfold dilutions were used for the inoculation. At least 5 titrations for each lot were performed. After 48 hours of incubation at 37⁰C the number of pocks per membrane was counted. The titre was calculated from the mean value of pock counts, the dilution and the amount of inoculum used, and expressed as PFU/ml. Other tests were done according to the WHO Requirements for Smallpox Vaccine (1966). The reactivity of each lot was tested in not less than 10 children. After a lot has successfully passed the state control it can be used for vaccination in the field.

Vaccination. Primary vaccination of not less than 10 children by each of our lots of smallpox vaccine is regularly performed at the Public Health Centre »Trnje« in Zagreb. Revaccination was done in the Medical Centre of Čakovec.

The vaccination was performed by the method of multiple pressure, on two sites of vaccination on the upper right arm in horizontal direction, with about 30 pressures on each site of vaccination. The space between the two sites was 3 to 4 centimetres. On coming for vaccination each child had a special card opened for it in which, beside general data, such as the child's name and surname, address, date of birth etc., also the data on postvaccinal reactions were later entered.

Infants of 6 to 24 months (75 per cent were between 6 and 8 months old) were covered by primary vaccination. On revaccination the average age of children was 7 years and the interval between the primary vaccination and revaccination was 3 to 6 years. The control of children after primary vaccination was performed from the 4th to the 14th day after vaccination, while the revaccinated children were observed on the 2nd, 4th, 6th and 8th day after vaccination. Examination of children after primary vaccination and revaccination was done by pediatricians of the »Trnje« Public Health Centre and a team of two epidemiologists from the Institute of Immunology (the same persons that vaccinated the children).

Clinical reactions

Clinical reactions of those vaccinees who after primary vaccination showed a typical primary vaccinia with one or two pocks after 7 days (the day following vaccination was counted as the first day) were followed up.

The appearance of minimum one pock was considered as successful vaccination. Only vesicular and pustular lesions (major reaction) were considered as successful revaccination (Cross 1961; WHO Expert Commity on Smallpox Vaccine 1964), while the appearance of papula (accelerated reaction) and macula (immediate reaction) were not taken into account. The reactions were definitively assessed on the 6th day.

Among clinical reactions particular attention was paid to the appearance of fever in order to assess its height and duration. The temperature was taken axillary twice a day beginning from the 4th day after vaccination. No child was given antipyretics. With regard to the fever, the successfully vaccinated children were divided into 3 groups. The first group marked »no fever« covered the children whose temperature was lower than 37⁰C. Children having the temperature from 37⁰C to 37.9⁰C were put in the second group marked »low fever«. The third group consisted of children whose temperature was higher than 37.9⁰C. This group was marked as »morbidity«. The rates were always expressed in percentages. Within the »morbidity« group we had two sub-groups in which the sub-group »high fever« represented the temperature higher than 38.9⁰C. The rate was expressed as the percentage of children with this high temperature from the total number of children in the »morbidity« group. The sub-group »prolonged fever« represented the duration of temperature for two days and longer. The rate was expressed as the percentage of children with the prolonged duration of temperature from the total number of children covered by the »morbidity« group.

Serology

For serological tests 0.3 ml of blood mixed with 0.3 ml Heparin solution (20 units in 1 ml) was taken. The first blood samples were taken on the day of vaccination and the second ones three to four weeks later. Sera were inactivated for 30 minutes at 56⁰C.

The sera were titrated by the haemagglutination-inhibition test (Kempe-Lennette 1964) using the micromethod* (Sever 1962). Double serial dilutions of sera with initial dilution of 1 : 8 were made. The test was performed with the antigen prepared from the virus inoculum of one lot of vaccine in HeLa cells.* In the test 2 haemagglutination units of the antigen were used. After one hour incubation at 37⁰C a suspension of 0.5% chick erythrocites was added.

After 1 hour at room temperature the test was read. Each test included the controls of sera, erythrocites and units of antigen and one positive and one negative serum. All dilutions were performed in Veronal buffer (DGV) pH 7.2.

At least a fourfold rise in titre of heminhibiting antibodies in primary vaccination and at least a twofold titre rise in revaccination was considered as a positive result.

* Microtitrator – American Hospital Supply Corp., New York.
* The antigen was received by Dr Mozetić, Institute for Public Health, Ljubljana.

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RESULTS

Clinical examinations were carried out in 340 children primarily vaccinated with 26 of our regular vaccine lots in the period from 1964 to 1969. Total results of these examinations are presented in Table 1.

It can be seen from the table that in 326 of 340 vaccinated children, i. e. 96 per cent, the vaccination was successful. Seventy-nine of these 326, i. e. 24 per cent, in the group successfully vaccinated had a temperature lower than 37°C (no fever). The temperature between 37°C and 37.9°C (low fever) was recorded in 154 children, i. e. 47 per cent, and only 93 children or 29 per cent had the temperature higher than 37.9°C, this being the »morbidity group«. Of these 93, 9 children, or 10 per cent, had the temperature for two or more days – the »prolonged fever«. The »morbidity group« represented about one third of the total successfully vaccinated rate.

In table 2 are presented the results separately of each one of the 26 tested lots.

In table 3 we have collected all results of vaccination with vaccine lots of high titres (A) and compared them, according to the same criteria, with the results obtained with vaccine lots of low titres (B). Under column A are the results of vaccination of children with 6 vaccine lots with the titre ranging from $10^{8.00}$ to $10^{8.45}$ PFU/ml; in column B the results of vaccination of children with 8 lots of vaccine with the titre ranging from $10^{6.80}$ to $10^{7.40}$ PFU/ml.

As can be seen from the table the only difference can be found in the »no fever« rate, which is 12 per cent in Group A and 27 per cent in group B, while the »low fever« rate is 54 per cent in group A and 42 per cent in group B. It follows that there is no difference either in the successful vaccination rate or in the morbidity rate between the lots with high and with low titres respectively. Here too the morbidity group represented one third of the total successful vaccination rate.

Table 4 shows the same indexes in one single lot with the titre of $10^{8.00}$ PFU/ml, which by its titre should belong to group A (according to Table 3) but which by its »morbidity rate« of 45 per cent differs from the average and amounts to nearly half of the total number of the successful vaccination rate.

Revaccination was carried out in children 6 to 7 years old. This was the first revaccination and it was performed 3 to 6 years after primary vaccination. We used a lot of vaccine with the titre $10^{8.00}$ PFU/ml. As

seen from table 5, in 32 of 98 children, i. e. 32.7 per cent, vaccination was successful. Of this number 8 children, or 8.2 per cent, reacted with pustular lesions and 24 or 24.5 per cent with vesicular lesions. In the »minor reaction« group there were 66 children or 67.3 per cent. The »morbidity rate« here was 6 per cent. There were two children with the temperature above 37.9°C and with pustular lesions.

The titration of sera was performed by using the haemagglutination inhibition test. Twenty-nine successfully vaccinated children (primary vaccination) were covered. In 21 or 72.4 per cent of children we had a four-fold or higher rise of titre of heminhibiting antibodies, in six children or 20.7 per cent a two-fold rise, and only in two children or 6.9 per cent there was no rise of titre.

We have also examined 21 paired sera taken from the successfully revaccinated children (from the group of 98 revaccinated children, the remaining data on which are being presented in table 5). Conversion of heminhibiting antibodies was observed in 20 children, i. e. 95.2 per cent. Only in one child with vesicular lesion there was no conversion of antibodies.

DISCUSSION

The aim of our study was the characterization of the Berne-Zagreb strain. We have based our results on clinical reactions which are the most significant, although often varied. Of the clinical criteria we have set apart particularly the »morbidity rate«. This was very low in the vaccination of 340 children with our 26 series of vaccines, and amounted to 29%, and was considerably lower than that for the Berne strain in the tests of Polak and collaborators (1963). However, in comparison with the vaccination results of only one separate vaccine lot (table 4) the resulting 45% are almost equal to those of Polak. This lot belongs to the high titre group.

To return to table 2, we shall see that the lot 5 with a titre of $10^{8.00}$ also has a very high morbidity rate – 50%. This could lead to the conclusion that a high morbidity rate is accompanied by a high titre. But, looking at the lot 10 with a titre of $10^{8.45}$, we can see that the morbidity rate is only 30%. Likewise, the highest morbidity rate of 55% can be found in lot 11 with a titre of $10^{7.20}$ PFU/ml. This is one more proof that the morbidity rate does not depend upon the height of titre in an individual lot.

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Individual lots of the vaccine differ in the »morbidity rate« which varies from 10–55⁰/o. It follows that the properties of a strain cannot be evaluated by the results of one lot exclusively, since there must be also other factors responsible for the differences in the degree of clinical reactions within a group of successfully vaccinated children.

These other factors could be the differences in the work of individual vaccinators, the age of the children, their nutritional state, the epidemiological circumstances at the time of vaccination (Mravunac, B. personal communication) and others.

In revaccination, the reactivity in spite of a high titre lot of vaccine was rather low. We think that the reason for this is that a high percentage of the children had been primovaccinated only 3 to 4 years before. A repeated revaccination of children with a negative reaction was not done.

As we can see, the Berne-Zagreb strain gives mild clinical reactions, and postvaccinal complications, such as encephalitis, are rare· We think that these mild reactions can be ascribed to the history of our strain which has not changed its host for more than 30 years.

Table 1

Reactivity of 26 lots of vaccine prepared from Bern–Zagreb Strain in primary vaccination of 340 children

	⁰/o	
Successful vaccination[1] rate	96	(326/340)
No fever[2] rate	24	(79/326)
Low fever[3] rate	47	(154/326)
Morbidity[4] rate	29	(93/326)
High fever[4a] rate	10	(9/93)
Prolonged fever[4b] rate	26	(24/93)

1 Minimum one pock
2 Temperature lower than 37.0⁰ C
3 Temperature up to 37.9⁰ C
4 Temperature higher than 37.9⁰ C
4a Temperature higher than 38.9⁰ C
4b Fever lasting 2 days and longer

Table 2

Reactivity of each of 26 lots of vaccine prepared from Bern–Zagreb strain in primary vaccination of 340 children

Lot	Virus titre PFU/ml	Successful vaccination rate %	No fever rate %	Low fever rate %	Morbidity rate %	High fever rate %	Prolonger fever rate %
1	—	100 (10/10)	50 (5)	40 (4)	10 (1)	00 (0)	00 (0)
2	—	100 (17/17)	29 (5)	53 (9)	18 (3)	00 (0)	33 (1)
3	—	100 (10/10)	00 (0)	60 (6)	40 (4)	00 (0)	25 (1)
17	$10^{6.80}$	90 (9/10)	00 (0)	67 (6)	33 (3)	00 (0)	00 (0)
23	$10^{7.10}$	100 (10/10)	20 (2)	40 (4)	40 (4)	00 (0)	25 (1)
18	$10^{7.10}$	100 (10/10)	30 (3)	40 (4)	30 (3)	00 (0)	00 (0)
11	$10^{7.20}$	92 (11/12)	18 (2)	27 (3)	55 (6)	33 (1)	00 (0)
22	$10^{7.30}$	86 (12/14)	25 (3)	50 (6)	25 (3)	00 (0)	33 (1)
24	$10^{7.39}$	92 (11/12)	00 (0)	64 (7)	36 (4)	17 (1)	00 (0)
25	$10^{7.40}$	100 (30/30)	50 (15)	30 (9)	20 (6)	25 (1)	17 (1)
28	$10^{7.40}$	100 (14/14)	29 (4)	42 (6)	29 (4)	25 (1)	50 (2)
15	$10^{7.50}$	87 (13/15)	46 (6)	23 (3)	31 (4)	00 (0)	25 (1)
20	$10^{7.50}$	100 (18/18)	39 (7)	39 (7)	22 (4)	50 (1)	50 (2)
8	$10^{7.60}$	100 (10/10)	30 (3)	50 (5)	20 (2)	50 (1)	100 (2)
4	$10^{7.65}$	100 (10/10)	00 (0)	80 (8)	20 (2)	00 (0)	100 (2)
6	$10^{7.65}$	91 (10/11)	20 (2)	50 (5)	30 (3)	00 (0)	33 (1)
7	$10^{7.70}$	100 (13/13)	31 (4)	46 (6)	23 (3)	00 (0)	83 (1)
19	$10^{7.70}$	88 (14/16)	21 (3)	50 (7)	29 (4)	20 (1)	50 (2)
21	$10^{7.80}$	94 (16/17)	25 (4)	44 (7)	31 (5)	00 (0)	40 (2)
12	$10^{7.90}$	100 (10/10)	30 (3)	50 (5)	20 (2)	20 (1)	00 (0)
5	$10^{8.00}$	91 (10/11)	00 (0)	50 (5)	50 (5)	00 (0)	20 (1)
13	$10^{6.10}$	100 (10/10)	20 (2)	50 (5)	30 (3)	25 (1)	33 (1)
14	$10^{6.10}$	88 (14/16)	14 (2)	57 (8)	29 (4)	00 (0)	25 (1)
26	$10^{6.10}$	100 (14/14)	07 (1)	72 (10)	21 (3)	00 (0)	00 (0)
27	$10^{8.30}$	100 (10/10)	30 (3)	20 (2)	50 (5)	00 (0)	00 (0)
10	$10^{6.45}$	100 (10/10)	00 (0)	70 (7)	30 (3)	00 (0)	33 (1)
Total		96 (326/340)	24 (79)	47 (154)	29 (93)	10 (9)	26 (24)

Table 3

Comparison between the reactivity of vaccine lots with highest titres (A) and those with lowest titres (B) in primary vaccination of 183 children

	A %/0		B %/0	
Successful vaccination rate	96	(68/71)	96	(107/112)
No fever rate	12	(8/68)	27	(29/107)
Low fever rate	54	(37/68)	42	(45/107)
Morbidity rate	34	(23/68)	31	(33/107)
High fever rate	09	(2/23)	09	(3/33)
Prolonged fever rate	17	(4/23)	15	(5/33)

A = 6 vaccine lots (5, 10, 13, 14, 26, 27) with titres ranging from $10^{8.00}$ to $10.^{8.45}$ PFU/ml

B = 8 vaccine lots (11, 17, 18, 22, 23, 24, 25, 28) with titres ranging from $10^{6.80}$ to $10^{7.40}$ PFU/ml

Table 4

Reactivity of one lot of vaccine prepared from Bern–Zagreb strain in primary vaccination of 183 children

	%/0	
Successful vaccination rate	97	(178/183)
No fever rate	42	(75/178)
Low fever rate	13	(23/178)
Morbidity rate	45	(80/178)
High fever rate	44	(35/80)
Prolonged fever rate	03	(2/80)

Table 5

Reactivity of one lot of vaccine prepared from Bern–Zagreb Strain in revaccination of 98 children

	%/0	
Successful vaccination rate	33	(32/98)
No fever rate	85	(27/32)
Low fever rate	09	(3/32)
Morbidity rate	06	(2/32)
High fever rate	00	(0/2)
Prolonged fever rate	—	

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SUMMARY

On the basis of clinical reactions observed in 340 children primarily vaccinated and 98 children revaccinated with 26 regular lots of smallpox vaccine the Berne-Zagreb strain was characterized. Particular attention was paid to morbidity rate. It appeared that there was no difference either in the successful vaccination rate or in the morbidity rate between the lots with high and with low titres respectively. As a rule clinical reactions were mild and complications, especially postvaccinal encephalitis, were very rare. It is considered that this is due to the history of our vaccinia virus strain which never changed its host for over 30 years.

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SIMULTANEOUS ADMINISTRATION OF SMALLPOX AND DIPHTHERIA, TETANUS AND PERTUSSIS VACCINE

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From 1948 to the end of 1958 in the whole of the country the smallpox vaccine was administered simultaneously with the DT vaccine, both in primary vaccination and the revaccination of children. Only from 1959 when the DTP vaccine had been introduced was the administration of these two vaccines separated and an interval of 4–6 weeks was introduced between the administration of the smallpox vaccine and the DTP vaccine.

The mutual effect of antigens – the positive and the negative one – in combined vaccines has been extensively tested in laboratory and field trials (Barr 1960; Barr, Glenny and Butler 1955; Dick and Horgan 1952; Ikić 1956; Lin 1965; Mayer and all. 1967; Weibel and all. 1966).

Taking into consideration the advantages of simultaneous administration of smallpox vaccine and combined DTP vaccine it emerged as a matter of particular interest to ascertain the mutual effect of these antigens with respect to the antigenic effect and to find out whether it causes a higher incidence of reactions and complications, especially postvaccinal encephalitis. The mutual effect of antigens was studied in the laboratory, and the effect of simultaneous administration of smallpox vaccine and DT vaccine on the incidence of postvaccinal encephalitis was studied in the field.

MATERIAL AND METHODS

Laboratory studies

In a series of eight tests DTP + vaccinia prophylactic was compared with DTP prophylactic (produced at the Institute of Immunology, Zagreb).

1 cc of prophylactic: 40 Lf Di
                      8 BU Te
                 20×10^9 Per
                 0.04 cc Vaccinia virus
                 10 mg $ALPO_4$
                 1/10,000 merthiolate.

As reference served the same batch of DTP adsorbed without vaccinia.

The antigenicity of the pertussis component was tested in the active mouse-protection test (Kindrick et al. 1947).

In each test a group of ten mice were vaccinated with three doses of each vaccine.

Doses: 10^9 bacteria 0.5 cc
               2×10^8 bacteria 0.5 cc
               4×10^7 bacteria 0.5 cc

B. pertussis strain 214–E Glaxo was used as the challenge strain. The infective dose contained 200 LD_{50} doses on an average.

The influence of vaccinia virus on the immunogenicity of the tetanus and diphteria antigens was tested with the same prophylactics on guinea-pigs by the one-stimulans method. The method has been described in detail elsewhere (Higy–Mandić 1965).

Field studies

The influence of simultaneous application of DT vaccine and the vaccine against smallpox on the frequency of postvaccinal encephalitis (PE) after primary vaccination, was studied in patients with a clinical picture corresponding to PE, who had been treated between 1948 and 1969 at the Fever Hospital of Zagreb and the Department for Infectious Diseases of the General Hospital Šibenik. Only those patients were taken into consideration who had been vaccinated in territories which administratively belong to the hospitals mentioned. The patients were divided into two groups.

The first group (1948–1958) included those patients who had been primarily vaccinated simultaneously with the DT vaccine and the smallpox vaccine and the second group (1959–1969) patients who, on the occasion of their primary vaccination, had received separately the DTP vaccine and the smallpox vaccine with an interval of 4 to 6 weeks.

RESULTS OF LABORATORY STUDIES

The influence of vaccinia virus on the immunogenic potency of pertussis antigen

As shown in table 1, the DTP adsorbate afforded the strongest protection at the largest dose (1.10^9) and at the lowest dose (4.10^7), whereas at the middle dose (2.10^8) stronger protection was conferred by the DTP

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adsorbate + smallpox. The statistical analysis shows no difference in protection (MD: 0.4 P 0.5) between those two prophylactics, i. e. DTP adsorbed and the same prophylactic with addition of smallpox vaccine.

The influence of vaccinia virus on the immunogenic potency of tetanus antigen

In Table 2 is shown the influence of vaccinia virus on the level of antitetanus immunity in guinea-pigs vaccinated with DTP adsorbed prophylactic and with the same prophylactic with addition of vaccinia virus. The assays were performed by the one-stimulans method. The geometric mean of antitoxic units in the sera of guinea-pigs by the one-stimulans method amounts to 2.28 and 0.61 (DTP adsorbate and DTP adsorbate + vaccinia virus) respectively. The statistical analysis shows the difference in the level of the antitoxic titre attained by this method to be significant (0.01 P 0.02).

The influence of vaccinia virus on the immunogenic potency of diphtheria antigen

With the intention of ascertaining whether an addition of vaccinia virus would influence the immunogenicity of the diphtheria component in combined prophylactic, investigations were planned by the same design as were the investigations of the influence of vaccinia virus on the immunogenicity of the tetanus component.

In Table 3 is shown the influence of vaccinia virus on the level of antidiphtheria immunity determined by the one-stimulus method.

By the one-stimulus method the geometric mean amounts to 3.388 A.U. and 1.445 A.U. (DTP adsorbate: DTP adsorbate + variola) respectively. The difference between the antidiphtheria levels attained by both prophylactics is statistically highly significant (P 0.001).

RESULTS OF FIELD STUDIES

Table 4 shows that in the group vaccinated simultaneously with DT and smallpox vaccines 10 patients acquired PE and 3 died, while in the group vaccinated only with smallpox vaccine 3 fell ill and 2 died.

By age, the first group had 6 patients up to 4 years old, and 4 older than four years. In the second group all the patients were less than 4 years old as from 1959 onwards primary vaccination after reaching the age of 3 was no longer permitted.

Since in the territories sending patients to the above hospitals the vaccination was well carried out, it can be taken that annually 80% of the total number due for vaccination undergo primary vaccination. Our

Institute being the only manufacturer of smallpox vaccine in Yugo-slavia, this vaccine is the only one which is used in the whole of the country. Between 1945 and 1969 30 million doses of vaccine against smallpox were distributed. For the S. R. of Croatia the number of doses is 7,500.000 of which one third has been used for primary vaccination which makes an average of 80,000 doses per year.

This enables us to calculate that in the first group in a period of 11 years (1948–1958) there was one case of PE to 88,000 doses, and in the second group in the same period (1959–1969) one case of PE to 290,000 doses of smallpox vaccine. In the first group there was one death to 290,000 doses of smallpox vaccine, and in the second group one death to 440,000 doses.

DISCUSSION

On the basis of laboratory data the addition of vaccinia virus to DTP prophylactic suppresses immunity to both tetanus and diphtheria, whereas the negative influence of vaccinia is the least expressed in the case of pertussis immunity.

In view of the fact that vaccination of children with DT prophylactic is twofold and with DTP threefold and vaccinia is given simultaneously only with the first dose, the initial suppression of immunity to the other components caused by vaccinia is compensated in the vaccinated orga-nism after the last dose when the expected immunological effect of all components is achieved.

In the first years after the introduction of the DT vaccine – in 1948 – owing to shortage of staff, it was permitted to use the DT vaccine and smallpox vaccine simultaneously. In 1958 this was changed by a decree which stipulated that each of these vaccines was to be applied separa-tely with an interval of six weeks. This separate vaccination was started in 1959 when the DTP vaccine was introduced.

An analysis of the ratio of PE to a certain quantity of vaccine doses is always difficult. It is in this light that we have to observe and accept the formerly mentioned data. We are not enthusiastic about the simul-taneous application of smallpox vaccine and other vaccines as long as the vaccinal strains from which the vaccine is prepared maintain their present virulence. We had therefore separated smallpox vaccination from other vaccinations as soon as our medical service was able to put this suggestion into effect. The simultaneous application of smallpox vaccine and other vaccines has both many advantages and many pro-blems. The medical services of individual regions have to consider both advantages and problems and make a decision bearing in mind their own specific circumstances.

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Table 1

	Summarized results of a series of 8 tests				*Statistical analysis*	
Vaccine Dose in germs	DTP adsorbed		DTP adsorbed + vaccinia		DTP adsorbate	DTP adsorbate + vaccinia
	D/T*	%	D/T	%		
1.10^9	3/71	4	5/78	6	MD	0.4 P 0.5
2.10^8	10/76	13	4/74	5	MR	P 0.001
4.10^7	12/78	15	30/79	38	MP	0.05 P 0.1

* Deaths/Total number of immunized mice.

Table 2

Level of tetanus antitoxin (AU cc) in the sera of guinea-pigs after single immunization
(one-stimulans method)

Sera classified according to antitoxic contents (AU cc)	DTP adsorbate		DTP ads. + vaccinia	
	No of sera	%	No of sera	%
<0.01	—	—	—	—
0.01—0.1	—	—	2	12.5
0.1 —1.0	5	29.4	8	50.0
1.0 —10.0	11	64.7	6	37.5
>10.0	1	5.9	—	—
Total number of sera	17		16	
log mean	0.358		—0.213	
S. D.	2.736		3.632	
geometric mean	2.280		0.613	
t		2.559		
Difference		$0.01 < P < 0.02$		

Table 3

Level of diphtheria antitoxin in the sera of guinea-pigs after single immunization (one-stimulans method)

Sera classified according to antitoxic contents (AU cc)	DTP adsorbed		DTP ads. + variola	
	No of sera	%	No of sera	%
<0.01	—	—	—	—
0.01—0.1	—	—	—	—
0.1 —1.0	1	5.0	7	35.0
1.0 —10.0	19	95.0	13	65.0
>10.0	—	—	—	—
Total number of sera	20		20	
log mean	0.530		0.160	
S. D.	1.225		1.866	
geometric mean	3.388		1.445	
t	4.353			
Difference	P < 0.001			

Table 4

Comparison of the frequency of pe-like CNS disorders in simultaneous and separate application of samllpox vaccine and DT vaccine

Period of vaccination	PE ofter primary vaccination	
	Affected	Died
Simultaneous application of DTP and smallpox vaccine 1948—1958	10 (4)	(3) 3
Separate application of DTP and smallpox vaccine 1959—1969	3	2

( ) = older than four

SUMMARY

The work reports on the influence of smallpox vaccine on the immunogenicity of diphtheria, tetanus and pertussis antigens when these four components were given and tested simultaneously by the corresponding laboratory tests in experimental animals. In AMPT (the active mouse protection test) it was not possible to observe any kind of influence of vaccinia on the pertussis antigen. In guinea-pigs (one stimulus method) it could be concluded that vaccinia exerted an antagonistic effect on both diphtheria and tetanus antigens. In the field studies which have lasted for twenty-one years one persons of those who had received simultaneously DT + smallpox vaccine developed a fatal PE in a period of eleven years. In the next period of ten years also one fatality was recorded among those who received smallpox vaccine separately from the DTP. The implications of these findings are discussed.

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PROGRESS IN ANIMAL-LYMPH
VACCINE PRODUCTION

RECENT ADVANCES IN PRODUCTION OF SMALLPOX VACCINE IN CZECHOSLOVAKIA

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This will be only a short information on recent progress in the production of smallpox vaccine in Czechoslovakia. Most of the work has been done by Dr. Slonim and his colleagues; this document was compiled during his stay abroad.

The first step in production consists in obtaining virus-rich material from infected calves. The main factors are the technique of the procedure, the using of seed virus of stable properties, optimal titre of inoculated virus, the sanitary regime during the all procedure and the timing of harvest. Yields such as 10^{10} PFU/g with less than 20000 nonpathogenic bacteria can be obtained under appropriate conditions (Slonim et all. 1968d).

In the second step, i. e. processing the material, we wish to stress the importance of maintaining the temperature of the virus suspension as low as possible, the low speed centrifugation for removing balast materials, the time of exposure and the concentration of phenol for selective inactivation of bacteria (Slonim et al. 1968a, c) and finally the high speed centrifugation for concentration and partial purification of the virus suspension.

As an alternative method the production of smallpox vaccine in chick embryos has been also developed (Slonim et al. 1967c).

In liquid vaccine production it is necessary to control the properties of glycerine because it was shown that some fatty acids which might be present inactivate the virus in direct proportion to their concentration and to the temperature (Slonim et al. 1966b, Slonim 1968).

Besides quantitation of the routine estimation of CAM–PFU (Slonim et al. 1967b, 1968b) also certain conditions for the titration of vaccinia virus in chick fibroblasts culture were investigated (Slonim et al. 1966a).

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The control of our vaccine corresponds to WHO Requirements (1966) with a few exceptions. Our regulations (PNY 30–102/67, ON 86 3610) estimate the content of bacteria to be less than 100/ml, but most of the batches of our smallpox vaccine in last years were bacteriologically sterile. We enclose also some control tests which are not obligatory according to the WHO Requirements: test for absence of M. tuberculosis, the control of pH and of the content of phenol and proteins in the vaccine, estimation of pH of the diluent for freeze-dried vaccine.

The content of virus in the vaccine required in Czechoslovakia is more than 5.10^7 PFU/ml. This is based on long previous experience that with our vaccinal strain and in our conditions of application the vaccine this number of PFU is sufficient for evoking positive responses in 100% or almost 100% of primovaccinated and in a relatively high percentage of revaccinated persons. The ten-fold diluted vaccine gives still close to 100% takes. Nevertheless, routinely produced batches of our smallpox vaccine contain usually $1–3.10^8$ PFU (or the desired quantity up to 10^9 PFU) and can therefore be used under conditions of WHO Requirements.

The vaccine is distributed in three different forms which all have been thoroughly clinically controlled and investigated (Kratochvílová et al., to be published).

1. One-dose liquid vaccine in special polyvinylchlorid tubes filled and sealed in a semiautomatic apparatus constructed in our institute.

2. Classical glass capillaries with 10 doses of liquid vaccine.

3. Freeze-dried highly stabile vaccine sealed in vacuum or in an innert gas, containing in the ampoule 20 or 50 doses; in the near future also the 5 or 10 dose freeze-dried vaccine will be introduced. The decrease in titre after 4 weeks at 37^0 is usually only 0,3 log or less and the stored vaccine is stable so far for many years (Slonim et al. 1967a, 1968e).

Much work has been done on biological and pathogenic characteristics of the vaccinal strain ÚSOL–V used in Czechoslovakia. It was compared with the Lister Institute and the USSR strain in respect to plaque formation under various conditions, to the influence of temperature on reproduction dynamics and lethal properties in chick embryo and in different tissue cultures and by using some other markers (Slonim et al.: a series of papers in press). No significant difference of the three strains studied was found. Our strain can be therefore considered as strain with good and satisfactory properties. The finding of laboratory correlates to the behaviour and properties of various smallpox vaccines in men represents however still an open problem.

In conclusion, I believe that we have succeeded to reach a good standard level in smallpox vaccine production and also to contribute to the research of vaccinia virus and smallpox vaccine in general.

SUMMARY

A short information is given on progress in research and in routine production and control of smallpox vaccine in Czechoslovakia.

Standard methods developed enable to produce a highly effective and practically sterile vaccine in various application forms.

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BRIEF HISTORY OF EPIDEMIOLOGY AND CONTROL OF SMALLPOX IN TURKEY AND RECENT DEVELOPMENTS IN VACCINE PRODUCTION

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EPIDEMIOLOGY OF SMALLPOX IN TURKEY

Smallpox is not an endemic disease in Turkey. It has been eradicated since 1952, and last outbreak which occurred in 1957 and caused by an imported case from one of the south-eastern neighbouring countries, limited to 128 cases with 7 deaths.

As will be seen from Table 1., the total number of smallpox cases in Turkey from 1938 to 1957, was 24,012 and fatality ratio 11.6%. For the 10-years period before 1938, these figures were 3,573 and 32.9%, respectively.

As smallpox persisted in other countries in the Region, there had been repeated introductions resulting in localized outbreaks in border areas.

Vaccination against smallpox has become compulsory in Turkey since the 19th century and immunity level of the population has got higher year by year. This may be the possible cause of the decrease in the fatality ratio for the years 1938–1957, as compared with 32.9% for 1928–1937 period.

Vaccination programmes have been quite beneficial in taking the endemic disease under control from time to time in the past; but the reasons why smallpox could not be eradicated in this country earlier than 1952 in spite of extensive vaccinations possibly were: a) the vaccines not kept under proper conditions, b) shortage of trained vaccinators, and c) the traffic on the vast boundaries with the southern and south-eastern neighbours of Turkey; the cause of the 1957 outbreak was also imported cases from one of the southern neighbours of Turkey and the outbreak limited to 3 towns in this region.

Table 1
Number of Reported Cases of Smallpox and Case Fatality Rations in Turkey,
1938–1957[a]

Years	No. of cases	No. of deaths	Percent fatal
1938	641	168	26.2
1939	438	81	18.5
1940	958	130	13.6
1941	898	113	12.7
1942	1 871	174	9.3
1943	12 395	1 380	11.1
1944	6 093	678	11.1
1945	309	34	11.0
1946	8	1	12.5
1947	2	—	—
1948	39	7	17.9
1949	73	14	19.2
1950	7	—	—
1951	152	3	2.0
1952–1956	—	—	—
1957	128	7	5.5
Total	24 012	2 790	11.6

CONTROL OF SMALLPOX IN TURKEY

Turkey is the first country on the western hemisphere which used an immunizing method against a communicable disease, the smallpox. The history of smallpox vaccination in Turkey goes back to the 17th century (Ünver, 1948). Though in some old Turkish books it was stated that the smallpox vaccine produced from cowpox and applied by the Jennerian method existed in Anatolia as early as 1679, we do not have any written paper about this practice. However, there are enough data for that variolation carried out in Turkey was first described by the great physician Emmanuel Timonius in 1713. Four years after Timonius, Lady Mary Wortley Montague, the wife of the British Ambassador to Turkey, intraduced the method of variolation to the western countries.

Three years after the publication of Jenner's work, Dr. Mustafa Behçet was the first who wrote about vaccination (1801). First experiments on calf vaccine were done in 1811. In the 19th century, as mentioned above, vaccination has become compulsory and took place in our health organizations and regulations.

Until 1890–91, the vaccination had been carried out with the material imported from foreign countries. When the material was not enough at any time, the vaccinators used to take pus from the local reaction of vaccinated children and apply it to the others for vaccination.

In 1890–91, an Inoculation House was established in Istanbul and the vaccine prepared in calf skin was sent to all parts of the country. Until

1923, the material used as seed virus had been imported from the Pasteur Institute, Paris. Since then, the vaccinia virus has been passed through donkey after every second passage in calf, and the vaccine lymph obtained from second calf passage used for vaccine production.

In 1934, the Smallpox Vaccine Production Laboratory was transferred to the Refik Saydam Central Institute of Hygiene, Ankara, founded by the Ministry of Health and Social Assistance. This Laboratory prepares 5 to 10 million doses of glycerinated smallpox vaccine a year, according to the need of the country, and distribute them in refrigerated containers to the Health Centres of every province in Turkey (Table 2). The Laboratory has started the production of freeze-dried smallpox vaccine since 1965. The dried vaccine is sent to the warmer regions of Turkey in summer months.

Besides as a compulsory immunizing method, smallpox vaccination can be counted as a tradition in Turkey and therefore people are mostly cooperative. Parents of babies usually take their children of 6 months to 1 year old, to a vaccination station, preferably during the spring months, probably the main reason being that they believe that the time when trees blooming is the most suitable one for smallpox vaccination, as this disease is called »çiçek« in Turkish and means »flower«. Civilians are vaccinated in Health Centres and dispansaries in cities; villagers and their children are vaccinated by the mobile teams of sanitarians, in their village.

Table 2

The Glycerinated and Freeze-dried Smallpox Vaccine Doses Produced and Distributed, 1964–1969 (by July)

Year	Glycerinated vaccine (dos.)		Freeze-dried vaccine (dos.)	
	Produced	Distributed	Produced	Distributed
1964	4 543 750	4 602 140	—	—
1965	9 013 450	9 007 300	136 600	3 750
1966	6 492 350	6 580 420	680 750	36 675
1967	13 757 900	12 992 520	393 400	768 425
1968	4 420 250	5 273 320	472 650	138 600
1969 by July	1 964 050	2 364 770	280 200	166 750

In Turkey, a vaccination certificate is necessary at school entry, for military service, before entering a business and going abroad. Besides, everyone has to be vaccinated when a mass vaccination programme, connected with the smallpox cases in the neighbouring countries, is being carried out. The visitors coming from the countries where smallpox is endemic, should have a valid vaccination certificate. All the ports to Turkey have facilities for the isolation and quarantine of suspected cases and vaccination of contacts.

Table 3. may give an idea for the numbers of smallpox vaccinations carried out in Turkey during the last 9 years. According to the figures in the Tables 2. and 3., it seems that about only half of the vaccine doses distributed have been used in the field and/or the amount of vaccine dose used per single vaccination may have been more than estimated and recommended by the production laboratory, for example against the 39 569 900 distributed doses of vaccine, only 19 223 353 persons were vaccinated in the years 1964–1968.

PRODUCTION OF SMALLPOX VACCINE

Smallpox vaccine used in Turkey is prepared at the Refik Saydam Central Institute of Hygiene, Ankara, from virus grown in the skins of calves. The female calves are examined and tuberculin-tested by a veterinarian and kept in quarantine for at least two weeks before use. After clipping and washing, animal is secured on its left side on the table special for the purpose; the whole flank and abdomen are shaved, washed thoroughly and then scarified with a sterile instrument. The seed virus is uniformly spread over the scarified area, and after drying

Table 3

Number of the Smallpox Vaccinations in Turkey, 1960–1968[b]

Year	Number of vaccinated
1960	4 202 633
1961	3 329 268
1962	11 966 555
1963	2 729 109
1964	2 046 609
1965	3 245 730
1966	2 693 983
1967	8 521 380
1968	2 715 649

up of the seed, vaccinated side of the animal is covered with a sterile compress; then calves are returned to their special pens where suspending belts prevent their lying down on the dirt. Their temperature are recorded and compresses are changed with sterile ones daily during four-day incubation period. On the 4th day of vaccination the lymph is harvested after vaccinated area scrupulously cleaned and animal sloughtered. The collected pulp is transferred to a sterile jar previously labelled and weighed. After recording the weight of the pulp on the label the jar is stored at $-15^0- -20^0C$ until it is required for vaccine production.

Since the beginning of 1961, the technique used in the production of smallpox vaccine has been changed basing on the methods of the Lister Institute, England. The differences are mainly in the preparation and number of animals inoculated weekly, the technique used for homogenizing the pulp, the kind of suspending solution, antibacterial agent used, incubation period for eliminating bacteria, bacteriological controls and titration of vaccine(Table 4.).

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Table 4

Recent Changes and Developments in Smallpox Vaccine Production and Distribution in Turkey

Material and method used	Before 1961[c]	Since 1961[d]
Cleaning of animals before vaccination and harvest	With soap and hot water	With soap, soft soap, ether soap and water during definite periods of time
Animal in incubation period	Tied only from neck	Suspensors used for preventing the animal from lying down on its dirt
Emulsifyng the pulp	By a grinder and then a mill for two hours, container being in ice tubes	In an electric-mixer for ten minutes, container being in ice-water mixture
Suspending solution	Glycerine containing 20% distilled water	McIlvaine's sodium phosphate-citric acid buffer (pH 7.2). containing 0.4–1% phenol
Time of the glycerine addition	Partly during homogenizing, partly after	After homogenizing and bacteriological control
Antibacterial agent	Glycerine	Phenol and glycerine
Incubation period for eliminating bacteria	6 months at low temperature and, if necessary, few days at room temperature	15–24 hours at 22^0–24^0 C.
Bacterial content	Less than 1000/ml.	Less than 500/ml.
Bacteriological control	In each batch	In every single harvest, then repeated in the batch
Titration method and acceptable titre of vaccine	By intradermal rabbit test; 0.1 ml. of 1/1000 dilution of vaccine should produce a distinctive papule within 3 days	By pock counting on the chorioallantois of the chick embryo;[e, f, g] it should contain vaccinia virus not less than 10^8 PFU/ml. (rabbit skin scarification test is used in the National Control Laboratory)
Distribution	In ordinary boxes and transport	In refrigerated containers (since 1965) and by the quickest mail
Expiration date (for wet vaccine)	6 months in winter and 3 months in summer	2 months

The strain of vaccinia virus in use at the Refik Saydam Central Institute of Hygiene for smallpox vaccine production was obtained from the Pasteur Institute, France, about 46 years ago. This virus strain has since been maintained by cutaneous passages on calves and, after every second calf passage, on donkey. As mentioned above, the pulp from second calf passage is used for vaccine production. The sequence of virus transfer is shown in Figure 1.

Fig. 1
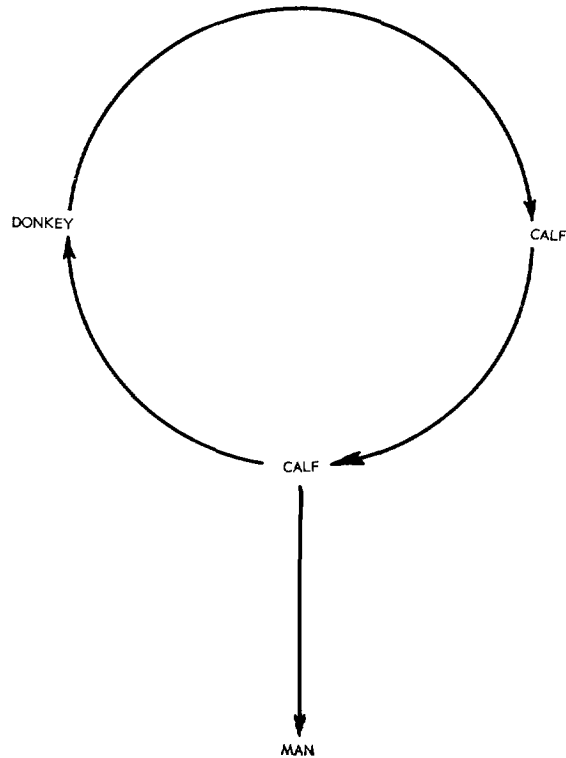
The sequence of passages of the vaccinia virus

Since 1965, we have started to prepare freeze-dried smallpox vaccine by the method used in the Lister Institute, England, and recommended by the WHO.

The laboratory and field studies we carried out helped us in improving our vaccine. In a study with the smallpox vaccine prepared by the old method, it was found out that the take rate was parallel with the titre of vaccine and in order to obtain 10% major reaction in primary vaccinations we had to prepare a vaccine of higher potency (Özlüarda et al., 1960). In another study performed for finding out the relation between the factors effecting the production of smallpox vaccine, we

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came to the conclusion that to produce a vaccine of good quality and quantity, it was necessary to use a seed of high titre of virus and low bacterial content, and, the pulp obtained from the second passage on vaccine animal was more economical than the first passage pulp, as its weight and virus content was much more (Özlüarda, 1964).

As sheep are more easily kept clean than calves and do not suffer from tuberculosis, we investigated the possibility of using sheep in small-pox vaccine production. We planned a study on five different breds of sheep available in Turkey and compared them with regard to their suitability for the purpose. Tests showed that none of the 5 sheep vac-cines was as active as calf vaccine (Özlüarda, 1967a). Later we observed that even the dried vaccines prepared in our laboratory from sheep lymph were less stable than that prepared from calf lymph.

During the mass smallpox vaccination campaign when more than 11 million persons were vaccinated, we had the opportunity of finding out the ratios of successful vaccinations and postvaccinal complications, and also the opinion of vaccinators about the vaccine in use and its packing and distributing system, by the help of questionaires sent by the Ministry of Health to all of the local health authorities. The filled questionaires gave us a rough idea about the above mentioned points. The average rate of takes among the persons of more than 25 years of age was 55.5%, in school children 66%, in pre-school children and in-fants 87%. As postvaccinal complications, the ratios for generalized vaccinia and postvaccinial encephalitis were 14 per 10,000 and 1 per 480,000 vaccinated individuals, respectively (Özlüarda et al., 1963). We thought that the term of generalized vaccinia must have been misunder-stood by the observers mostly and used for all types of transfer of in-fection on the skin and also for eczema vaccinatum.

After starting to produce freeze-dried smallpox vaccine and before distributing it to the vaccination stations for routine use, a pilot field study was arranged to compare the dried and glycerinated vaccines in the field, their stability and the relationship of their titres to frequency of vaccine take. It would be also useful to see whether any difficulty would appear in the field with this new type of smallpox vaccine pre-paration with which the vaccinators in our country were not familiar (Özlüarda, 1965). As a result, the average success rate with dried vaccines under study was found to be 97%, and with the glycerinated vaccine 95% in primary vaccinations. On the other hand, dried vaccines stored 9–10 weeks at 37°C. and with which a total of 819 persons were vacci-nated and controlled, were giving 92% success rate, while only 26% of the primary vaccinations with glycerinated vaccine kept at 37°C. for 11 days were successful. The average success rate in revaccinations with dried vaccines was 94% and with glycerinated vaccine 89%. No severe complications have been encountered in this study which was carried out on 2,469 (controlled) persons. In one case vesiculation occurred in nostrils of a girl due to the transmission of vaccine by hand, and in 4

other cases satellite pustules came out around the main reaction, due to the rubbing of the vaccination site with a piece of cotton soaked into acetone to clean the skin. With one ampoule of dried vaccine of 0.3 ml. 35–40 persons were successfully vaccinated and a single linear scratch of 5 mm. long was enough for primary vaccination. The sanitarians who carried out vaccinations and controls were the skilled staff of the BCG Campaign and they had been subjected to a two-day course on the small-pox vaccination with dried and glycerinated smallpox vaccines before the trial. We think that this training is necessary for all vaccinators in order to obtain more successful results in vaccinations and less compli-cations.

One of the first batches of our dried vaccine was controlled by the Rijk Instituut voor de Volksgezondheid, Netherlands, and favourable results were obtained.

In order to prove the efficacy of the dried and glycerinated vaccines used in the above mentioned study, and at the same time, to determine the success rate of our dried vaccines in revaccination, we challenged the successful primary vaccinations by a potent dried vaccine 1 year after the previous study, As a control for vaccine used, a small group of unvaccinated children were vaccinated primarily; this would be a test of efficacy for the batch used in the field as well. At the end of the study the bulk of the responses to challenge were of the accelerated type, indicating substantial protection, the control group vaccinated with the same dried vaccine giving 100% positive response showing »major re-action«. No complication was encountered in 422 persons vaccinated and controlled. From this study we came to the conclusion that a) the dried and glycerinated smallpox vaccines produced in our laboratory ensured satisfactory protection in successfully vaccinated persons, b) there was no difference in the degree of protection one year after vaccination between those vaccinated by the glycerinated and dried vaccines, c) our dried vaccine was highly successful in revaccinations as well (Özlüarda, 1967b). As had done the previous study, this one also confirmed that the proper application of the vaccine minimized the possibility of compli-cation. All of the field trials carried out so far showed that the vaccinia virus strain in our smallpox vaccine is satisfactory without producing severe local reactions and marked systemic disturbance and any other complication, when applied properly.

In 1965, at a Meeting on the activities of the BCG Campaign, the Ministry of Health had decided to have a pilot study performed on the simultaneous administration of the smallpox and BCG vaccines. A small-scale field trial was carried out on 1095 children (apart from the children BCG vaccinated only as a control group) in 0–6 age group in the 5 villages of Nevşehir Province in December 1965 (Özlüarda et al., 1966). The success rate in primary and revaccinations was found to be even higher in those who were vaccinated simultaneously by the smallpox and BCG vaccines than in those vaccinated by smallpox vaccine only.

It was also found out that the allergy rate after simultaneous vaccination with these two vaccines was higher than that after BCG vaccination only. No complication occurred after simultaneous BCG and smallpox vaccinations at different sites, or administration of each vaccine alone. These satisfactory results encouraged the BCG Campaign of Turkey in starting to perform smallpox vaccinations during their routine activities.

A long-term study on the stability of our freeze-dried vaccines is now being performed, in order to find out the true expiration date in our local conditions. The samples from all of the batches kept at -15^0, 4^0. 24^0 and 37^0C. are being titrated at weekly or monthly intervals. From the titrations made so far we concluded that, to ensure the stability at 37^0C. for 4 weeks (i. e. to keep a titre of at least 10^8 PFU/ml.), we have to prepare a dried vaccine of quite a high potency (almost 5×10^3 PFU/ml.), and that when kept in an ordinary refrigerator, our dried vaccine is almost as stable as in deep freeze, while glycerinated vaccine does not keep more than two months when stored in refrigerator. The results of this study will be evaluated after the laboratory tests have been completed.

SUMMARY

Smallpox is not an endemic disease in Turkey. It has been eradicated since 1952, and last outbreak occurred in 1957 caused by an imported case from one of the southern neighbours of Turkey. Vaccination has been compulsory since the 19th century in this country and is being performed in the first year of life, at school entry, before entering military service or an official business and going abroad. Mass vaccination campaigns are carried out when smallpox outbreaks occur in neighbouring countries. The smallpox vaccine is prepared at the Refik Saydam Central Institute of Hygiene from virus grown in the skins of calves, and vaccinia virus is passed through donkey after every second passage on calf. The origin of the vaccinia virus in the vaccine is the Pasteur Institute, France, from where it had been obtained about 46 years ago. The field studies carried out so far showed that smallpox vaccines prepared in this laboratory do not cause much complications if applied properly by trained vaccinators. Some alterations have been made in the production procedures and quality of the vaccine developed since 1961, according to the results obtained from the laboratory tests and field trials, and basing on the methods used in the Lister Institute, England. The smallpox vaccine production laboratory prepares 5 to 10 million doses glycerinated vaccine a year. The freeze-dried smallpox vaccine production started in 1965 and the dried vaccine produced has since been distributed to the health centres in the warmer regions of Turkey.

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PROGRESS IN CALF LYMPH VACCINE

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A report on the status of calf lymph vaccine today from the viewpoint of the individual laboratory must necessarily present a limited view of rather parochial interests in view of the dynamic state of smallpox prevention today around the world. The activities of all the laboratories are undoubtedly affected and influenced by these common factors. A few of the relationships between such events and Wyeth's program of providing improved smallpox vaccines and their delivery systems are the subject of this brief report.

Eradication Programs

The progress of lymph vaccine technology is brightly highlighted by the increasing effectiveness in many parts of the world of vaccination procedures upon large population groups. As manufacturers of the Dryvax brand of dried smallpox vaccine, which has been used extensi·vely in some of these campaigns, we have been considerably heartened by the expanding control of smallpox by the eradication programs now under way over the globe. Where these campaigns have been carried out vigorously, with good organization and adequate population coverage, and utilizing vaccines of good quality, these areas have become declassified as endemic centres of the disease. Thus, little more than eight years after the beginning of the world's first successful regional campaign by

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the Pan American Health Organization, the disease has been virtually eliminated from the Americas so that only Brazil has reported smallpox cases thus far in 1969[1], and even in that country the program has been intensified with almost 35 million vaccinations administered during the past year since the program began. Similarly, in West and Central Africa, where more than 80 million persons of a population estimated at 119 million have been vaccinated since January of 1967, only three countries out of the original group of nineteen have reported cases through the end of May of this year[2]. The 348 cases reported in 1969 represent a decrease of about 90% in comparison with the 3,500 cases in the area of the comparable period in 1968, in which year the average also declined to about half of the 1960–1967 average.

A good part of the success of these programs is due to the use of heat-stable freeze-dried vaccine with standardized potency. While the real development of this vaccine has been under way for more than twenty years, when one considers the recent expansion of use in preference to liquid vaccine whose quality deteriorates so rapidly in warm, humid climates, the conversion to the longer-lasting, lyophilized product fistr developed in 1939 may in effect be considered new. This must be the consideration of the present major endemic countries which are just now approaching a degree of self sufficiency in vaccine production to the extent that recently Dr. D. A. Henderson could announce that more than 95% of the vaccine presently in wolrd-wide use is being prepared by lyophilization[3]. Most of this supply has been propagated on animal skin of one or another species.

Development of a Standardized Production Technology

Since the freeze-dried vaccine of 45 to 50 countries is presently being produced by as many as 70 laboratories, obviously considerable variations in quality are possible, and a survey of these procedures has shown that less than half of the products conform to WHO requirements[4] for quality, potency and stability. In an effort to remedy this situation a working committee of experts in vaccine production from several countries was convened by the WHO to exchange information on their respective production techniques and other research and development experience to yield a uniform production method which could be reasonably applied to good effect by those countries still experiencing difficulty in regular manufacture. During early 1968, this committee visited and

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reviewed production in the several laboratories represented by the consultants–Conaught Laboratories, University of Toronto; Laboratory for Virus Preparations, Moscow; National Institute of Public Health, Utrecht; Wyeth Laboratories, Philadelphia; and the Department of Microbiology, University of Reading. The summary of this traveling seminar[5] is a document which provides all the information needed to establish an effective technology for freeze-dried smallpox vaccine production on animal skin.

Vaccine Delivery Systems

Lyophilized vaccines are most effectively and economically prepared in multiple-dose units for mass use. Containers generally range from 10 to 100 doses per unit for multiple puncture or pressure and scarification procedures, and the jet-gun method of administration, as favored in the WHO and West African campaigns, has used multiples of 100 to 500 doses. It has been much more difficult to prepare lyophilized vaccine in single dose units for private-patient use, where the single-dose presentation still depends upon liquid vaccine. We have attempted to fill this gap by developing a system for a single dose of dried vaccine which can also benefit from the stability advantages of the dried form. The system utilizes our bifurcated needle with a minute drop of vaccine dried between the prongs, and the assembly is housed in a tubular container which converts into an application device. Since the dried plug of active material represents a volume of the order of magnitude of 0.002 ml, the problems of quantifying dose volume, moisture content, drying times, and the like have been profound. This development has been under way for several years; however, we believe that most of the difficulties have now been eliminated and the system should be available for general use in the very near future.

The bifurcated needle above has also been used to considerable advantage in the two WHO campaign as an alternative system to the jet-gun injector. Although the latter method can produce as many as 1,000 vaccinations per hour, the fairly high cost of equipment creates a problem in those areas where it is difficult to bring together enough people at one time to make the jet-injector method of application financially acceptable. The use of the bifurcated needle successfully fills this gap by providing an inexpensive device that transfers a reproducible quantity of vaccine, and it is so designed that it can prevent excessive penetration

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of vaccinees' skin by careless vaccinators. This needle has been used to perform multiple puncture and multiple pressure vaccinations in literally millions of subjects in Pakistan and several African countries with a success record matching the jet-injector.

Vaccine Standardization and Potency Criteria

In the United States the regulatory agency of the Public Health Service is currently establishing revised standards for vaccine production from inoculated calves or chicken embryos. The preliminary publication of these criteria[6] defines the conditions of production in either system at all stages from animal facilities and substrate preparation through final containers of virus. Safety-testing of the calf product continues to exclude pathogenic organisms, and for vaccines not considered for jet injection a limit of the equivalent of 200 other viable organisms per ml is suggested.

Potency testing continues by rabbit »scarification« with the alternative of titrating the virus by the CAM »pock-count« method.[7] In either case, potency is established in comparison with a simultaneously titrated reference vaccine. For other than jet vaccine, the rabbit potency cannot be less than a ratio of 0.7 of the reference, corresponding to not less than $10^{8.0}$ PFU's per ml on the CAM when the reference yields its assigned titer of $10^{8.1}$ PFU's per ml in a simultaneous test. The jet-injected product must show by either test a titer equivalent to 0.1 ml of the reference vaccine diluted 1 : 30 for each human dose.

There is experimental evidence that vaccines meeting these potency criteria actually contain a built-in »cushion« of surplus activity. When such vaccines are used to re-vaccinate volunteers in a procedure wherein comparisons are made on a blind basis between responses in opposite arms of individual subjects, it is evident from Table 1 that through ten-fold vaccine dilutions which yield rabbit-potency ratios in the range 0.2–0.6, or corresponding CAM titers of 7.2–7.7 log 10 PFU's per ml, the human responses are indistinguishable from those to the undiluted system. It is noteworthy that the CAM titer corresponding to a take rate of 99% in primary vaccinees has a recommended minimum level of $10^{7.7}$ PFU's per ml;[8] however, the precise reproduction of CAM assays can be significantly affected by the method of titration[9] – the greater the volume of infectious material applied to the membrane, the possibly smaller the number of lesions.

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Table 1

The Effect of Dilution of Smallpox Vaccine on Clinical Reaction.
CAM and Rabbit Potency

Dilutions of Vaccine Tested	Date	No. Vaccinated	No. of Major Reactions		Vaccine Potency Tests			
			Control Arm	Test Arm	Test Vaccine		Control Vaccine	
					CAM	Rabbit	CAM	Rabbit
Undiluted vs. Undiluted	8/5/68	14	9	9	8.4	1.02	8.4	1.03
	12/9/68	21	11	9	8.2	0.86	8.2	0.87
	3/4/69	24	18	17	8.3	0.92	8.3	0.96
		59	38 (64%)	35 (59%)				
Undiluted vs. 1:2.5 Dilution	8/19/68	6	4	4	8.0	0.61	8.3	0.97
	6/2/69	21	14	13	8.1	0.46	8.4	0.84
		27	18 (66%)	17 (63%)				
Undiluted vs. 1:5 Dilution	7/14/69	49	82	28	7.7	0.46	8.3	0.82
	6/30/69	25	16	13	7.5	0.24	8.2	0.96
	2/3/69	20	14	12	7.2	0.29	8.2	1.0
	10/28/68	24	17	14	7.7	0.34	8.3	0.97
	9/16/68	19	9	10	7.7	0.48	8.4	0.85
	7/22/68	16	11	7	7.7	0.44	8.6	0.91
		153	99 (65%)	84 (55%)				
Undiluted vs. 1:10 Dilution	7/8/68	14	9	7	7.4	0.27	8.3	0.93
	9/30/68	21	14	13	7.4	0.21	8.5	0.91
		35	23 (66%)	20 (57%)				

Total Vaccinees = 274.
All individuals were young adult re-vaccinees in a penal institution.
All vaccinations by multiple pressure with bifurcated needle; performed, observed and classified by M. Z. Bierly, M. D.
All samples were coded and used blind by clinical and laboratory personnel.

SUMMARY

The superior quality of modern dried, stabilized animal-lymph vaccines has accounted in large part for the increasing efficacy of worldwide smallpox vaccination programs. A guide to production of the improved product has been placed within reach of the remaining countries where the disease is endemic. The availability of adequate supplies of vaccine and improved vaccine delivery systems, including jet-injection and use of the bifurcated vaccinating needle, offers a promise of success in the WHO's current efforts to eradicate smallpox from all the countries of the world.

Some factors of potency standardization of calf-lymph vaccine are discussed; and the relationship between potency titration criteria and vaccine effectiveness in terms of ability to elicit »major« reactions in re-vaccination procedure is evaluated.

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TISSUE CULTURE-GROWN
SMALLPOX VACCINE

THE STABILITY OF FREEZE-DRIED VACCINIA VIRUS PREPARATIONS DERIVED FROM CELL CULTURES AND ANIMAL SKIN

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INTRODUCTION

For many years smallpox vaccine has been successfully prepared with vaccinia virus propagated in the skin of animals. The almost invariable contamination of the vaccine with non-pathogenic bacteria was not a cause for concern. The need for a bacteria-free smallpox vaccine be-- came clear with the advent of the jet injector and the success and obvious importance of jet injection as a technique of vaccination in the smallpox eradication scheme of the World Health Organisation. The virtual impossibility of consistently obtaining bacteriologically sterile vaccinia virus suspensions from virus grown in sheep skin encouraged us to consider the feasibility of tissue culture as an alternative substrate for virus growth. In our experience, the tissue culture vaccines were less stable than vaccines prepared from sheep skin. The following work attempts to elucidate some of the factors influencing the stability of vaccinia virus suspensions derived from cell cultures, with a view to developing a smallpox vaccine for jet injection that fulfils the World Health Organisation's recommendation that »Vaccine to be used for jet injection must be as stable as conventional dried vaccine«.[1]

MATERIALS AND METHODS

Virus

The Lister Institute strain of vaccinia virus was used in all experiments. Seed virus for inoculation of W. I. 38 and chick embryo cell cultures was prepared from the first passage of a production batch of freeze-dried smallpox vaccine in the chorioallantoic membranes of leucosis-free hens eggs.

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Cell cultures and virus propagation

W. I. 38 cells were obtained from Dr. Perkins, Director of the Division of Immunological Products Control of the National Institute for Medical Research, London. The cells were grown in Eagle's Basal Medium (B. M. E.) + 10% calf serum in 4 oz prescription bottles. At the 27th passage level 3 days after splitting, the growth medium was replaced with B. M. E. without serum and the cultures were inoculated with vaccinia virus at a multiplicity of infection of 5. After 4 days incubation at 37^0 the residual monolayers were removed from the glass into the maintenance medium by shaking with glass beads. The cell suspensions were treated with ultrasonic waves at 18–22 Hz at 0^0 and stored at -70^0.

Chick embryo cells, obtained by tryptic digestion of decapitated eviscerated 11-day chick embryos, were grown in B. M. E. + 10% calf serum in roller bottles rotating at 8 r. p. hr. at 37^0. When the monolayers were confluent the cells were washed in Earle's balanced salt solution and inoculated with serum-free B. M. E., containing vaccinia virus to give a multiplicity of infection of 1. After 4 days the virus was harvested in a similar manner to the W. I. 38 cell virus suspension and stored at -160^0.

A vaccinia virus suspension was prepared by extraction of the virus from sheep dermal pulp in 0.003 M phosphate buffered saline pH 7.2 containing 10% Arcton 113 (v/v) and 0.4% phenol (w/v). After centrifugation at low r. c. f. the supernatant was separated and incubated at 22^0 overnight.

Virus Assay

Virus infectivity was assayed by pock count in the chick chorioallantois (Westwood et al.)[2] and by a plaque technique in chick embryo cell cultures. Virus titres in eggs were expressed in pock-forming units per ml. (pk. f. u./ml.). In cell cultures the test samples were assayed against a reference preparation and the titres expressed as log potency ratios.

Protective agents

In the first experiment a degraded gelatin solution was added to the virus suspensions at a final concentration of 5% (w/v). The agents used in the second experiment are shown in table 2.

Freeze-drying

After the addition of the appropriate protective agents the virus suspensions were filled into ampoules in 0.25 ml. volumes and dried by sublimation from the frozen state in an Edwards 30P2TS freeze drier. The primary drying cycle began when the product temperature reached -40^0. When the vacuum reached 0.1 Torr. heat was applied to the pro-

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duct. When the product temperature and shelf temperature coincided the secondary drying cycle over P_2O_5 was started and continued for a further period of 18–20 hrs.

All the samples in each of the two experiments reported here were dried simultaneously in a single freeze-drying run.

RESULTS

Three vaccinia virus suspensions were prepared, respectively in the human diploid cell strain W. I. 38, in chick embryo cell cultures and in sheep skin. A fraction of each virus suspension was partially purified, and both purified and unpurified fractions were appropriately diluted to anticipate roughly comparable virus infectivity titres after freeze-drying. The diluted purified fraction of W. I. 38 derived virus contained 1.45 mg/ml. of N_2 and the purified virus suspension of chick and sheep origin contained 16.3 and 14.5 mg/ml. of N_2 respectively. After the addition of degraded gelatin as a protective agent the six virus suspensions were freeze-dried in 0.25 ml. aliquots as described. Samples of each freeze-dried preparation were held at 37^0, 22^0, 4^0 and $—70^0$ and their residual virus infectivities were assayed after 4 and 8 weeks.

The results on samples held at 37^0 and 4^0 are shown in table 1. No change in titre was found in samples stored at $—70^0$ whilst samples at 22^0 gave titres intermediate between those at 37^0 and 4^0.

Effect of substrate on stability

Unpurified virus preparations derived from W. I. 38 cells and chick cells were less stable than sheep derived freeze-dried virus at 37^0 and 22^0. The stability of the tissue culture preparations at 37^0 and 22^0 was significantly improved by partial purification of the virus suspensions before freeze-drying.

Effect of protective agents on stability

A purified suspension of vaccinia virus grown in W. I. 38 cells (362.5 μg/ml. N_2) was divided into six aliquots and freeze-dried in the presence of mixtures of sorbitol and glycine, cysteine, glutathione, protamine, and glutamate respectively; the sixth protective agent was peptone alone. Samples held at 37^0 and $—15^0$ were assayed for residual live virus after 5 weeks. The results are shown in table 2.

DISCUSSION

Many reactions of biopolymers, such as oxidation and protein denaturation may occur at solid/liquid, liquid/vapour and solid/vapour interfaces, i. e. these are surface reactions. The usual »freeze-dried« vaccine consists of a sponge-like material providing an enormous surface

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area for such reactions to occur. Under such conditions even trace amounts of inactivating agents could promote large areas of surface activity.

Virus stability in relation to freeze-drying is a two-fold problem. Firstly the virus may be inactivated during freeze-drying; this may be due to factors such as oxidation or rupture of H-bonds. Secondly the virus may be inactivated during normal storage after freeze-drying. We have attempted to protect the virus against both forms of inactivation.

Potency assays on virus preparations derived from chick embryo cell cultures were done in chick choioallantoic membranes, because potency ratios computed from plaque counts in chick cell monolayers do not accurately reflect titres in chorioallantoic membranes or plaque assays in heterologous cell systems.

Although peptone is the most effective of the protective agents tested (table 2) the relative effectiveness of cysteine and glutathione suggest that oxidation may play a part in the thermal inactivation of the freeze-dried virus. In each of the two experiments reported here the virus suspensions were freeze-dried simultaneously in a single freeze-drying run and are therefore comparable. Recent work by Grieff and Rightsel[3] indicates the complexity of the factors influencing the stability of virus suspensions during freeze-drying, and we believe that further work on the stability of freeze-dried vaccinia virus must relate stability with residual water estimations, with product temperature profiles and with estimations of oxidation products. These important measurements are lacking in the present study because we have not yet perfected our method of determining residual water content. When virus stability is the only factor investigated, the search for suitable protective agents is entirely empirical. It is probable that the optimal level of residual water giving a stable vaccine varies with the type and concentration of the protective agent used.

Although we have produced a relatively stable freeze-dried preparation of vaccinia virus derived from tissue culture, we have not yet found a suitable protective agent for vaccine to be given by jet injection. Animal peptone is unsuitable for injection in man and the degraded gelatin used in the first experiment was found retrospectively to contain tryptophane and tyrosine and cannot therefore be regarded as a suitable constituent of a vaccine to be administered parenterally.

The production of a stable potent smallpox vaccine in animal skin is relatively simple and inexpensive and control testing is comparatively undemanding, whilst current standards of this type of vaccine remain acceptable. On the other hand, vaccine for jet injection should conform to higher standards since part of the inoculum invariably enters the subcutaneous tissue. In our opinion cell cultures, possibly the human diploid cell strain W. I. 38, will provide the logical substrate for virus propagation in the preparation of a stable freeze-dried vaccine for jet injection.

Table 1

The stability of freeze-dried vaccinia virus preparations derived from W. I. 38 cells, chick embryo cells and sheep skin after 4 weeks and 8 weeks storage at 37⁰ and 4⁰

Substrate for virus growth	Nature of the suspension dried	Temp.	Log_{10} Virus Titre (pk. f. u./ml.)		
			Initial	4 wk	8 wk
W. I. 38	Unpurified	37^0	7.27	5.04	4.76
		4^0	7.27	6.73	6.61
	Purified	37^0	7.81	6.62	6.59
		4^0	7.81	7.44	7.09
C. E. C.	Unpurified	37^0	7.77	6.51	5.87
		4^0	7.77	7.25	7.23
	Purified	37^0	7.69	7.37	6.12
		4^0	7.69	7.39	7.19
Sheep	Unpurified	37^0	8.16	7.83	7.04
		4^0	8.16	7.99	7.95
	Purified	37^0	7.91	7.61	6.22
		4^0	7.91	7.72	7.91

Table 2

Stability after 5 weeks storage of freeze-dried purified vaccinia virus preparations derived from chick embryo cell cultures, freeze-dried in the presence of six protective agents

Protective Agent	Potency Ratio (log_{10}) & (Fiducial Limits)*				
	-15^0			37^0	
5% glycine + 5% Sorbitol	0.08	(−0.06	0.21)	−1.89	(−2.01 −1.77)
0.1% cysteine + 5% „	−0.23	(−0.35	−0.11)	−2.78	(−2.89 −2.66)
0.01 glutathione + 5% „	−0.49	(−0.62	−0.37)	−2.32	(−2.44 −2.20)
1.0% protamine + 5% „	−0.63	(−0.75	−0.51)	−3.93	(−4.13 −3.74)
2% glautamate + 5% „	−0.26	(−0.38	−0.14)	−1.88	(−2.01 −1.74)
5% peptone	0.05	(−0.09	0.18)	−1.31	(−1.43 −1.18)

* (Log_{10} potency of test preparation) / (Log_{10} potency of a reference preparation).

SUMMARY

Freeze-dried preparations of vaccinia virus propagated in W. I. 38 cells and chick embryo cells were less stable than freeze-dried virus derived from vaccinial dermal pulp. The stabilities of the tissue culture preparations were improved by partial purification of the virus suspensions before freeze-drying.

Of a number of substances examined for their capacity to protect vaccinia virus during freeze-drying, peptone proved to be the best.

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TISSUE CULTURE VACCINE FROM PRIMARY EMBRYONIC CALF MUSCLE CELLS

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During the last years traditional methods of preparing smallpox vaccines in living mammalian hosts have been abandoned in some institutes because of the obvious disadvantages of these methods.

For the purpose of getting a smallpox vaccine free from contaminating bacteria and moulds and with the intention to exclude the individual reaction of animals the vaccinia virus has been cultivated on the chorioallantoic membranes of the developing chick embryo with good results (Goodpasture et al. 1933, Herzberg 1949, Espmark 1962).

On the other hand attempts have been made by propagating the vaccinia virus in tissue cultures for vaccine production. The advantage of this method is to get a primary sterile vaccine which contains less unspecific protein especially in monolayer tissue culture vaccines.

The tissue culture vaccine production was started in plasma clots (Wesslén 1953, 1956, Ramon et al. 1954, Frenkel et al. 1954) and in monolayer cell cultures (Herrlich et al. 1957, Cutchins et al. 1958, Sureau et al. 1959, Bartell et al. 1960, Bonitz et al. 1961, Kaplan et al. 1961, Shaw et al. 1964).

Different materials from animals have been used in both systems such as skin- or tonguetissue from cattle, kidney cell cultures from rabbits and calves and chick-embryo fibroblasts. During the very last years experiments have been made with human diploid cells with good results (Ikić 1968).

Since 1959 we are producing in Hamburg smallpox tissue culture vaccine in the fluid and dried form from monolayer cultures of primary embryonic bovine muscle cells. The advantage of this cell system lies in

the fact that calves are very suitable for vaccine production. Contaminations of the bovine foetuses with extraneous viruses are very rare (Mayr 1962, Pöhn 1964, Adlinger 1966) and high virus titers are easily obtainable (over log 1.0×10^9). It is up to the producer to get the titer needed by adding smaller or larger amounts of resolving fluids to get the recommended titer.

<div align="center">MATERIALS AND METHODS</div>

Seed virus

The vaccinia strain Hamburg is used routinely and the vaccinia strain Elstree was obtained for experimental purposes.

The seed virus is always passaged once on a rabbit. Calves are inoculated afterwards. Subsequently the calf pulp is transmitted to primary embryonic bovine muscle cells in nine passages. In view of the WHO recommendation of passaging the seed virus only about five times we have started to fulfil this requirement (WHO 1966). The passaged seed virus is used for smallpox vaccine production in tissue cultures.

Preparation of the cultures

Uteruses with about four months old embryos are obtained from tuberculin negative cows from the slaughterhouse. The uterus is desinfected and opened carefully as well as the foetal integuments. The extruded embryo is being prepared in a sterile box. After cutting off the skin of the embryo the skeletal muscles are minced into small pieces with scissors. The particles of about 3–5 mm in length are rinsed once in Hanks salt solution and treated with a 0.25% trypsin solution. The splitting -off of the cells occurs in one litre Rappaport bottles on magnet stirrers within 15 min. at 37°C. The supernatant from the first trypsinization is discarded, only the 2nd–5th one is used. The muscle cells in the supernatant are filtered through gauze to retain mucous substances and they are centrifuged for 15 min. at 1000 g to eliminate the trypsin. The sedimented cells are solved in yeast medium with 10% calf serum and filtered again. The cell-suspension is diluted to 1.0 million cells and sowed in Roux bottles. The cells grow during three days at 37°C. On the average we are getting 4–5 l cell-suspension in one experiment.

Infection of the cultures

Before infecting the cells the medium of the cell-cultures is discarded. Later on the cells are inoculated with a mixture of medium TCM 199 with 10% calf serum (inactivated in 30 min. at 56°C) and seed virus log 8×10^6 pfu/ml cell-culture. The infected cell-cultures are kept 30–33 hours at 37°C. The cultures are now frozen at —20°C until further preparation.

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Preparation of the vaccine

The cell-cultures are thawed at room-temperature and they are centrifuged 15 min. at 1000 g. The supernatant is discarded and the cell sediment is removed and ground with an »ultraturrax« in order to obtain a high virus concentration. When producing a fluid vaccine we are removing in 10 ml of a glycerine-agar-saline-solution and when preparing a dried vaccine 5 ml of a 10% pepton-TCM-solution is used. Later the vaccine is kept at —20°C until further use.

Titration

Titrations of samples are performed on the chorioallantoic membranes of 12 day old chick embryos by the pock counting method (Burnet 1936, 1938, Overman et al. 1956, Westwood et al. 1957) as recommended by WHO (1966) and by the ID 50 in primary embryonic bovine muscle cells (Dulbecco 1952, Dulbecco et al. 1954, Noyes 1953, Bonitz 1960).

Additional titrations are made in the skin of rabbits (Herzberg 1935, 1955) and the potency is further investigated in 12 year old revaccinees.

Sterility-tests and virus-titrations on the chorioallantoic membranes follow at all stages of preparation.

For the potency test on rabbits according to Herzberg from the prepared vaccine and a reference vaccine three different dilutions are made. 0.1 ml of each dilution is given on a field of scarified skin on each of three rabbits. The number of the pustules formed is evaluated four and five days after challenging.

The sterility-test is done in glucose broth and thioglycolate broth at 37°C and at room-temperature during 14 days. In questionable cases different media for isolation of fungi, PPLO and for differentiating of bacteria are used. Infected tissue culture vaccines are discarded immediately but that has happened very seldom.

Investigations for extraneous viruses are made in the following way: Samples of cell cultures of every charge are kept and observed for 2–4 weeks for plaque-forming viruses.

After finishing the titrations and the sterility-tests some vaccines (1–2 l) will be mixed and diluted later for its special use. Now a recheck is made on the virus content (CAM, ID50, Herzberg).

For the preparation of a fluid vaccine the virus suspension will be diluted to a titer of log 2.5×10^8 pfu/ml. For the preparation of a dried vaccine the virus suspension shall have log 3.5×10^8 pfu/ml before drying.

The fluid vaccine is filled in capillaries and tubes and the dried vaccine is freeze-dried in ampoules with the apparatus of »Texvac« or the freeze-drying maschine of »Kniese« respectively.

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DISCUSSION

Experiments with tissue culture vaccines have been carried out since 1959 in our laboratory and about 120 000 persons have been vaccinated until 1967. Good vaccination results have been obtained during that time (Bonitz et al. 1961, Herrero et al. 1969). In Munich about 70 000 persons have been vaccinated with tissue culture vaccines from embryonic bovine muscle cells too with good results (Munz 1967).

During the last two years we changed the smallpox vaccine production more and more from dermo vaccines to tissue culture vaccines of embryonic bovine muscle cells. Nearly all of our fluid and dried vaccines are tissue culture vaccines now and more than 300 000 portions have been distributed from 1967–69.

The reasons for using primary tissue cultures from the bovine foetuses for vaccine production are the following:

The production of smallpox vaccines in monolayer tissue cultures has the advantage of getting a vaccine free from bacteria and moulds and with less unspecific protein.

Contaminations of muscle cells from bovine foetuses with extraneous viruses are very rare. Calves are used for smallpox vaccine production since more than 150 years and there has not been much trouble with extraneous viruses in the past (Munz 1968). When using cultures from bovine tissues the same conditions as in calves are given. Experiments have been done in finding different kinds of extraneous viruses in the lymph. Mayr (1962) tested smallpox vaccines prepared from experimentally infected calves with ECBO-viruses. He could not find any ECBO-viruses in these vaccines Pöhn (1964) has been looking for ECBO-viruses in the lymph too and Adlinger et al. (1966) for parainfluenca 3 and rhino viruses. Both authors had negative isolation results.

Using primary tissue cultures the risk of the transformation of cells is reduced (Kaplan 1964) as well as the possibility of inducing tumors. Furthermore antigenicity of the different vaccines is not altered in tissue culture vaccines because the same seed virus is used for the production of many batches.

Finally the vaccine production in tissue cultures seems to be much more economical than the lymph won by animals. From one calf one can obtain about 0.8 litre smallpox vaccine with a titer of 1.0×10^8 pfu/ml. From one batch tissue culture 400 ml vaccine with a titer of log 3.0×10^8 pfu/ml can be obtained. Only about two batches are necessary to get the same amount of vaccine as from one calf (Munz 1968, own experiments).

We had no difficulties in achieving high virus titers in tissue cultures when the infection was carried out with the strain Hamburg in the above mentioned way. The average titer of the different batches of

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vaccines prepared in the last two years was log 3.0×10^8 pfu/ml. The vaccines have been diluted to 2.5×10^8 pfu/ml, because this dilution gave about 90% major reactions in revaccinees by experienced vaccinators.

The stability of fluid tissue culture vaccines is as sufficient as with dermo vaccines after using in tubes for 20 and 50 portions. The titer of the tissue culture vaccines does not remain stable at the desired level when the vaccine is sold in one portion capillaries in the country. For distribution we are filling all tubes with tissue culture vaccines and all capillaries with dermo vaccines until now. We started experiments to find a suitable medium for stabilization of the vaccinia virus in tissue culture vaccines.

When preparing dried tissue culture vaccines we had good results when using log 3.5×10^8 pfu/ml before drying. Normally after drying we obtain the desired titer of log 2.5×10^8 pfu/ml or more. The relative high drop in titer of freeze-dried vaccines results only in tissue culture vaccines whilst freeze-dried dermo-vaccines show only a very low drop. A heat resistance test on dried vaccines is done at an incubation temperature at 37°C for four weeks and boiling for one hour. Our dried vaccines mostly fulfill the WHO-requirements. The freeze-dried tissue culture vaccines are tested in 12 year old revaccinees and the average value of the take-rate from the vaccines of the last two years was 88,3%.

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TISSUE CULTURE VACCINE IN CLINICAL PRACTICE

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In the preceeding paper Dr. Wokatsch has already outlined the various advantages of tissue culture vaccines. We want now to give a review of the work performed until now, as far as the *practical application of tissue culture vaccine* is concerned.

Rivers was the first who successfully experimented with chick-embryo culture vaccine on 3 children in 1931. Other pioneers in this field were *Herzberg* (1933, 1935) as well as *Rivers* and *Ward* (1933). Their methods did not gain access into the vaccine production, until antibiotics were available and until *Enders* and his collaborators demonstrated reproduceable methods for tissue culture.

The following phase has been initiated by the production of an embryonic bovine skin cell-vaccine by *Wesslén* (2000 persons had been immunized, 1953, 1955). Differences in the vaccinal reactions between the tissue culture vaccine and the traditional dermo-vaccines have not been observed. The average take-rate was 90%. Revaccination carried out one year later resulted in equivocal reactions. Bovine tongue tissue culture vaccine was used 1957 by *Herrlich* and *Mayr* for the primary vaccination of 12 children. A human diploid cell tissue culture vaccine by *Ikić* et al. (1969) proved to be safe.

A stronger indicator of the potency of a vaccine, besides the measurement of titer, is the *take-rate in revaccinees*. The former vaccinators were already aware of this fact. But the first to demonstrate a definite relationship between the titer of the vaccine and the take-rate in revaccinees were *Bonitz* and *Seelmann* from the Vaccination Institute Hamburg in 1960 (see also *Hobday* et al., 1961).

Bonitz and *Seelmann* showed too, that with a tissue culture vaccine from embryonic calf-muscle cells comparable vaccination results from dermo-vaccines can be achieved, if the titer of the vaccine is higher.

A study of *Kaplan* and *Micklem* (1961) with chick-embryo-culture vaccine demonstrates too that tissue culture vaccines meet the necessary requirements: a 93% take-rate in revaccinees; a control dermo-vaccine showed 97%. The vaccinations were performed by *Cross*, an experienced vaccinator. One year after the primary vaccination with tissue culture vaccine, 50 persons were challenged with potent sheep-lymph; there was only one take, 22 indurations, but no vesiculation! These could have been either immediate or weak accelerated reactions.

A field experiment with chick-embryo fibroblasts was later conducted by *Shaw* and *Kaplan* (1964). The differences in the success-rate with the trial vaccine (41,7% take versus 59,9% with the conventional vaccine) were statistically significant. Revaccinations were carried out by different vaccinators, thus the wide variation in the success-rate (17,4% to 78,5% with the same experimental vaccine) may reflect the skill of the vaccinators or the immunity status of the revaccinees in a higher degree than the potency of the tissue culture vaccine; for instance, the lowest take-rate resulted from immunizations in a Tropical Institute. We have shown previously that, using the same lymph, differences in the take-rate of 30% from vaccinator to vaccinator may exist *(Ehrengut,* 1968).

Nevertheless, it is a fact that also *De Risso* and *Soares* (1968), who used rabbit kidney-cells as a vaccine substrate, had seen in 75,8% of 1257 revaccinees equivocal and negative reactions with a titer of $1,2 \times 10^7$ p.f.u./ml.

Experiences with tissue culture vaccine during a 10-year-period

Already in the first report on tissue culture vaccine of the Vaccination Institute Hamburg *(Bonitz* and *Seelemann,* 1960), favourable results from comparative trials between dermo-vaccines and tissue culture vaccines (bovine embryonic muscle cells) could be observed (Table 1). The authors demonstrated that the tissue culture vaccine has to have a titer of around 0,5 log. and more, in order to give identical results. In a subsequent study made in our Institute, *Herrero* and *v. Mutzenbecher* (1969) demonstrated the same relationship on a still more embracing material. Among 5038 twelve-year-old revaccinees an increase of the titer of the tissue culture vaccine (from log. $10^{8,10}-^{8,94}$ p.f.u./ml, see Table 2) is followed by a similar increase in the percentage of major reactions (from 55% up to 94%).

Clinically there was no obvious change in the *incidence of post-vaccinal complications,* though the dermo-vaccine in the public vaccinations was substituted more and more by the tissue culture vaccine. The decline

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of post vaccinal encephalopathies, observed in most of the European countries during the last years, cannot be connected with the introduction of the tissue culture vaccine *(Ehrengut,* 1969).

In Germany, compulsory vaccinations are carried out by scarifications. The *tenacity of the tissue culture vaccine* is therefore of some importance. Though this vaccine is a little more liquid than the usual dermo-vaccines (even after the addition of agar), no difficulties arose for the various vaccinators, who adjusted themselves very quickly to the other consistency of the new preparation.

In general, we used *liquid tissue culture vaccines* in 20 and 50 portions for public vaccinations (expiry – date: 4 weeks, storage as usual in the coldest part of the refrigerator), also *lyophilzed tissue culture vaccine* was applicated (see *Herrero* and *Meggers,* 1968). It remained stable under 2 months storage too, in a tropical environment. The clinical experiment we made in India convinced us that there is no difference between the number of major reactions maintained by the new vaccine *in comparison to dermo-vaccine.*

One important aspect is the *immunogenicity* of the tissue culture vaccine. An indication that tissue culture vaccine produces a good immunity against smallpox as well as the dermo-vaccine is the observation of immediate reactions following a challenge with potent dermo-vaccine after one year *(Wesslén,* 1955, *Kaplan* and *Micklem,* 1961), and furthermore the animal experiment. *Kaplan* and *Micklem* (1961) observed just as we did, equivocal reactions in revaccinated rabbits, immunized previously with tissue culture vaccine and challenged with potent dermovaccine. Our report of a high take-rate (of 72% major reactions with a tissue culture vaccine of 10^9 p.f.u./ml) in the staff of the Tropical Institute, which has been vaccinated at least 10 to 14 times against smallpox in yearly intervals, is in complete harmony with the above assumption. A field trial with these vaccines should be conducted at any rate in smallpox endemic areas so as to give a definite reply to questions still unanswered.

Tissue culture vaccine for jet-injection (Preliminary experiment)

The primary freedom of bacterial contaminations makes tissue culture vaccines especially suitable for jet-injection. Already in 1967 *Payne* made an appeal for the production of purified vaccines, free from pathogenic organisms, suitable for jet-injection. Success and failure in eliciting vaccinal reactions by scarification methods depend largely on the skill of the vaccinator. On the other hand, the jet-gun may be used by relatively untrained personnel. A further advantage of the jet-injection method lies in the fact that *uniform quantities of vaccine are always introduced.*

There are only a few reports of smallpox vaccinations by jet-injectors with different routes of applications (subcutaneous injections with tiny

quantities intracutaneously: *Elisberg* et al., 1956) intracutaneous method: *Millar* and *Neff*, unpublished observations). Simultaneous vaccinations (e. g. in combination with measles- or yellow fever vaccines) are not being discussed here. In Brazil *Millar* and *Neff* used a freez dried-calf vaccine (dilution 1 : 10, respectively 1 : 50). The success-rate was 95,2% in primary vaccinees; with a dilution 1 : 50 of a lymph (titer 10^8 p.f.u./ml) they obtained in 88,2% major reactions (US-vaccine) in contrast to 76,3% with a vaccine of Brazil.

As far as we know, intracutaneous application of tissue culture vaccine by the jet-gun have not been published so far. The following experiment on 36 medical students (20–30 years of age), who had been revaccinated at least once, sometimes twice a few years ago, may therefore be of interest:

The intracutaneous injections (0,1 ml of a liquid tissue culture vaccine, strain Hamburg, titer 17 millions p.f.u./ml) were made with the jet-injector of the Scherer Comp., Detroit, Mich., USA. The vaccine was kept at —20°C. It was thawed first before use and adjusted with Hanks solution to the above mentioned titer. No other stabilizing agents were added.

Before the operation, the sub-deltoid area of the students was sponged with ether. After the injection, 10 to 20 seconds later, a blood-tinged fluid oozed from the puncture, but no special measures, just a plaster in a few cases, were neccessary.

Results: In a few patients, a control of the *local reaction* was possible on the *third day after vaccination*. It is interesting to note that, e. g. one student, who had been vaccinated 6 times before, showed an infiltration 0,5 cm in diameter (as all other vaccinees did), with a marked area of $8,3 \times 8,5$ cm.

Already on the second day, he felt an itch on the injection spot, but no swelling of the local lymph nodes. The symptoms subsided after the third day. The patient was seen on the seventh day with only a tiny intracutaneous infiltration (hardly to be felt), and no area was visible any longer. We are prepared to accept this type of reaction in analogy to the scarification method as an *equivocal reaction*.

The students seen on the *seventh day of infection* with a *vesicular* or *pustular take* showed the above mentioned infiltration on the third day too, but in the centre of it a blueish-coloured vesicle respectively, a very small pustule, which did not rise very markedly above the level of the epidermis, could be observed. As in revaccinees with the scarification method, the area in major reactions was more or less apparent on the seventh day. Only a few patients claimed to have had fever of a mild nature. In every case, the pustules were much flatter than in cases following scarifications with pustular take. 33% of our revaccinees had equivocal reactions (according to our suggested nomenclature), 66% *major reactions*. Serological investigations have not been carried out.

DISCUSSION

Though we had the impression that also students with equivocal reactions seemed to have some kind of interaction with the vaccinia virus due to the wide area, we cannot prove it serologically, *Elisberg* et al. (1956) also described such types of reaction, varying from *3,5* to *10,0* cm in diameter on the second day following jet-injections.

Our limited experience with tissue culture vaccines given intracutaneously suggests that, for obtaining a reasonable take-rate in revaccinees, our vaccine should have a titer between 25 to 30 millions p.f.u./ml. Taking into account that a tissue culture vaccine has to have a higher titer than dermo-vaccines – as already outlined –, there seems to be no great difference economically.

For eradication programmes, freeze-dried vaccines are necessary. Freeze-dried tissue culture vaccines can be used without great loss even under tropical conditions *(Ehrengut,* unpublished observation). As a stabilizing agent, peptone is used in most preparations.

From experiences gained with tetanus toxoid we know that a small amount of *peptone may provoke a peptone shock* in immunized persons. Vaccinators should therefore be seriously warned against using lyophilized vaccine for jet-injection without exactly knowing the detailed composition of the vaccine. In the directions for use of a dried smallpox vaccine (*»Dryvax,* Weyth Laboratories, Marietta, Pe., USA), the injection of the product was expressily warned. We can only support this plea. We therefore used no ordinary vaccine for our study and we have to look in the future for a suitable stabilizer.

To accomplish smallpox eradication, further progress in technology must be awaited. *By the technique of jet-injection,* a rapid immunization is possible. Since we have to inject the vaccine intra-dermally, *it is questionable, whether the immunity conferred lasts as long as that following epi-dermal application of the vaccine. Bickel* et al. (1966) were able to get 42% major reactions in a group of patients successfully vaccinated 7 months before by the intracutaneous route. Under this point of view, a longer experience with the jet-injection method is necessary, before it can be generally accepted as an equivalent to the scarification method.

The *safety of jet-injection* is also guaranteed, if one selects the vaccinees carefully. The effortless handing of the jet-gun can easily tempt us to »shoot«, but the absolute well-being of the vaccinee is a prerequisite. The time necessary to take the case history in primary vaccinees is certainly sufficient to load the lancet at the same time with the lymph for scarification. In *primary vaccination therefore there is no absolute need to use the jet-gun,* even in undeveloped countries. In contrast, under adverse conditions, the thorough questioning of the revaccinee is not so important (postvaccinal encephalitis according to our figures in only 1 case among 1,5 million revaccinations), the jet-injector is time-saving.

Table 1

Comparative results of revaccination with derma-vaccine and tissue culture vaccine (bovine muscle cells, strain Hamburg): Bonitz and Seelemann (1960)

Vaccine	Titer (p. f. u./ml.)	Revaccinees 12 year of age reactions		altogether
		equivocal	major	
Dermo-vaccine	7,03	1640 (57%)	1251 (43%)	2891
Tissue culture vaccine	7,52	109 (59%)	77 (41%)	186

Table 2

Comparative results of revaccination in 12 year old children, vaccinated with dermo-vaccine and tissue culture vaccine in Hamburg: Herrero and v. Mutzenbecher (1969)

Vaccine	Titer (p. f. u./ml.)	No. of revaccinees	among them % major reactions
Dermo-vaccine	7,16	178	48
	7,20	245	58
	7,24	351	59
	7,30	165	65
	7,66	126	82
	7,71	194	74
	7,75	788	74
	7,83	119	72
	8,01	126	86
	8,03	116	90
	8,26	17	94
	8,29	124	97
Tissue culture vaccine	8,10	135	55
	8,26	99	69
	8,34	818	66
	8,36	276	75
	8,38	315	72
	8,44	342	83
	8,44	396	78
	8,48	556	80
	8,51	483	81
	8,58	228	81
	8,62	106	90
	8,67	83	94
	8,94	147	91

Since blood-tinged fluid sometimes oozes from the injection-spot following the vaccination, the nozzle of the jet-gun should be immediately withdrawn from the dermal puncture, to avoid *transmission of blood-borne infections.*

ADDENDUM

A recent experience on a large scale vaccination (72000 subjects in Puerto Rico, see Internat. Med. Digest 85, 6, 303 (1969) with avianized vaccine by jet-injection revealed in a survey in 11,5% of the primary vaccinees *ulcers* (versus 0,03% among revaccinees). The incidence of complications was significantyly greater with avainized vaccines than with calf vaccine.

SUMMARY

After a review of the clinical application of tissue culture vaccines in literature, personal experiences with such vaccines from bovine embryonic muscle cells over ten years have been given. The tissue culture vaccines are well tolerated, no untoward reactions have been observed. The take-rate in revaccinees is equivalent to dermo-vaccines, if one uses a higher titer. Preliminary experiments with tissue culture vaccines injected intradermally by the jet-gun are reported. The pros and cons of the jet-injection method and rather unusual local reactions have been discussed here. For this purpose only freeze-dried vaccines which do not contain any peptone, should be used.

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HUMAN DIPLOID CELL GROWN SMALLPOX VACCINE

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In the late second half on the 19th century special institutes for the large-scale production of smallpox vaccine in the scarified skin of live animals were established in many countries. In some of the following decades, the preparation of smallpox vaccine did not experience any essential changes. Then the freeze-dried vaccine was developed representing the most significant progress since Jenner's vaccine has been used.

Today as the result of the improvement of tissue culture techniques, the interest in the development of a new substrate for the preparation of smallpox vaccine increases from day to day.

The tissue culture that we decided to choose as a substrate for vaccine production were the human diploid cells Wi–38 (Hayflick and Moorhead 1961). First data on the possibilities of using this tissue for the preparation of vaccine were presented at the Symposium in Opatija (Ikić et al. 1963a), and experiences with the vaccine prepared from the Lister (Elstree) strain (Ikić et al.) at the Symposium in Zagreb.

In this country human diploid cells (HDC) as a substrate for virus vaccine production have been placed on the same plane as primary cells. Our Licensing Authorities have accepted live oral polio vaccine prepared in HDC, registered it and licensed for use on a large scale. Live measles vaccine prepared in HDC has been also accepted, registered and licensed for massive use.

Thus, in Yugoslavia the way has been paved for the manufacture of vaccines in HDC and for their application by either oral or parenteral route.

The decision to equalize HDC and primary cells has been made after several years of consideration of results of our laboratory and field investigations of HDC (Wi–38) and the vaccines made in this substrate (Ikić et al. 1963a, b, c; Ikić 1964; 1965; 1968a, b, c, d, e; Ikić and Juzbašić 1968b).

This was the main reason why we in two last years started to consider more intensly the possibility of preparing smallpox vaccine in this substrate.

WORKING PROGRAMME, MATERIAL AND METHODS

Working Programme

We have planned to adapt vaccinia virus to HDC by passing it in this substrate and using plaque-technique to obtain a pure virus generation freed from all extraneous agents which may be present in the smallpox vaccine prepared from calf lymph (Ikić et al. 1968a). Our next goal has been to get a high virus titre on this tissue and to assess the reactivity of vaccine.

Tissue culture and media

In all experiments the human diploid cells Wi-38 were used. The number of cells in the confluent sheet ranged between 1 and 3 million in baby-bottles, and 5 to 12 million in Roux bottles. The interval from the splitting of the cells to their infection – i. e. the age of cells – was between 2 and 8 days.

Eagle's medium containing 10% calf serum was used as growth medium, and Medium 199 containing 1% peptone (Difco) as maintenance medium. Baby-bottles contained 20 ml and Roux bottles 100 ml of medium. Trypsin was used as 0.25% solution.

Seed virus

All the experiments described were performed with the Lister strain of vaccinia virus obtained from the Institut Serotherapique et Vaccinal, Berne. The virus was adapted to the Wi-38 cells where it underwent 8 passages including 3 plaquings. The seed virus represented the 10th passage.

The quantity of inoculum used contained about 2,000.000 virus particles per baby-bottle and 8,000.000 per Roux bottle (about 1 virus particle per 1–2 cells). The temperature for growth as well as the incubation temperature was 37°C. Until titration the virus harvest was kept at —20°C.

Titration and control of vaccine

The titration was done by inoculating chorio-allantoic membrane (CAM) of 12 days old chick embryos (Leghorn breed) (Burnet and Faris 1942). The inoculum was 0.1 ml of virus suspension per egg. Not less than 10 eggs for each of two tenfold dilutions were used for the inoculation. At least 5 titrations for each lot were performed. After 48 hours of incubation at 37°C the number of pocks per membrane was counted. The titre was calculated from the mean value of pock counts, the dilution and the amount of inoculum used, and expressed as PFU/ml.

The control of the substrate was performed according to the Minimum Requirements for the Selection and Use of Human Diploid Cell Strains in the Production of Virus Vaccines (Opatija 1963), and the control of the vaccine according to WHO Requirements for Smallpox Vaccine (1966).

Vaccine

Three lots of smallpox vaccine were prepared in HDC (Wi–38). Their titre after freeze-drying amounted to: $10^{7.20}$ PFU/ml, $10^{7.60}$ PFU/ml and $10^{8.00}$ PFU/ml. The routine vaccine (lot 25, titre $10^{8.00}$ PFU/ml) prepared from calf lymph was used as reference vaccine.

Vaccination

Primary vaccination of children with smallpox vaccine prepared from the Lister strain grown in human diploid cells Wi–38 was performed in the Medical Centre of Krapina and in two Public Health Centres in Zagreb, while the revaccination was carried out in the Medical Centre of Čakovec. Vaccination was performed by a team of the Institute of Immunology with assistance of the local health service. Vaccination was carried out always by the same person – an epidemiologist from the Institute of Immunology. For vaccination the method of multiple pressure was used. Inoculation was performed on two sites of the upper part of the right upper arm with 30 pressures on each site. The space between the inoculated sites was 3–4 cm. On coming for vaccination each child had a special card opened for it in which data on postvaccinal reactions were later entered.

For primary vaccination we have taken children aged 10–30 months, the age of most children was about 14–17 months. At revaccination children were on the average 7 years old so that the interval from primary vaccination amounted to 4–6 years. The control of children after primary vaccination was performed from the 4th to the 14th day after vaccination, while the revaccinated children were observed on the 2nd, 4th, 6th and 8th day after vaccination. The control of children both after primary vaccination and revaccination was performed by the same persons – two epidemiologists from the Institute of Immunology.

Clinical reactions of those vaccinees who after primary vaccination showed a typical primary vaccinia with one or two pocks after 7 days (the day following vaccination was counted as the first day) were followed up.

The appearance of minimum one pock was considered as successful vaccination. Only vesicular and pustular lesions (major reaction) were considered as successful revaccination, while the appearance of papula (accelerated reaction) and macula (immediate reaction) were not taken into account. The reactions were definitly assessed on the 6th day.

Among clinical reactions particular attention was paid to the increase and duration of fever. The temperature was taken axillary twice a day beginning from the 4th day after vaccination. No child was given antipyretics.

With regard to the fever, the successfully vaccinated children were divided into 3 groups. The first group marked »no fever« covered the children whose temperature was lower than 37⁰C. Children having the temperature from 37⁰C to 37.9⁰C were put in the second group marked »low fever«. The third group consisted of children whose temperature was higher than 37.9⁰C. This group was marked as »morbidity«. The rates were always expressed in percentages. Within the »morbidity« group we had two sub-groups in which the sub-group »high fever« represented the temperature higher than 38.9⁰C. The rate was expressed as the percentage of children with this high temperature from the total number of children in the »morbidity« group. The sub-group »prolonged fever« represented the duration of temperature for two days and longer. The rate was expressed as the percentage of children with the prolonged duration of temperature from the total number of children covered by the »morbidity« group.

RESULTS

The relation of virus titre to the age of cells, maintenance medium and concentration of virus inoculum is shown in Table 1.

The highest virus titre was achieved on lower passage levels up to the 27th (passages from 20th–34th were used) with 3–4 days old cells (the age of cells ranged from 2 to 8 days), using Medium 199 with 1⁰/o protone and the inoculum containing 1 virus particle per 1–2 cells.

The concentration of virus in the inoculum is in close relation with the time of incubation, i. e. the interval between inoculation and a certain stage of CPE of cells. If the concentration of virus is too small, incubation is too long, so at the temperature of the thermostate of 37⁰C and a reduced pH, the virus generation – liberated from the cells earlier – deteriorate, and this decreases the titre. A too large inoculum will not lead to the desired results, the reproduction being low because all cells are attacked as early as in the first generation.

We achieved the best titres when the time of incubation ranged between 48 to 72 hours. If we keep the ratio between concentration of virus particles in inoculum and amount of cells in bottle – 1 virus particle per 1–2 cells – the virus multiplication is about 2.4 logs i. e. 250 times.

Under optimal conditions we obtained a titre of $10^{7.70}$ PFU/ml in our experiments. This titre being unsatisfactory, we concentrated the virus using the technique of interruption of incubation at a certain stage of CPE. Part of the medium was decanted after 48–72 hours of incubation at the temperature of 37^0C and incubation continued with the remaining small quantity of medium (10–15 ml per 1 liter bottle) at the temperature of $+4^0$C for additional 4 to 5 days. After that the bottle was stored at $—20^0$C. After the material had been three times frozen and thawed, it was homogenized and then centrifuged at 2000 r. p. m. in order to remove the cell debris. The supernate without further additives represented the vaccine.

Reactivity of the three lots of vaccine was followed up in primary vaccination of 130 children. The results are presented in Table 2. The lots differed in virus titre (7.20, 7.60, 8.00 logs). All the results are summarized in this table because the titre did not influence the reactivity of vaccine.

From the table it can be seen that 95% of children were successfully vaccinated (124 from 130); 46% of the successfully vaccinated children did not develop fever, and only 35% of children (i. e. 1/3 of the successfully vaccinated children) had a temperature higher than 37.9^0C. In no infant this temperature lasted 2 or more days.

In Table 3 reactivity of vaccine in revaccinated children is presented. The titre of vaccine amounted to 7.6 log. A total of 96 children was followed up. The table indicates that from 96 children 23 were successfully vaccinated, i. e. 24%. In the successfully vaccinated children reactions were very mild. From 23 children 21 i. e. 91% did not develop fever.

DISCUSSION

HDC (Wi-38 strain) is a well defined substrate for the preparation of virus vaccine, carefully tested using all methods available at present to exclude the possibility of cell transformation, oncogenic action and presence of extraneous agents.

The last versions of suggested methods for the management and testing of a diploid cell culture used for virus vaccine production (Hayflick and Jacobs 1968) as well as criteria for the acceptibility of a diploid cell population as substrate for virus vaccine production (Perkins 1968) are published.

For the preparation of vaccine in HDC (Wi–38) the Lister strain was chosen because in this substrate it gave a higher titre than the Berne–Zagreb strain.

In the course of nine passages in human tissue, including 3 plaquings. the virulence of the Lister strain did not increase. The intensity of local and systemic reactions both in primary vaccination and revaccination complied with our expectations. The titre of the vaccine ranged between 7.2 and 8.0 log.

The concentration of infective units in the vaccine is only one of the factors responsible for the success or failure in primary vaccination.

A very important factor is the technique of vaccination. The vaccinator decides on the size of the skin area to be injured; whether to inoculate in one or several sites and how long and deep the scar must be. It is certain that the vaccination technique has an influence on the intensity of postvaccinal reactions.

Considering all these factors, we may say that we are satisfied with the results achieved in primary vaccination which have shown that 95% of the children were successfully vaccinated and that 46% of the successfully vaccinated children had no fever.

The challenge of skin resistance after primary vaccination by means of subsequent infection is an important method for the testing of acquired immunity and for the study of the efficacy of various vaccinal strains and vaccines. In our studies the percentage of successfully vaccinated children upon revaccination amounted to 24%. In 91% of successfully vaccinated children no increase in temperature was observed. This percentage of successfully vaccinated children is not particularly high, but it had not been high in our last study of the Berne–Zagreb strain either.

The results of such examinations do not depend only on the concentration and virulence of the strain, on the vaccination technique, but also on the degree of immunity of the group which is being revaccinated.

Thus in a hospital staff revaccinated in 1961 with the vaccine the titre of which was lower than in our present vaccines, out of 385 observed revaccinated adults, 305 had a reaction similar to the primary, i. e. 79% (Tudorić 1962). The reactions were particularly strong in older persons with the long interval between the vaccination and revaccination.

In another study with the same vaccine the percentage of negatives in revaccinated adults was 38.7. After repeated revaccination of these persons the percentage of negatives fell from 38.7 to 23.5 (Emili, unpublished data), presumably due to longer and deeper cuts into the skin on the part of the vaccinator. Thus the final percentage of successfully revaccinated persons amounted to 76.5.

If we wish to compare and choose the most adequate strains for the preparation of vaccine by this method we must randomize all factors known and unknown which could affect the results. Therefore, such studies ought to be conducted only according to the design of strictly controlled field trials.

Finally we consider that HDC may be suitable substrate for the preparation of smallpox vaccine. We are also satisfied with the properties of the Lister strain propagated in this substrate.

We would endeavour to further attenuate the strain in this substrate. However the way to further attenuation of the strain can only be opened by reasonable requirements for the protective value of the vaccine and a laboratory method for measuring the degree of immunity. At present, however, we are still far from this aim.

Table 1

Virus titre in relation to the age of cells, maintenance medium and virus inoculum

Age of cells	Virus titre*	Maintenance medium	Virus titre*	Inoculum per bottle	Virus yield per bottle
2 days	$10^{7.30}$	Medium 199 0.4% glucose 0.5 peptone	$10^{7.10}$	$10^{5.00}$	$10^{8.10}$
4 days	$10^{7.60}$	Medium 199 0.5% peptone	$10^{7.30}$	$10^{6.00}$	$10^{8.45}$
8 days	$10^{7.40}$	Medium 199 1% peptone	$10^{7.60}$	$10^{7.00}$	$10^{8.80}$

* Average titre expressed in PFU/ml

Table 2

Reactivity of 3 lots of vaccine prepared from Lister strain in primary vaccination of 130 children

	%	
Successful vaccination[1] rate	95	(*124*/130)
No fever[2] rate	46	(57/*124*)
Low fever[3] rate	19	(24/124)
Morbidity[4] rate	35	(43/*124*)
High fever [4a] rate	14	(6/*43*)
Prolonged fever[4b] rate	00	(0/*43*)

1 Minimum one pock
2 Temperature lower than 37.0° C
3 Temperature up to 37.9° C
4 Temperature higher than 37.9° C
4a Temperature higher than 38.9° C
4b Fever lasting 2 days and longer

Table 3

*Reactivity of one lot of vaccine prepared from Lister strain
in revaccination of 96 children*

Successful vaccination rate	24	(23/96)
No fever rate	91	(21/23)
Low fever rate	09	(2/23)
Morbidity rate	00	(0/23)
High fever rate		—
Prolonged fever rate		—

SUMMARY

As substrate for smallpox vaccine preparation the Wi–38 human diploid cells were used. The Lister strain of vaccinal virus was adapted to this substrate by passaging it 10 times in HDC, including here three plaque passages.

Three experimental lots of vaccine were prepared, differring in titre. In a controlled field trial 130 children were primarily vaccinated, 95 per cent of which successfully.

Ninety-six children were revaccinated, 24 per cent successfully. The reactivity did not depend on titre. General reactions, as a rule, were mild.

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EGG-GROWN VACCINES
AND KILLED VACCINE

EGG VACCINE AGAINST SMALLPOX: APPRAISAL OF EFFICACY AND INNOCUITY

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INTRODUCTION

The initial works of Goodpasture and coworkers on cultivation of vaccinia virus in the chorioallantoic membrane (CAM) of the chick embryo for vaccine use (Goodpasture and Buddingh 1933; Goodpasture & al. 1935) elicited a considerable interest in this technique for vaccine production and for virus titration. The egg technique was thoroughly investigated and further developed by Burnet who showed it to be useful for the cultivation of a wide range of viruses (Burnet 1936).

Further works on smallpox vaccine production in the CAM were reported in the 1940 by Buddingh (Buddingh 1943) who demonstrated that vaccinia virus upon long-term passage in eggs lost part of its virulence for the mammalian host. This finding seems to have created some reluctance against the use of egg vaccine despite the fact egg vaccines used in the field thereafter have generally been low egg passage material.

The vaccine cultivation in eggs is associated with some features which constitute certain advantages from the production point of view. Sterile vaccine of high potency can be produced in a short period of time, and at a low cost. For technical details of production procedures reference is made to Cook and coworkers (Cook & al 1953), Jackson and coworkers (Jackson & al 1956) and to Espmark (Espmark 1962).

Only in some areas of the world has egg vaccine been used extensively for routine immunization for a considerable number of years i. e. in Texas (Cook & al 1953), Sweden (Espmark 1967) and in Brazil (Clausell 1963; J. Fonesca da Cunha, personal communication). On the whole, experiences in these areas have been good, a fact that has encouraged the continued use of egg vaccine.

The aim of the present report is to review and discuss data which are pertinent to the evaluation of the practical efficacy and innocuity of egg vaccine as compared to calf lymph vaccine. Details of production methodology will be omitted unless obviously needed for the clarity of discussion of these topics.

EFFICACY OF EGG VACCINE

Stability in liquid and dried form

As to the heat stability of liquid egg vaccine the opinion was early expressed by several authors (e. g. Gastinel & Fasquelle 1941, Fasquelle & Barbier 1950), that egg vaccine was less stable than calf lymph vaccine. These observations can be confirmed by many collegues, but are mainly pertinent only if no special precautions are taken to stabilize the vaccinia virus suspension. It has now been confirmed by several authors that glycerol as an additive makes more harm than benefit to the stability, and so does adult cattle serum which usually has a tendency to cause aggregation of tissue fragments and virus particles in the vaccine. Calf serum, especially fetal, and rabbit serum exerts a stabilizing effect (c. f. Buddingh 1938; Cook & al 1953; Goodpasture & Buddingh 1936), but may confer other disadvantages. More inert substances such as sorbitol (Cabasso & al 1958; Espmark 1962) and peptone (Collier 1954; Jackson & al 1956) also seem to be good stabilizers and should be preferred over serum. The storage and shipping stability of such liquid vaccine in non-tropical areas are quite satisfactory as has been reported by Espmark (Espmark 1967).

The stability of egg vaccine upon *freeze-drying* was intially reported to be moderate or poor (Jackson & al 1956). Better results have been obtained during the last few years by the use of suitable buffers, peptone and/or low percentage of sorbitol and scrutinous control of vacuum and sealing conditions (e. g. K. G. Hedström, Statens Bakteriologiska Laboratorium, Stockholm, personal communication). A very extensive program for production of freeze-dried smallpox vaccine of egg origin is being conducted at the Instituto Oswaldo Cruz in Rio de Janerio (J. Fonseca da Cunha, personal communication). It seems very probable that methods being worked out at this institute will serve as standards also for egg vaccine production elsewhere, intended for mass vaccination campaigns in tropical areas.

QUALITATIVE AND QUANTITATIVE RESPONSE

If the effects of different culture media for vaccine are to be compared it is evident that other factors that might likewise influence the response, e. g. the strain of virus and the infectivity titer, must be carefully con-

trolled. The following response comparisons have been performed with ordinary calf lymph and egg vaccines derived from the same strain (the Beaugency strain), the egg vaccine being no more than three egg passages away from the calf lymph. These studies have been reported in detail elsewhere (Espmark 1965a, 1965b).

It is of some interest to see if qualitative and quantitative responses to vaccination are such as might be predicted from *in vitro* potency tests only.

Figure 1 shows quantal responses – per cent positive reactions – obtained with dilution series of one calf lymph vaccine, two egg vaccines and one calf skin tissue culture vaccine. The calf lymph and the two egg vaccines had approximately the same in vitro titer ($10^{8.0}$–$10^{8.15}$ ID_{50}/ml). The curves representing these vaccines are approximately parallel sigmoid curves. The curve of egg vaccine No. E13 is situated about 0.25 logs to the left of the calf lymph curve, i. e. less than a 2-fold difference. In view of the combined titration errors in vitro and in humans, this difference is probably insignificant. It thus seems that only in vitro potency but not growth medium of virus *per se* determines the type of quantal response in human vaccinations.

In order to compare quantitative (or at least semi-quantitative) responses it was necessary to estimate the approximate degree of dose-dependence of such responses. Eighty-five recruits, previously vaccinated more than 10 years ago, were each inoculated on three sites with three eight-fold dilution of calf vaccine. A rough scoring of each reaction was made at readings 7 days later. The distribution of reactions according to size is seen in Figure 2A, from which it appears that quantitative responses are dose dependent, i. e. a potent vaccine is more apt to give strong local reaction than are weak vaccines. In Figure 2B a calf vaccine and an egg vaccine of similar potency have been compared by the same type of scoring on 52 late revaccinees (top and middle part of the diagram). There is no evident difference in the average severity of local reactions, indicating again that potency but not origin of culture medium influences the quantitative response. It might be brought to mind in this context that if different vaccinia strains are used, differences in severity of reactions may be expected even if infectivity titers are the same (Polak & al 1963).

Immunity conferred by egg vaccines

A large amount of viral particles and antigen is produced in the vaccination pock. It is mainly this material, derived from human host cells, rather than the inoculated vaccine, that is the direct inducer of immunity. Unless the vaccine virus is extensively adapted to a foreign host it thus seems unlikely that the vaccine culture medium would be of crucial importance to the immunogenic property of the vaccine.

With respect to serological response, small trials have been made to compare calf and egg vaccine without any significant difference being

found in neutralizing antibodies (Espmark, unpublished data). In a comparison made in Brazil, on the other hand, a slightly lower level of neutralizing antibodies was found in a group vaccinated with egg vaccine than in those given calf lymph (Fonseca da Cunha & Mack, Rio de Janeiro, personal communication 1968). In this study, however, calf lymph and egg vaccine represented two different vaccinia strains, a fact that might have accounted for the difference. Observations reported from a smallpox endemic area in India suggest that egg vaccine and calf lymph are equally efficient in conferring protection against smallpox (Pandit 1946). Reports are also available on the successful use of egg vaccine to combat smallpox outbreaks in non-endemic areas (Irons & al 1953; Ström & Zetterberg 1963).

INNOCUITY OF EGG VACCINE

Reports on complications

In Sweden a total shift from calf lymph to egg vaccine was carried through on March 1, 1961. Comparison of complication rates during three years prior to and three years after that date do not suggest any differences.

Egg allergy

No reports on complications due to egg allergy are available. In fact, after preliminary skin tests, excessively egg-sensitive patients have been deliberately challenged with full strength egg vaccine without any adverse effects being noted (E. B. M. Cook; Å. Nilzén; personal communications).

Adventitious agents

The safety of calf lymph vaccine is taken for granted not because of strict control regulations and scrutinous safety tests but due to tradition. The only factors that may presently prevent approval of calf or sheep vaccine batches are obvious clinical signs of illness in the animal, gross contamination of the vaccine with pathogenic bacteria or with some of the few, as a rule relatively innocent, viruses which may show up in the animal tests or the *in vitro* tests. No attention is paid in international or national regulations to the possibility that calf vaccine might be hazardous due to the presence of hidden and hitherto undectable contaminating agents as, for instance, oncogenic viruses. Such viruses have been detected lately, e. g. the bovine adenovirus type 3 (Darbyshire 1966). Evidence has also been produced to suggest that the bovine leukosis may be caused by a transmissible agent (Götze & al 1956; Olson 1961). It is not possible to evaluate epidemiologically at the present time the impor-

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tance of such factors, since comparative data from reliable morbidity statistics are not available from the time before calf vaccine came into general use all over the civilized world.

Regulations pertaining to egg vaccines are more rigid. Also in fowl there are oncogenic viruses, for instance, avian leukosis, Marek's disease (Payne & Biggs 1967) and the CELO adenovirus (Sarma & al 1965). The main attention is paid to the leukosis group which consists of several variants and is very common in uncontrolled flocks and eggs. Supposedly leukosis-free flocks exists now for the commercial supply of eggs for vaccine production. Nevertheless it seems advisable to have leukosis tests available in the production control (see below).

It is very difficult to estimate the danger connected with leukosis viruses contaminating vaccines. However, in serological studies of sera from vaccinees who received vaccine containing avian leukosis virus (Sarma & al 1964A; Markham & Levine 1965; Piraino & al 1966) no leukosis antibodies were found, indicating that the virus probably does not multiply in the human host.

Some epidemiological inferences may be derived from tumor morbidity statistics in areas where egg vaccine has been in common use for many years. In Texas egg vaccine was introduced in 1939 and by 1947–48 practically all vaccine used in the state (population 10–12 million) was of chick embryo origin. The awareness of the need of leukosis control was not generally developed until after 1960. Therefore it may be assumed that a high percentage of egg vaccine batches issued before that time contained avian leukosis virus.

In figure 3 the incidence of leukemia, lymphoma and Hodgkins disease in Texas is matched against the number of issued egg vaccine doses in the state. In states other than Texas the main type of vaccine used has been calf lymph. For comparison the nationwide incidence of the mentioned diseases as well as the incidences in two other states are given. (The data on vaccine distribution have been kindly provided by Dr. J. V. Irons of Austin, Texas and the official tumor morbidity data were kindly put at disposal by Dr. Eleanor J. Mac Donald at the M. D. Anderson Hospital and Tumor Institute, Houston, Texas). It is seen in the diagram that, from the time egg vaccine came into general use (1947–48), there is no increase in the malignancy incidence in the state, but after 1949 the curve rather remains below the figures for the whole country.

A very virulent relative of the avian leukosis complex, the Schmidt-Ruppin strain of Rous sarcoma virus has been shown, unlike the leukosis agents, to induce tumors in mammalian hosts and, *inter alia,* in mormoset monkeys (Deinhardt 1966). A group of newborn monkeys were injected with 0.25 ml of a 10 percent chicken tumor extract, undiluted, 5 times concentrated and 20 times concentrated. The latent times before tumors developed were 90 days, 30 days and 23 days respectively. Later experiments with mormoset monkeys (F. Deinhardt, personal communication 1969) indicate that the minimal tumor-forming dose is of the order of
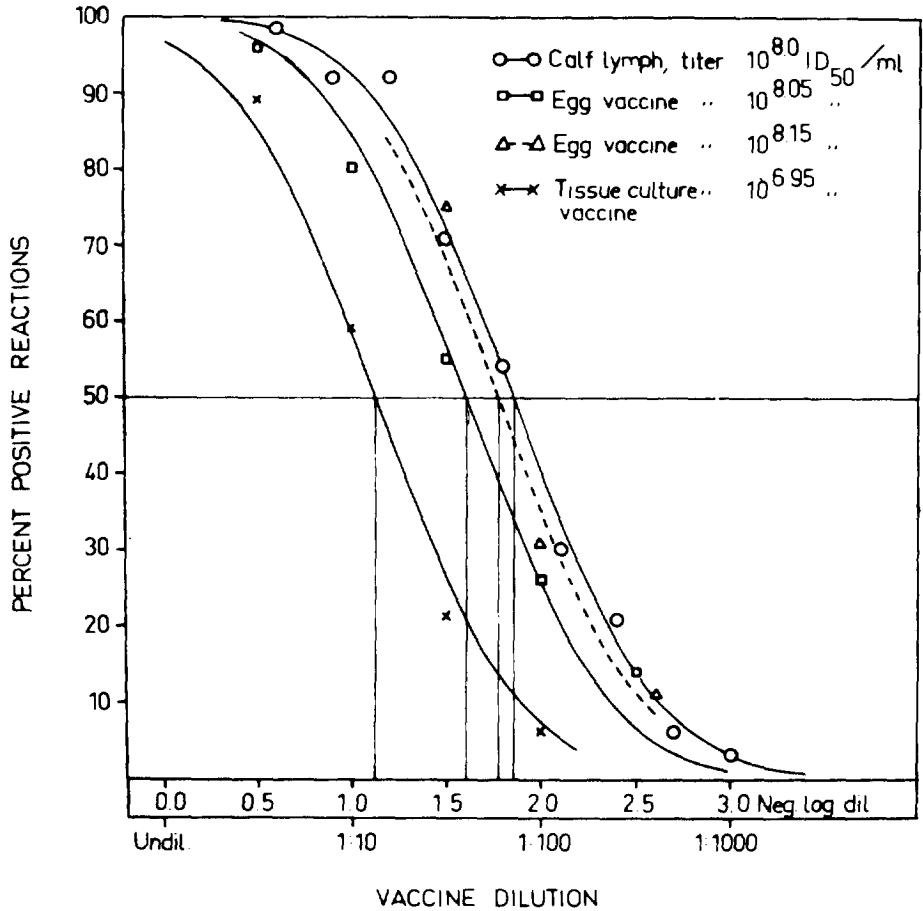
Figure 1

Quantal responses obtained with dilution series of four different smallpox vaccines. All Vaccines were 19–21 years of age and had previously been vaccinated in infancy, i. e. more than 15 years previously.

10^3 focus-forming units. Faced to this reasoning should be the facts that leukosis viruses have not yet been found to induce tumors in mammals; moreover, concentration of such agents in uncontrolled smallpox vaccine batches of egg origin (pools of numerous membranes) are probably low, the volume of the vaccination dose at percutaneous application is very small and the vaccine is not usually applied to immunologically incompetent hosts.

Technical details on leukosis control

Seed virus may be freed from leukosis agents by a 2–6 hours treatment with ether, a procedure that reduces the infectivity of vaccinia virus only slightly. Seed lots should be controlled for leukosis virus. Since this requires cultivation, the vaccinia virus must be partly separated by differential centrifugation, and the rest of vaccinia virus neutralized

Dilution of calf vaccine # 8/51 Titer, ID_{50}/ml	Frequencies (percent) of reactions of indicated size			
1st site: 1:4 Titer $10^{7.4}$		1%	25%	56% / 18%
2nd site: 1:32 Titer $10^{6.5}$	33%	30%	35%	2%
3rd site: 1:256 Titer $10^{5.6}$	72%	20%	8%	0%
	Neg.	+	++	+++
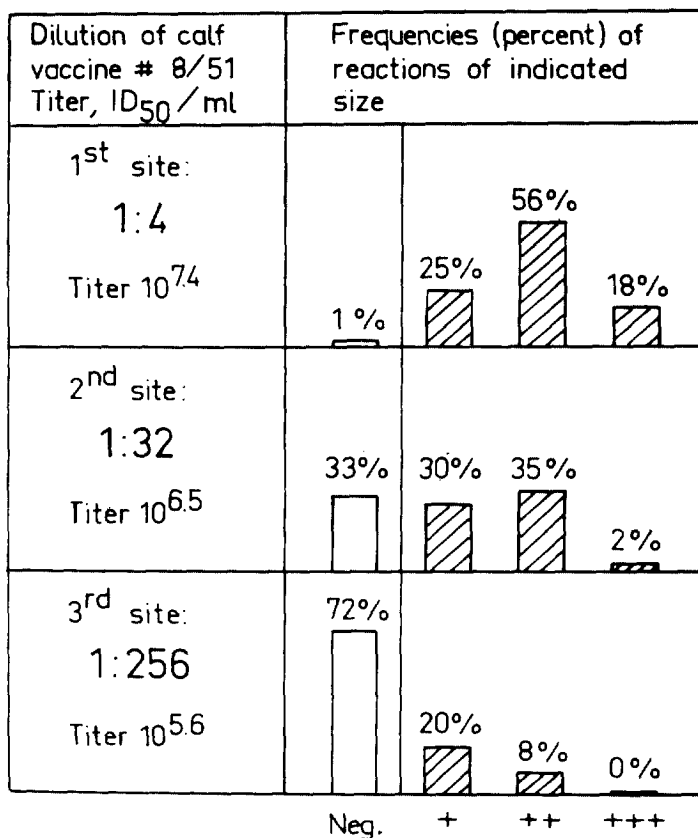
Figure 2 A

Comparison of size of local reactions obtained with vaccines of different potency (dilution series). Eighty-five vaccines were each inoculated with three vaccine dilutions: 1 : 4; 1 : 32 and 1 : 256. At the 7-day reading each reaction was scored semiquantitatively as follows:

Neg.: transient reaction or no reaction
*: pock 5 mm in diameter or less
**: pock size 6–9 mm, moderate erythema
***: pock 10 mm or more either surrounded by large erythema or resembling a primary reaction.

with a potent vaccinia immune gammaglobulin. To prevent adverse effect of traces of vaccinia virus that may still be present, the cultivation is performed under agar to prevent spread of vaccinia virus.

Individual batches of vaccinia need not be tested separately but a representative sample of uninoculated eggs (10–25%) of the batch used for vaccine production should be saved for the preparation of a culture pool on which the leukosis test is performed.

The best known methods for the test-proper are the COFAL test using sera from tumor-bearing hamsters (Sarma & al 1964B) or pigeons (Sarma & al 1969), and the immunofluorescence technique (Payne & al 1966). Lately the mixed hemadsorption technique (Fagraeus & Espmark 1961) has been used to detect avian leukosis virus in tissue cultures with a very high degree of sensitivity (Rossi & Segre 1969).
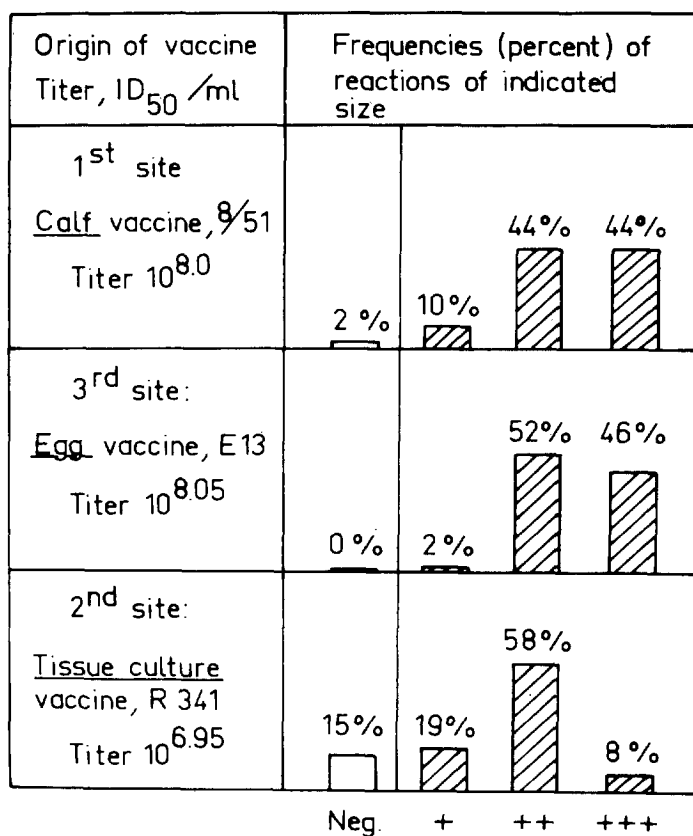
Figure 2 B

Comparison of size of local reactions obtained with three undiluted vaccines of indicated potency in 52 vaccinees. Method of test and scoring of reactions as in Figure 2 A.
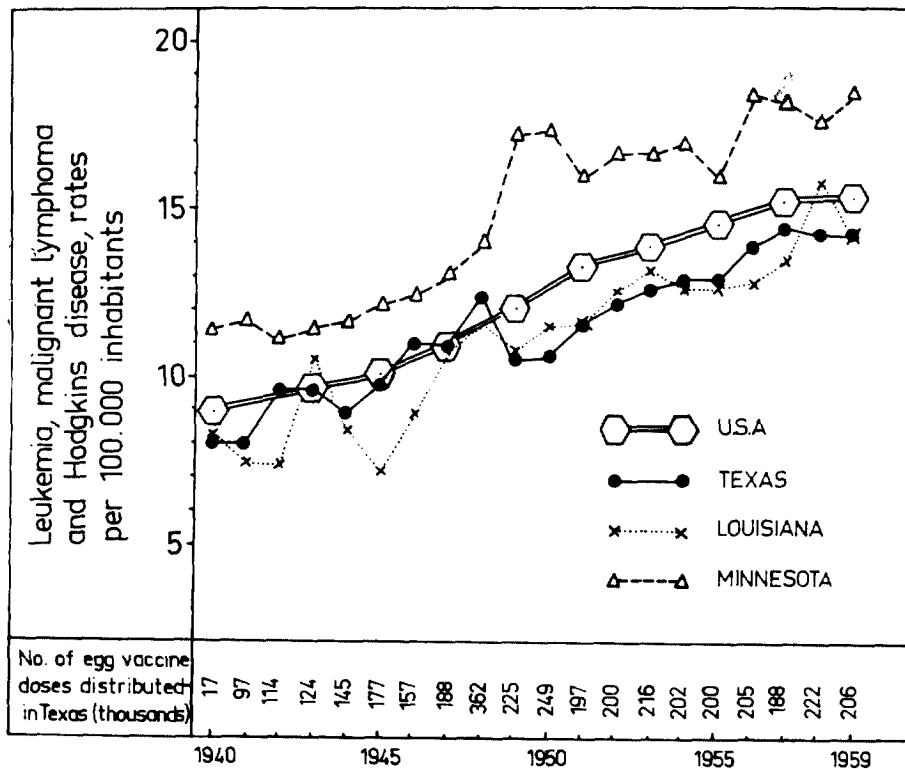
Figure 3

Morbidity data of leukemia, malignant lymphoma and Hodgkins disease in Texas 1940–1959. The number of egg vaccine doses issued in the state during this period is indicated. Camparative morbidity data are given for two states where egg vaccine is not generally used. For sources of data, see text.

SUMMARY

Smallpox vaccine of chick embryo origin was tested in small vaccination trials already at the beginning of the 1930s. Presently it is used on a large scale mainly in Texas, Brazil and Sweden. For vaccines at a low egg passage level the efficacy, as judged from quantal and quantitative responses, serological responses and protection against smallpox, seems to be as good as that of traditional calf lymph vaccine.

As to safety control the possible danger of contamination with avian leukosis virus has posed the most important problems, although other potentially hazardous contaminants are also known. Data are presented to illustrate that this risk is probably small. Nevertheless leukosis control should be incorporated in the routine control procedure. It is proposed that the COFAL or the immunofluorescence techniques be used to control uninoculated sublots of egg batches used for production.

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THE CLINICAL EVALUATIONS
OF THE CVI VACCINIA STRAIN
BACKGROUND AND STATUS

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In the areas of the world where smallpox is endemic and where as many as 100,000 cases of variola major per year carry a fatality rate of approximately one-third, vaccination continues to be of infinite importance as a public health measure. On the other hand, in countries where the disease has been eradicated and new introductions are prevented or limited by adequate quarantine measures, the complications of vaccination represent a far greater liability in morbidity and mortality than exists in the risk of incurring the disease itself.

Between 1961 and 1965 there were 31 deaths in the United Kingdom from smallpox vaccination while there were only 26 cases of smallpox (Dick, 1967). In the United States, a national survey of complications from vaccination for the year 1963 (Neff, et al, 1967a) showed 7 deaths in that year alone of more than 240 since the last fatal smallpox case in 1948 (Kempe, 1968b). Of about 6 million primary vaccinations and nearly 8 million revaccinations in 1963, 433 vaccine-associated illinesses were reported, including 12 with postvaccinal encephalitis, 9 with vaccinia necrosum, 134 with generalized vaccinia, 111 with eczema vaccinatum, 115 with accidental infection and 52 with other complications. Age was an important factor in this survey since the highest complication rate, at about 150 per million, was among those under one year of age. There was evidence in a companion study (Neff, et al, 1967b) that the incidence of generalized vaccinia and eczema vaccinatum may actually range as much as ten-fold higher, and accidental inoculations perhaps thirty-fold higher.

Approximately two-thirds of these complications were considered theoretically preventable, including most of the cases of eczema vaccinatum and vaccinia necrosum, by more careful screening for known

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contraindications for vaccination and by the exercise of a more sanitary technique in the vaccination process. In this respect, the principal risks lie with infants and young children with a current or past history of eczema or other skin diseases. For these individuals, the essential prophylactic has been to avoid vaccination and restrict contacts with vaccinated individuals. The former is a difficult practice in the face of the requirements for vaccination certificates for travel; the latter is virtually impossible in the intimacy of the family situation.

As a possible alternative to simply avoiding vaccination of these individuals and continuing their risk, it has been reasoned that if their first exposure to vaccina is transformed to a situation simulating revaccination, then the incidence of complications will be minimized. The active or passive induction of a basic immunity prior to regular vaccination appears to have accomplished this effect. Nanning (Nanning, 1962) has shown that a low level of passively induced antibodies can yield a four-fold decline in the number of cases of postvaccinal encephalitis after regular primary vaccination. This procedure mimics the effect by which an active transplacental maternal immunity is known to prevent reactions to primary vaccination in the very young infant.

A comparable approach has been taken through active immunization, although the success with killed vaccines has sometimes been equivocal (Kaplan, et al, 1965; Kaplan, 1969). The use of live vaccinia virus of low pathogenicity has alternatively shown promise of supplying the needed basic immunity to these individuals, and to this end, derivatives of the Rivers strain, attenuated by serial passage in chick embryo explant cultures (Rivers, et al, 1933) have been effective in actual clinical trials. The second-revived strain, CVII, with approximately 180 additional passages in ckick embryo cultures, has been used in the Netherlands for primary immunization by scarification of 60,000 military recruits (van der Noordaa, et al, 1967), producing one mild case of postvaccinal encephalitis; whereas the lowest frequency obtained with the contemporary lymph vaccine was at least three to four-fold higher. Less fever and milder reactions were seen with the CVII vaccine than with lymph strain, and the level of neutralizing antibody was reduced; however immunity was adequate to meet a later challenge with regular vaccinia in terms of the comparability of the post-challenge skin reactions.

In the United States Dr. Kempe of the University of Colorado has been carrying out extensive comparable studies for many years, primarily in infants and children, with a further-passaged derivative of the first-revived Rivers strain (CVI). In a preliminary study (Kempe, 1968a) approximately 600 children with eczema were vaccinated either by multiple pressure ($10^{8.4}$TCID$_{50}$ per ml) or subcutaneously with doses varying from 1,000 to 30,000 TCID$_{50}$. Compared with normal children given regular vaccination with standard lymph, they showed less febrile reactions and no complications; the antibody conversions were comparable. A second study (Kempe, et al, 1968c) expanded the population to appro-

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ximately 1,000 children and confirmed the earlier results. Under the screen of demonstrable seroconversion, some of these children were given regular vaccine six months later and again there were no complications.

Wyeth Laboratories have been collaborating with Dr. Kempe in the continuing expansion of this clinical trial designed to enforce the confidence that this strain can indeed be used effectively for elective preliminary vaccination of children with eczema and other skin disease for their protection against the complications attending elective or accidental contamination with standard lymph vaccine. This report describes the features of this continuing program and reports its progress to date. We are grateful to Dr. Kempe for his permission to present the interim data which have been collected and analyzed to the present time.

Vaccine Preparation and Characterization

After the first revival of the Rivers strain by testicular passage, the CVI strain was passaged 59 times in chick embryo explants, followed by 19 passages in the chick embryo choriallantoic membrane (CAM). This derivative, called CVI-78, comprised the starting virus pool for the preparation of production seed in fertile eggs taken from a closed, leukosis-free chicken flock. For each passage, approximately 10^4 pock-forming units (PFU's) are inoculated on the CAM of 12-day old embryos. The infected membranes are harvested after 48 to 56 hours at 36^0 C and held at -20^0 C until tests of the embryos and extraneous embryonic fluids from each egg are negative for COFAL antigen. The membranes are then processed in an appropriate stabilizing medium by homogenization and eventually lyophilized in the same way as the corresponding calf lymph preparation. The titers of the harvest pools are generally between $10^{8.0}$ to $10^{8.5}$ PFU's per ml and the pools are tested at these concentrations for sterility and safety by procedures which are generally consistent with those employed in the safety evaluation of contemporary egg and calf-lymph vaccines

The identity of the CVI strain as a reflection of its high-passage history can be clearly distinguished from calf-lymph strains, or early egg passages of these strains, in terms of decreased pathogenicity for human and rabbit skin and several tissue culture characteristics. In a standard rabbit potency test the CVI strain titers from 6- to 100-fold lower than the calf-lymph standard, even though the CAM titrations of the strains are equivalent. The analogous situation in human skin may be seen in Table 1 comparing the responses of subjects vaccinated in one arm with comparable titers of the standard calf lymph vaccine (CLV) and on the other arm with titers either CVI or the calf-lymph strain after 31 CAM

Table 1

Response of Vaccinated Adults to Revaccination with CVI Strain,,
Low Egg-Passaged Lymph (CLV-P31) and Control Calf Lymph Vaccine

	CLV P31	CVI
Number vaccinated on both arms	9	61
Major reaction with both vaccines	6	11
Major reaction with Control Vaccine[a] *only*	0	33
Major reaction with test vaccine *only*	1	0
Equivocal reaction with both vaccines	2	17

[a]Routine market production lot of lyophilized calf lymph vaccine.

passages (CLV P31). With allowance for the subjective nature of re-cording these responses, it is evident that the CAM-lymph strain read against the lymph control clearly showed a significantly reduced incidence of major reactions: CLV P31 evoked 7 major reactions, and only 6 of the 9 subjects also showed a major reaction to the control vaccine; whereas only 11 of 61 showed a major reaction with the CVI strain, a level representing one-third of the lymph control rate.

A marker correlating with this decreased skin avidity has been established in tissue culture systems (Ellis, et al, 1967). A study was made of the comparative sensitivities of vavious primary and cell-line tissue cultures from different sources to the CVI and lymph strains (CLV), including other low egg-passaged derivatives. Generally in these sy-

Table 2

Comparative Tissue-Culture Titers
of CVI Strain versus Calf Lymph Virus

Cell	Titers ($\log_{10}$)	
	CVI	CLV
HEK	4.15	8.28
BK	6.12	7.62
WI-38	6.16	7.73
CEF	6.84	7.61
HA	6.95	8.31
GMK	7.34	8.43
PRK	7.15	8.20
RK13	7.52	7.52

stems the strains showed (Table 2) equivalent titers in the serial rabbit cell (RK 13), or constant titer ratios favoring somewhat the calf-lymph virus (bovine kidney, WI-38, chick-embryo fibroblast, human amnion, primary rabbit kidney, and green monkey kidney cells). On the other

hand, in human embryo Kidney cells (HEK) there was a marked reduction in the CVI titer as compared to those of the unattenuated or low-passage strains. In routine use as a marker, the 3–4 $\log_{10}$ difference in CVI titers between HEK and green monkey kidney cells (GMK) is quite reproducible in respect to replicate testing (Table 3) and over at

Table 3

Comparison of the Sensitivity of HEK to Various Vaccinia Virus Preparations

Virus	Experiment 1		Experiment 2	
	HEK	GMK	HEK	GMK
CVI–79(Wyeth)	3.7	7.4	4.3	7.5
CVI–85 (Wyeth)	4.7	8.2	4.3	8.2
Lederle (avianized)	9.0	8.8	8.6	8.8
Texas (avianized)	9.0	8.7	>9.0	8.8
Dryvax (Lot 209102)	8.3	8.8	8.5	8.8

HEK = Human embryonic kidney
GMK = Cercopithecus monkey kidney

Table 4

Response of Chicken Embryo CAM-Propagated Vaccinia Viruses to the HEK/GMK Marker System

	HEK	GMK
	$Log_{10}TCID_{50}$	
CVI	3.5	7.5
CVI P10	4.0	7.6
CVI P34	3.7	8.5
CLV P10	8.2	8.4
CLV P31	8.4	8.8
LE	8.9	8.7
TS	8.9	8.8

CVI   = Wyeth attenuated vaccinia virus (2nd passage from Kempe seed)
CVI P10 = Wyeth attenuated vaccinia virus (10th passage from Kempe seed)
CVI P34 = Wyeth attenuated vaccinia virus (34th passage from Kempe seed)
CLV P10 = Wyeth calf lymph vaccine (10th passage in chicken embryo CAM)
CLV P30 = Wyeth calf lymph vaccine (31st passage in chicken embryo CAM)

least 34 passages of the CVI strain (Table 4). In other culture systems, the CVI strain gives rise to smaller pocks on the CAM than lymph strains or low egg-passaged derivatives, whereas CVI plaques on RK13 cells are nearly twice in size as those produced by the corresponding less-passaged strains. These findings suggest that the CVI strain uniquely has an altered capacity to replicate in certain tissue systems.

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The plan for studying immunization of unvaccinated subjects with the CVI strain has included the search for possible differences in clinical reactions to vaccination by routine multiple pressure versus subcutaneous administration and the effects on corresponding antibody responses; the evaluation of the reactions of the subjects to subsequent routine vaccination with smallpox vaccine lymph and determination of optimal time for this procedure; and the study of the duration of antibody response and immunity following the prevaccination process only. These answers would provide a working basis for carrying out the elective immunization of eczematous children with safety; and to this end, the clinical studies carried out to date by Dr. Kempe and his collaborators have considerably expanded those which have already been reported (Kempe, 1968a; Kempe, et al, 1968c). With the cooperation of approximately thirty physicians, mostly from Denver and surrounding areas but including also other points in the United States and abroad,

Table 5

Diagnosis	MP(1)	Route of Administration					NR(3)	Totals
		Jet-Gun		SC(2)				
		1:100	1:1000	1:100	1:1000			
Skin Disease								
Primary Vaccination	203	87	652	47	376		12	1377
Revaccination	41	10	90	21	67		0	229
Normal								
Primary Vaccination	248	7	114	3	86		4	462
Revaccination	20	0	41	6	32		0	99
Other								
Primary Vaccination	20	4	90	10	27		1	152
Revaccination	11	0	11	2	12		0	36
Diagnosis Not Reported								
Primary Vaccination	9	4	9	3	9		2	36
Revaccination	2	0	4	1	0		0	7
Totals								
Primary Vaccination	480	102	865	63	498		19	2027
Revaccination	74	10	146	30	111		0	371
NR(4)	12	2	29	3	5		2	53
Total	566	114	1040	96	614		21	2451

(1) Multiple pressure
(2) Subcutaneous
(3) Route of administration not reported
(4) Whether primary or revaccination not reported

and drawing upon the patient-panels of many more cooperating physicians, the results from approximately 2,500 subjects have now been assembled in a computer-based pool of data for examination and analysis.

The subject of these studies were usually outpatients, and generally the parents were provided with forms for recording post-vaccination responses. These follow-up data were then analyzed by Dr. Kempe who provided the assessment of the subjective comments on these reports, and the data were transferred to IBM cards for permanent recording of the key information in the vaccination process, the local and systemic responses, and in some cases the resulting antibody responses.

Table 5 provides a distribution of 2,451 subjects by diagnosis, route of vaccine administration and whether the CVI preparation was given as a primary or revaccination procedure. The skin-disease category primarily includes individuals with chronic or subacute eczema; however only a small number of these (10 subjects) were vaccinated when showing acute, weeping infantile eczema. A small percentage had other skin diseases, such as ichthyosis or allergic dermatoses, or only a history of eczema but were clear at the time of vaccination. The »other« category includes subjects with a history of allergic disease not diagnosed as eczema but for whom regular vaccination had been withheld.

Multiple pressure administration (5–6 pressures with the bifurcated needle) utilized fluid prepared by reconstituting a unit of lyophilized virus 0.3 ml of glycerinated diluent; for sub-cutaneous use, the same amount of virus in 30-ml of water provided a »1.100« dilution which was administered in either 0.1 or 0.3 ml volumes by syringe or jet-gun. A further 10-fold dilution yielded the »1.1000« preparation used in the same sub-cutaneous doses. CAM titrations of these dilutions have shown the following correspondence: undiluted, $10^{7.2-8.4}$; 0.3 of 1:100, $10^{4.7-5.4}$; 0.1 of 1:100, $10^{4.2-4.9}$; 0.3 of 1:1000, $10^{3.7-4.4}$; and 0.1 of 1:000, $10^{3.2-3.9}$. The range in each case reflects the variability of titers from containers kept in frozen storage over the three-year period during which these trials have been carried out. Of the subcutaneous doses, 0.1 ml of either the 1:100 or 1:1000 dilutions was most commonly employed.

The largest group of subjects in this study have been in the skin disease category and most of these received either undiluted vaccine by multiple pressure or 0.1 ml of the 1 : 1000 dilution sub-cutaneously by either jet-gun or syringe and needle. The subjects range in age from less than one year to 78 years; however approximately two-thirds of those given primary vaccinations were between 1 and 5 years of age; less than 5% were less than one year of age; and 30.5% were 6 years of age or older.

Local reaction are shown in Table 6 for the 1,377 subjects with skin disease receiving primary vaccination. A 0+ reaction was recorded

Table 6

Skin Disease – Primary Vaccination

Route	Local Reaction					Total
	0+	1+	2+	3+	NR(4)	
MP(1)	79	79	25	10	10	203
Jet–Gun						
1:100	31	36	14	0	6	87
1:1000	360	161	28	9	94	652
SC(2)						
1:100	33	10	3	0	1	47
1:1000	281	56	14	5	20	376
NR(3)	5	4	2	1	0	12
Total	789	346	86	25	131	1377

(1) Multiple pressure
(2) Subcutaneous
(3) Route of administration not reported
(4) Severity of local reaction not reported

where there was no evidence of skin response or it was »equivocal« by WHO criteria. At the other extreme, 3+ describes the usual response following multiple pressure administartion of regular calf lymph vaccine to primary vaccines. The other responses are intermediate in respect to vesicle size or induration and surrounding erythema. It is noteworthy in Table 6 that, by any route of administartion, most of the subjects for whom responses are recorded showed either a minimal or mild local response. Less than 5% showed a response as severe as that normally following routine calf lymph vaccination, and the greatest contribution to this category came from the multiple pressure group. This may possibly reflect the higher titer of administered virus.

In Table 7, the incidence of systemic responses in the same group of subjects is shown. The gradings of severity are generally arbitrary: a 0+ response reflects either no symptoms or minimal effects of short duration not interfering with normal activity; whereas a 3+ systemic response indicates anorexia, possibly vomiting, malaise and lassitude that interfere with normal activity for a day or more during the period of maximum response, which occurs 6 to 10 days following primary vaccination. Of the total 1,377 primary vaccinees in this category, 73% had either no manifestations or only minimal responses; whereas less than 1% (11 subjects) had a 3+ systemic reaction. Contribution to this latter category were seen for both routes of administration.

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Table 7

Skin Disease – Primary Vaccination

Route	Systemic Reaction					Total
	0+	1+	2+	3+	NR(4)	
MP(1)	152	32	5	3	11	203
Jet–Gun						
1:100	73	4	2	0	8	87
1:1000	474	70	7	2	99	652
SC(2)						
1:100	37	8	0	1	1	47
1:1000	261	75	14	5	21	376
NR(3)	10	0	2	0	0	12
Total	1007	189	30	11	140	1377

(1) Multiple pressure
(2) Subcutaneous
(3) Route of administration not reported
(4) Severity of systemic response not reported

Table 8

Skin Disease – Primary Vaccination

Route	Maximum Temperature Response					Total
	97.0–99.0	99.1–100.9	101.0–102.9	103.0+	NR(4)	
MP(1)	103	64	18	0	18	203
Jet–Gun						
1:100	33	37	8	0	9	87
1:1000	235	247	47	5	118	632
SC(2)						
1:100	28	10	6	2	1	47
1:1000	171	75	42	4	84	376
NR(3)	3	5	3	0	1	12
Total	573	438	124	11	231	1377

(1) Multiple pressure
(2) Subcutaneous
(3) Route of administration not reported
(4) Temperature not reported

Table 9

Primary Vaccinations

Post CVI Antibody Titer

Route	<10			10–39				40–159				160			Subtotals				Totals
	D[1]	N[2]	O[3]	D	N	O	NR[4]	D	N	O	NR	D	N	O	D	N	O	NR	
MP	2	0	0	28	2	5	0	50	8	3	0	4	2	0	84	12	8	0	104
Jet–Gun																			
1:100	0	0	0	4	0	0	0	2	0	1	0	0	0	0	6	0	1	0	7
1:1000	0	0	0	6	1	2	0	2	0	0	0	0	0	0	8	1	2	0	11
SC																			
1:100	0	0	0	14	1	5	2	24	2	5	1	4	0	0	42	3	10	3	58
1:1000	1	0	1	53	17	4	0	48	8	3	3	1	0	0	103	25	8	3	139
Subtotal	3	0	1	105	21	16	2	126	19	11	4	9	2	0	243	41	29	6	319
Total	4			144				160				11			319				

1. Disease
2. Normal
3. Other
4. Disease category not reported

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The febrile responses among the population of vaccinees with skin disease are shown in Table 8. Although the reliability of temperature recording by the parents as instructed in the program is open to some question; it is nevertheless highly significant that only 11 of 1,146 subjects for whom temperatures were actually recorded had a maximum elevation of 103° F or greater. This rate is considerably less than the frequency following standard smallpox vaccination and other commonly used immunizing systems such as pertussis vaccine or measles virus vaccine.

In Dr. Kempe's laboratory, serum neutralizing antibody determinations were carried out for a number of subjects in the study utilizing the disc plate assay method (Minamitani, 1968). In this test, a zone of inhibition produced by the immune blood applied with a paper disc to infected tissue cultures in Petri dishes is measured and the equivalent titer or corresponding serum dilution is then estimated by reference to a standard inhibition curve produced by dilutions of a reference serum of known titer determined by other conventional techniques. Dr. Kempe has compared titers obtained by this assay with those obtained by other standard neutralization tests and has shown comparable results. Primary vaccines were bled 1 to 3 months after CVI vaccination, and the antibody levels determined were compared with those in normal children vaccinated with standard lymph by multiple pressure and assayed ren vaccinated with standard lymph by multiple pressure and assayed by the same technique. Among the latter, the geometric mean neutralizing titer of 43 normal children was 41.5. This value may may be compared to the post-primary immunization antibody titers in Table 9 of eczematous subjects by route of administration. After vaccination, only four individuals had antibody titers of <1:10; of 319 subjects, 304 had antibody titers in the range of 1:10–1:159 regardless of the route of administration.

Approximately 400 subjects who received as a primary, prevaccination procedure have been revaccinated with standard calf lymph (Dryvax) and about 3/4 of these were in the skin disease group. All standard vaccinations were carried out by multiple pressure, some as early as 1 month following primary vaccination, but mostly, between 1 and 6 months (240 at 1–3 months; 147 at 6 months) and a few at 12 months (16). Local responses in 203 subjects were mostly classified in the 0+ or 1+ categories (97%) with only 6 showing a 2+ response, and only 1 at 3+. In the last case, the subject had been revaccinated 9 months after the primary treatment.

Only four complications have been observed in this entire study population: two children developed erythema multiforme following the primary course; one case was mild, the other moderate, lasting for four days (Kempe, et al, 1968c). One eczematous child of 5 years given primary vaccination with CVI developed an auto inoculation lesion of mild severity on the knee–the primary vaccination site had not been covered

in this case. Finally, an eczematous adult female was revaccinated with standard vaccine by multiple pressure one year after CVI vaccination, and a mild case of generalized vaccinia resulted which responded rapidly to a single dose of vaccinia-immune globulin. Her child, similarly vaccinated on the same schedule, showed no unusual response. Based upon current estimates of the frequency of eczema vaccinatum among patients with eczema and other skin diseases, the approximately 1,400 subjects at risk and undergoing primary CVI vaccination in this study might have been expected to yield 15 to 20 cases of this complication under regular vaccination, a number thus considerably higher than the actual incidence with the CVI strain.

The authors acknowledge with thanks the excellent technical services of Miss Helen L. Ellis and Dr. A. K. Fontes in tissue culture studies and viral preparation.

SUMMARY

The backgroung and current status of the clinical study by Dr. C. H. Kempe, University of Colorado Medical School, and his associates, on the elective vaccination of subjects with eczema and skin diseases with the CVI further-attenuated vaccinia strain is reviewed. The results to date continue to support the thesis that the risk of complications attending the standard immunization of this population with regular vaccine lymph may be significantly lessened if the procedure is carried out under the cover of immunity induced by the attenuated strain. Pre--vaccination with the CVI-78 vaccine is accomplished with a reduced incidence of the local and systemic reactions that generally occur in even normal individuals with standard non-attenuated vaccines, and the antibody levels which are induced by the preliminary procedure are equivalent and appear to be adequate to protect this population at greatest risk against the complications which can follow accidental or elective contact with the standard vaccine virus.

Some *in vitro* characteristics of the CVI vaccinia strain which correlate with its reduced avidity for human and rabbit skin and provide markers of the degree of its attenuation are reported.

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NON-INFECTIOUS SMALLPOX VACCINES

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Janson was the first who tried to induce immunity to vaccinia infection with heat-inactivated vaccine (1891). His work stimulated a series of investigations with the same issue. The results were mostly doubtful since the methods available to inactivate the virus or to prove the antigenicity of the non-infectious preparations were far from being sufficient. Either the vaccine had been exposed too long to the inactivation process, or too short. Tests in common use now to demonstrate the immunogenicity of the preparations are more sensitive than previously. Whilst the interest into inactivated vaccines seemed to be more academic (questions of immunity took priority), it was focused later into the prevention of postvaccinal encephalitis. Already in 1929 HOOKER suggested the application of inactivated vaccines for the prophylaxis of neural postvaccinal complications.

I. FREEDOM FROM LIVE VIRUS

A perequisite to achieve that goal is a potent *inactivated vaccine, free of residual living virus.* As the former requirement may be more easily fulfilled than the latter, it seems to be of interest to discuss this problem.

Most of the authors claimed that their inactivated vaccine did not contain any living vaccinia virus. AMIES drew attention to the fact that an extremely small dose of virus, far below the amount required to produce a clinical infection is sufficient to evoke an immune response. In studies on the kinetics of inactivation of the vaccinia virus by formaldehyde, he found that the rate of inactivation did not remain constant,

but decreased continuously in the course of the process, a phenomen already known from the work carried out with poliovaccine. According to AMIES, formaldehyde-inactivated vaccinia virus is of no practical value as an immunizing agent. As an argument for this thesis he takes the preparation of BEUNDERS et al. (1960), which lost its antigenic proparties when stored for a few weeks.

There is no doubt that the aim of a total inactivation of vaccinia virus and obtaining a good immunizing agent is problematic. *Kühn* and *Deutsch* (1964) observed pustule formations twice (with isolation of the virus in one case) after the application of the formalin-inactivated preparation. But if one considers the fact that this happened after the distribution of 100 litres of the vaccine, one will agree with the authors that the inactivation process has progressed to the point when it was measurable. The experiments of AMIES himself can be taken as a proof that this minimal amount of active virus is not harmful to the patient. It can only stimulate a still higher degree of immunity as suggested by his own experiments. *Collier* et al. (1955) showed, on the other hand, that even the addition of small doses of living vaccinia virus to their inactivated vaccine in excess of those that might have been given accidentally, did not modify the immune response; thus this effect was not due to traces of living virus that had escaped detection. From our own experience we can say that only at the very beginning of the production of our preparations we had a similar clinical problem only once.

II A. ANTIGENICITY OF NON-INFECTIOUS VACCINE

Of more concern is the antigenicity of the inactivated vaccine. In a recent study with *Wirahadiredja* (in the press), we compared 3 formaldehyde-inactivated vaccines from three European producers. Though we used many guinea-pigs, we were in general unable to obtain HI-antibodies after i. m. and i. p. immunization with 1 ml each of the vaccines and during a serological follow-up over a period of nine weeks. In contrast we could show marked differences by comparing the action of the vaccines in the rabbit following a challenge with living vaccinia virus. The comparison of 2 preparations concerning the *evolution of the vaccinal lesions on the 3rd and 4 th day after the vaccinia infection* by a neutral observer showed a marked acceleration of the vaccinal process in one of the preparations, thus proving a better antigenicity. We feel, therefore, that this method seems to be a good mean for testing the antigenicity of non-infectious smallpox vaccines (for details, see *Wirahadiredja* und Ehrengut, 1969).

Another attempt to prove the potency of inactivated vaccines is the *repeated immunization of animals* and a *check-up of the serological response*. A massive immunization programme (multiple intra-dermal injections followed by intravenous injections, see *Madeley*, 1968) is ne-

cessary to achieve an antibody spectrum comparable with that following live virus vaccination. Qualitative differences can only be detected by immunodiffusion tests (see *Madeley*, 1968).

Beunders et al. (1960) have shown in humans that only 5 to 4 injections of formalin-inactivated vaccines are necessary to achieve antibody formation; further injections being without additional effect. Allergic reactions may be expected.

A further approach to solve the problem is the *challenge of pre-immunized rabbits to the otherwise lethal action of rabbitpox virus. Madeley* (1968) has used this model to demonstrate the antigenicity of his preparation. None of his pre-immunized animals died following the challenge with the rabbit-pox virus, and none of them showed any viraemia, which normally follows the fatal virus infection.

In 1961 we performed *immunization with non-infectious vaccinia virus in previously vaccinated elderly persons* in order to attenuate their revaccination reaction. Thus their *antibody titre is boosted*. This action can be used also as a test of the immunogenicity of the non-infectious vaccine (see also Kaplan, 1962, Daneš, et al., 1966, Bonde and Hübert, 1968, Kaplan, 1969).

II B. REASONS FOR ANTIGENIC DIFFERENCES OF THE VARIOUS PREPARATIONS

a) *Excessive exposure to the inactivating agent* (formaldehyde, heat, U.V.-light)

According to Turner and Kaplan (1968) in the photo-dynamic inactivation the vaccinia virus will resist an even greater degree of »overkill« more than three times without complete destruction of the antigenicity. Therefore, this kind of vaccines seems to be very suitable, they were antigenically more potent, for they bound more antibody than formol-inactivated preparations *(Wallis* et al., 1967).

b) *Inadequate exposure to the inactivating agent* may be followed by residual living virus in the vaccine and thus simulate an antigenicity which normally inactivated vaccine does not posses.

c) *Insufficient potency of the living vaccine before inactivation. McNeill* (1966) claims that this is the main reason for the discrepancy in literature, as far as the antigenicity of the various preparations is concerned. According to this author, the initial titre of the product has to have aproximately 10^8 p. f. u./ml. before inactivation. We have used lower titres too, without obtaining insufficient vaccines.

III. MODE OF ACTION OF NON-INFECTIOUS VACCINES

Kaplan (1969) has expressed his doubts on the antigenicity of formol-inactivated vaccines, since it does not stimulate »much neutralizing antibody«. We were able to show that in children, preimmunized with

the formol-inactivated vaccinia virus, vaccinal antibodies can be detected after immunization with the live virus a few days earlier than in normal primary vaccinees. We suppose that immunocytes are sensitized due to the former contact with the dead virus, thus the earlier serological response might be explained. In a further experiment we checked the first appearance of the »vaccinal allergy« by applicating formol-inactivated virus intracutaneoulsy. A placebo-preparation (containing no virus antigen, but all the orther parts of the non-infectious preparation) was applied at the same time (Ehrengut, 1968). As we have demonstrated in a series of experiments »vaccinia antigen« is capable in a specific way, of inducing allergy of the delayed-type within 24–36 hours. One can see a marked local infiltration with red on the injection spot in primary vaccinees on the fifth to sixth day after infection with live virus (Ehrengut, 1968). The placebo preparation gave a traumatic reaction, which subsised already on the second day of post-injection.

In contrast, children pre-immunized with 0,3 ml. »Vaccinia antigen« before the normal Jennerian vaccination, in 10 cases (out of 24, see Table 1) showed the phenomenon of »vaccinal allergy« on the fourth day after infection. Due to the *more prompt appearance of the vaccinal allergy*, the *postvaccinal viraemia* may be shortened, as suggested by experiments of *Ehrengut* (1959) and *Spiess* et al. (1966). This could be of some importance in the prophylaxis of post-infectious encephalitis.

IV. CLINICAL REACTIONS FOLLOWING APPLICATION OF NON-INFECTIOUS VACCINE

An extensive study of the clinical reactions in immune and non-immune subjects against smallpox after the injection of »vaccinia antigen« has been made by *Ehrengut* (1968). The first precise description of the local reactions following smallpox vaccination in children, pre-immunized with the non-infectious vaccine has been given by *Herrlich* und *Ehrengut* in 1959, as well as by *Beunders* et al. (1960). Summarized, we find in general a *marked acceleration of the local evolution of the vaccinal lesions* (quicker scab and »area« formation), a picture, which can likewise be expected in revaccinees on the seventh day of post-infection. These phenomena can be seen also in the animal experiment (see above).

The *general reactions* do not differ too much from normal primary vaccinated individuals. One quarter to one third of them do not show any fever reaction at all. Using the Hamburg vaccine strain 9,8 to 19,3% of the individuals in different age-groups have high fever (over 40,5%, see Table 2a). The duration of the vaccinal fever in various age-groups is depicted in Table 2b (711 measurements by the parents). We recommend bed-rest to individuals over 5 years of age from the 4th to the 12th day and abstinence from sports until the 21st day after vaccination.

Besides, a certain percentage of pre-immunized subjects demonstrates *hyperergic reactions* like the »Hügelreaktion« (Ehrengut, 1959). Here

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we find a tiny »hill« surrounding the pustule due to a marked infiltration of the skin, which may look like an abscess, but which does not fluctuate. The hill-reaction occurs more often in older individuals (see Table 3) Around the 17th postvaccinal day, a *vaccinal ulcer* in some of the patients with »Hügelreaktion« develops. As in BCG-vaccinated patients the *incidence of ulcer development* is also dependent on age (see Table 3). This is the drawback of the combined method. But since we have found a new *therapy for this kind of complication (Ehrengut,* 1964), we are not too much concerned with it. In general, by *desensitization with non-infectious vaccines*, the ulcer heals promptly within 2–3 weeks, while in controls without therapy the healing can only be expected 6–8 weeks later. We have the impression that *postvaccinal exanthema* occur a little more often after the combined vaccination than after the normal immunization.

V. FIELD OF APPLICATION FOR THE NON-INFECTIOUS VACCINE

a) The domain of non-infectious vaccinia virus is the *older primary vaccinee who is much more prone to postvaccinal encephalitis* (see *Herrlich*, 1965). Over 300 000 portions of »vaccinia antigen« had been distributed in West Germany *(Herrlich,* 1967), and there were only 6 reports of neural complications, all of them without sequelae. In East Germany the dead vaccinia virus is already compulsory on children over 3 years of age. Around 200 000 patients have been vaccinated there with the combined method: 11 cases of encephalitis were reported, one of them fatal, another one with sequelae *(Rohde,* 1968). The last-mentioned child did not keep bed-rest and fell ill after some outdoor activities.

Lindemann and *Buser, Dostal* as well as *Kaplan* postulated a field trial with and without pre-immunization with killed non-infectious vaccine. The data of *Berger* and *Puntigam* (1954), of *Herrlich* et al. (1956) and *Seelmann* (1960) give an ambiguous indication that older persons are more prone to postvaccinal encephalitis. From the ethical point of view, therefore, a field trial cannot be performed anymore *(Ehrengut,* 1962, *Herrlich,* 1965). Among 15 600 primary vaccinations[*] we carried out with the combined method (since our report in 1959) we had only 1 case of a passing encephalitis (a 12 year old boy with drowsiness and increased cell count in the spinal fluid) without sequelae until now, whilst among about 60 primary vaccinees of the same age, who were vaccinated due to misinterpretation of the scar or the vaccinal status (falsified vaccination certificate), there was already one serious case of encephalitis.

[*] Among them were 4093 subjects 0– 3 years of age
                    3350 subjects 4– 6 years of age
                    1518 subjects 7– 9 years of age
                    1909 subjects 10–12 years of age
                    4730 subjects and more years of age

On the other hand no *field trial* has been made *in smallpox endemic areas* to prove the safety of the combined vaccination method. At least theoretically, untoward reactions could be provoked after a contact with smallpox in thus vaccinated persons. Fortunately, an experimental study was performed by *Danes* et al. (1966) in rhesus-monkeys. It could be shown that animals immunized with a single dose of non-infectious virus did not behave in an unusual way; they were susceptible to infection, yet no viraemia could be established and the variola virus could only be detected on the site of infection. It is therefore a justified hope that the combined method of vaccination will give no awkward reactions in contact cases and will confer immunity against smallpox in the same degree as the normal vaccinia infection is able to do.

The argumentation of *Kaplan* (1969) that the formalin-inactivated vaccines are probably without value since the incidence of encephalitides in Great Britain is less (1 : 30 000 without pre-immunization) than in East Germany (despite pre-immunization 1 : 20 000), does not seem to be justified. We know that in both countries different systems of notifications exist, therefore in East Germany a higher number of notified vaccinal complications may be observed. Our own figures point to a much higher incidence of postvaccinal complications and we are quite sure that »vaccinia antigen« is of definite value in the prophylaxis of neural vaccinal complications. It remains to be seen whether an improved and more antigenic preparation will give better protection.

b) We also use »vaccinia antigen« in the »child at risk« like prematures, twinbirth, ABO-incompatibility, birth trauma etc. *(Ehrengut,* 1968b). In problematic cases we give one immunization with the non-infectious vaccine, a second one after one year and recheck the vaccinability later. Thus we have immunized just a few children (in addition we gave gammaglobulin on the day of live vaccination) without any complications.

VI. UNTOWARD REACTIONS IN REVACCINEES

One argument which might prevent a wider use of vaccinia antigen is the possibility of the development of abnormal reactions following revaccination in patients, immunized previously with the combined method. If similar allergic phenomena would occur like those following the use of killed measles vaccine in subjects later immunized with the live measles vaccine *(Fulginiti* et al. 1967, *McNair Scott* and *Bonanno,* 1967) this would represent a serious drawback to a routine immunization. Fortunately, we have never heard of such an accident, though we have personally revaccinated at least 2000 persons, immunized 3 to 10 years before with the combined method. The number of major reactions achieved is – according to our clinical impression – smaller than in normal revaccinees. The serological study of *Oberdoerster* et al. (1968) also

showed that no special measures have to be taken for subsequent small-pox vaccinations. The *immunity attained towards vaccinia is therefore satisfying.*

On the other hand no *field trial* has been made *in smallpox endemic areas* to prove the safety of the combined vaccination method. At least theoretically, untoward reactions could be provoked after a contact with smallpox in thus vaccinated persons. Fortunately, an experimental study was performed by Danes et al. (1966) in rhesus-monekys. It could be shown that animals immunized with a single dose of non-infectious virus did not behave in an unusual way; they were susceptible to infection, yet no viraemia could be established and the variola virus could only be detected on the site of infection. It is therefore a justified hope that the combined method of vaccination will giveno awkward reactions in contact cases and will confer immunity against smallpox in the same degree as the normal vaccinia infection is able to do.

Table 1

The first appearence of »vaccinal allergy« in primary vaccinees, pre-immunized with 0,3 ml non-infectious vaccine 8 days before and tested intracutaieously with 0,15 ml non-infectious vaccine 2 days after the vaccination with live virus

No.	N a m e	Age (years)	Intracutaneous test Size of erythema (cm) on the day after vaccination	
			third	fourth
1.	W. Andrea	9/12	0,3 × 0,3	0,8 × 0,8
2.	D. Holger	9/12	0,6 × 0,6	1,0 × 1,0
3.	M. Manfred	9/12	0,6 × 0,6	0,8 × 0,8
4.	J-Klaus-Peter	11/12	**	0,5 × 0,5
5.	D. Horst	11/12	0,4 × 0,3	0,7 × 0,8
6.	F. Andrea	11/12	0,4 × 0,4	0,6 × 0,6
7.	P. Petra	4/12	negative	**
8.	J. Stephanie	5/12	0,6 × 1,2	2,0 × 1,7
9.	M. Thomas	5/12	0,4 × 0,4	0,4 × 0,4
10.	S. Patricia	11/12	0,6 × 0,6	0,8 × 1,5
11.	H. Danya	10/12	0,8 × 0,8	1,2 × 1,7
12.	D. Rosemarie	8/12	0,3 × 0,6	0,8 × 0,8
13.	H. Uwe	9/12	negative	0,3 × 0,3
14.	N. Manuela	11/12	0,6 × 1,5	2,2 × 1,7
15.	B. Claudia	11/12	**	**
16.	M. Christina	11/12	0,8 × 0,8	1,2 × 1,8
17.	B. Heidemarie	15/12	0,6 × 0,7	1,6 × 1,3
18.	E. Lane	16/12	0,3 × 0,3	0,3 × 0,3
19.	H. Michael	1 11/12	0,6 × 0,6	1,2 × 1,4
20.	K. Andrée	1 8/12	**	**
21.	A. Sylvia	1 3/12	0,6 × 0,6	0,8 × 1,3
22.	W. Detlef	1 2/12	negative	**
23.	T. Elvira	1 3/12	0,8 × 0,8	1,0 × 1,2
24.	H. Helmut	1 11/12	negative	**

** = just visible;
Vaccinees No. 1–13 had been pre-immunized with Vakzineantigen Impfanstalt Hamburg, No. 14–24 with Vacciniantigen, Behring-Werke Marburg/Germany.

Table 2 a

*The general reaction in 719 primary vaccinees, pre-immunized with
non-infectious vaccine, according to age*

Age (years)	No. of vaccinees		fever*		
			− 38,5⁰ C	− 39,5⁰ C	− 40,5⁰ C
0 − 3	135	34 25,19⁰/₀	37 27,41⁰/₀	38 28,14⁰/₀	26 19,25⁰/₀
3 − 6	143	49 34,26⁰/₀	39 27,27⁰/₀	41 28,67⁰/₀	14 9,79⁰/₀
6 and above	441	111 25,17⁰/₀	126 28,57⁰/₀	135 30,61⁰/₀	69 15,64⁰/₀

* The temperature recorded was in every case the *highest* measurement during the
general reaction.

Table 2 b

Duration of vaccinal fever in 711 primary vaccinees, pre-immunized with
non-infectious vaccine*

Days	0 − 3 years			3 − 6 years			6 and more yearse		
	till 38,5⁰	till 39,5⁰	till 40,5⁰	till 38,5⁰	till 39,5⁰	till 40,5⁰	till 38,5⁰	till 39,5⁰	till 40,5⁰
0 − 2	65,71	32,35	42,30	61,11	58,13	35,71	56,00	44,36	31,34
3 − 4	22,85	55,88	50,00	33,33	30,23	21,42	28,00	41,35	47,76
5 − 7	11,42	11,76	7,69	5,55	11,62	42,85	16,00	14,28	20,89

* A temperature of 40,5⁰ C does not necessarily mean that the patient had permanent
such a temperature. Only the highest measurement of the temperature had been
evaluated.

Table 3

*Incidence of »hill-reaction« and ulcer formation in 1397 primary vaccinees,
pre-immunized with formol-inactivated vaccinia virus*

Age (years)	0 − 3	3 − 6	6 and above
No. of vaccinees	497	274	626
No. of »hill-reactions«	3	4	53
One »hill-reaction« per . . . . . . vaccinees	165	68	12
No. of vaccinal ulcers	⌀	2	9
One ulcer per . . . . . . vaccinees	⌀	137	69

SUMMARY

A review of the present status of non-infectious smallpox vaccines is given. Among the freedom of residual virus the antigenicity of the preparation is of main importance. Differences in the immunogenicity of 2 commercial preparations were demonstrated on the evolution of the local response in rabbits, pre-immunized with killed vaccinia virus and later challenged by live vaccine. Though HI-antibodies were not detected in the pre-immunized guinea-pigs, it could be shown in the primary vaccinees pre-immunized with non-infectious vaccine that the vaccinal allergy develops 2 days earlier than in normal vaccinated subjects. On the whole the action of non-infectious vaccines seems to be due to stimulation of immunocytes, thus vaccinial antibodies appear earlier in the pre-immunized vaccinee and the viraemia is shortened. As a drawback to the combined vaccination method more hyperergic reactions occur. There is no doubt that non-infectious vaccines are of definite value in the prophylaxis of postvaccinial encephalitis in countries where this malady is prevalent.

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INVESTIGATION AND EXPERIENCE OF APPLICATION OF SMALLPOX VACCINE INACTIVATED BY GAMMA IRRADIATION

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One of the methods of prophylaxis of postvaccination encephalitis which is used more and more extensively consists in combined vaccination using inactivated smallpox vaccine (Herrlich, 1959; Beunders et al., 1960; Ehrengut, 1966; Rohde, 1968).

The available evidence indicates that application of this method leads to a reduction in the incidence of postvaccination encephalites after primary vaccination of older age groups (Kühn et al., 1964; Herrlich, 1965, 1968; Ehrengut, 1966; Rohde et al., 1968; and others).

Among the methods of inactivation of vaccinia virus, treatment of the virus with formalin is used most extensively (Ramon et al., 1948; Herrlich, 1959; Amies, 1961; and others). However, the possibility of virus inactivation with Roentgen rays of gamma-irradiation of Co^{60} has been demonstrated (Cowen et al., 1939; Salman, 1947; Bektemirov, 1961; Kaplan, 1962; Svet-Moldavskaya et al., 1966; Danes et al., 1966).

In our studies the calf pulp with a titer of $5 \times 10^8 - 2 \times 10^9$ PFU/ml (strain EM–63) purified with freon–113 (arcton–66) was irradiated with gamma-rays of Co^{60} in a dose of 1.5–1.7 megarad. After inactivation the preparation was lyophilized.

Study of the antigenic activity of the resulting preparation demonstrated that after a single immunization of white rats with the inactivated vaccine antibody titers during one year of observation were only $1^1/_2 - 2^1/_2$ times lower than those after immunization of animals with an equal dose of live virus. Similar results were obtained in rabbits.

The immunogenic potency of the inactivated vaccine was studied on the developed model of »fatal vaccinal disease« in irradiated rats and mice (Svet-Moldavskaya, 1967) and in rabbits.

The inactivated vaccine protected animals against a fatal challenge dose 10–12 months after a single immunization. Rabbits immunized subcutaneously with a single dose of inactivated smallpox vaccine were resistant to 100 LD_{50} of neurovaccine 2 months after vaccination.

By its antigenic and immunogenic properties the preparation was superior to formalin-inactivated tissue culture smallpox vaccine.

After trial of the vaccine in a group of volunteers in which safety and antigenicity of the preparation was confirmed, the vaccine was used for combined primary vaccination of children. For this purpose, the dose of the inactivated vaccine was reduced 10-fold as compared with that used for volunteers and was 1 ml of 1 : 10 dilution.

At 2–4 weeks after subcutaneous immunization with the inactivated preparation immunization with EM–63 live vaccine with a titer of 2×10^8 PFU/ml was carried out by means of epicutaneous scarification, the number of cuts being reduced to one.

The combined method of vaccination was used for a total of 50 children between $2^{1}/_{2}$ and 9 years of age who had contraindications to ordinary vaccination against smallpox (24 children with organic disorders of the central nervous system, 19 children weakened by acute and chronic diseases, 7 children with exudative diathesis and other allergic diseases.

No side-effects in response to inoculation of the inactivated smallpox vaccine were observed.

Forty-eight children were bled before and 4–5 weeks after immunization with the inactivated vaccine. Seroconversion after the first stage of vaccination was 92%, with the geometric mean titer of 1 : 9.

The »take« rate of live vacccine in children receiving the inactivated preparation was 100%. Some mitigation of the intensity of the general and local vaccinal reaction was observed: no fevers above 39°C or marked lymphadenites were noted, the average febrile period was shorter, lasting only 1.6 days.

Some children were bled 4–5 weeks after inoculation with live vaccine. In all the cases 32–64–fold increase in virus-neutralizing antibody titers was demonstrated, the geometric mean titer being 1 : 800 after live vaccine inoculation.

The foregoing data indicate that by means of gamma-irradiation of Co^{60} an inactivated vaccine with sufficient antigenic potency may be obtained, and that in combined vaccination it produces high immunologic response.

The first experience of practical application of the inactivated preparation in combined vaccination against smallpox has established that this method may be successfully used for vaccination of older children who have contraindications to ordinary vaccination.

SUMMARY

A method for preparation of smallpox vaccine inactivated by gamma-rays of Co^{60} and results of investigation of the antigenic and immunogenic properties of the inactivated preparation are described.

By the antigenic and immunogenic properties the resulting preparation was superior to the formalin-inactivated tissue culture smallpox vaccine.

The results of combined vaccination against smallpox (inoculation of the inactivated vaccine followed by immunization with live vaccine of EM–63 strain) of 50 children of older age groups who had contraindications to primary vaccination against smallpox by the ordinary method, including organic disorders of the central nervous system, are presented.

It has been demonstrated that inoculation of the inactivated vaccine produced antibody response in 92% of vaccinees.

Subsequent immunization with live vaccine increased per cent of seroconversion to 100, and geometric mean antibody titer at 4–5 weeks after vaccination exceeded the level of antibody produced after smallpox vaccination by the ordinary method.

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POSTVACCINAL REACTIONS
AND COMPLICATIONS

PROPHYLAXIS AND TREATMENT OF POSTVACCINAL ENCEPHALITIS

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The multiplication of the vaccinia-virus in the vaccinees releases different defence mechanisms. It is clear, that alterations in the immune defence lead to complications. The lesser complications are alterations in the local reactions. Besides this we find serious complications so the Eczema vaccinatum, generalized vaccinia, above all the vaccinal encephalitis. I would like to discus the different problems in connection with the prophylaxis and therapy of this illness.

There is no doubt about the fact that the vaccinia virus is connected with development of this complication. But it is still not clear what mechanism leads to this illness. Experience has shown that 4.000 to 1,000.000 vaccinations are necessary, before one case of encephalitis appears. This depends perhaps on the age of the vaccinees and the status of immunity against vaccinia virus and many other unknown facts. We cannot say with certainty if there are vaccinia strains which tend to lead to encephalitis. There is probably some relationship but we have no method to check this. There are several hypotheses on this development of encephalitis. One of them is in connection with the multiplication of vaccinia virus in the central nervous system and others are in connection with an encephalitis virus, which is activated in vaccinees or is inoculated with the vaccine.

In my opinion the theory of allergic mechanisms has great importance compared to the others. Above all the results of different experiments have shown some proof of this hypothesis.

When we discuss the immune pathogene mechanisms, we must begin with the analysis of the different antigens which are inoculated by the

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vaccination. We must decide between antigens of vaccinia virus and antigens of the different products in the vaccine, such as celldebris, proteins etc.

The composition of the antigens in vaccinia virus is very complex. First we have the NP-nuclearproteinantigen which can be precipitated with antisera of all pox viruses. This is a common antigen of the whole pox group. Further it was possible to isolate 15 other antigens with polyacrylamid-gel-electrophoresis *(Joklik* et al. 1966) and immuno-diffusion-technic *(Zwartouw* et al. 1965). In extracts of cell homogenates which were infected by vaccinia virus 17 precipitation lines were found, 7 of which identical with the antigens which had been got from purified elementary bodies *(Appleyard* et al. 1962). Appleyard et al. (1964) isolated an antigen which reacts with neutralising antibodies. This antigen can also induce the production of neutralising antibodies in vivo. Not to be forgotten are antigens of the celldebris, also of bacteria. Through the inoculation of vaccine only a small amount of the substances mentioned enter the body. The main part of the different antigens are produced during the virus multiplication in the body. The vaccination is an experimental infection and can be compared with another virus infection. We therefore find a relatively great multiplication of virus in certain cells in the organism. As a result of the multiplication in the cells, defence mechanisms are developed. The survival of this »illness« is in connection with the proper function of the different defence mechanism. Normally the multiplication of vaccinal virus is stopped and the resulting alterations in the tissue are eliminated within few days.

The heterogenity of a smallpox vaccine is shown in the first figure.

The diagram shows a separation of different components by density gradient centrifugation *(Dostal,* 1968). Besides vaccinal elementary bodies, celldebris, bacteria, soluble fractions can be seen. They have a different antigenic composition. It is not surprising that these antigens induce antibody response in the organism. Under certain circumstances they can inhibit the reaction of the immunological response. When after vaccination pathogenic immune phenomena appear and lead to complications it is not yet possible to say whether this phenomenon is connected with the important antigen antibody system or if it is not in relation with this system. The immune response is connected with the different immunoglobulins and with immune cells which are responsible for the immune response of type of the delayed reaction. Even after vaccination we can normally demonstrate both types of reactions. As far as immunopathogenic points are concerned we do not yet know which antibody fraction is responsible for a possible immunopathogenic reaction in the central nervous system (see *Steffen,* 1967). Antibody fractions, such as neutralizing antibodies, which react with different antigens on the elementary body or with the soluble antigens, are the ones which can be considered. It is probably the soluble antigenes which react with the antibodies which are responsible for the immunopathogenic phenomena.

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Experience has shown that the postvaccinal encephalitis does not develop when the antibodies are completly missing or when there are sufficient amounts of them in the vaccinees, but when the production of antibodies is reduced or delayed an encephalitis can appear.

This supposition is proved by the presence of a long viremia and reduction of antibody production. It is also possible to check the development of encephalitis by an active or passive increase of antibodies. This phenomenon can be clearly shown by example of the serum-illness. The post vaccinal encephalitis has probably the same mechanisms.

The figure 2 explains the reason of this illness. The immunopathogenic reactions result when there is an excess amount of antigens, a soluble antigen antibody complex develops. This complex is responsible for the immunopathogenic reactions. We already find by small anaphylactic shock cerebral and slight neurological symptoms. Apart from that, we have urticaria and nephrogene and myocardic symptoms. We can therefore interpret the symptoms of postvaccinal encephalitis as a type of a serum illness.

To support this opinion, I would like to mention again the prolonged viremia and too increased production of elementary bodies and soluble antigens. Vaccinated persons never get encephalitis and the same applies to the passive supply of antibodies. The active immunisation with killed vaccine virus can also inhibit the development of encephalitis. As these explanations have shown, the immune phenomena play an important part in the development of post vaccinal encephalitis. The application of vaccine antibodies for the prophylaxis or therapy is today the only way to get a result. Intense investigations on the effect of the passive supply of antibodies on the prophylaxis and therapy on encephalitis have been published by *Nanning* and *Marennikova*. The condition is that the gamma globulin has to contain a high concentration of antibodies.

We get the original material for the preparation from students who have been vaccinated 4 weeks earlier. In order to obtain larger quantities of plasma we use the method of plasma phoresis. In this way we obtain from each donnor $2^{1}/_{2}$ ltrs of plasma. During the donation the antibody titer is constantly checked. The gamma-globulin is produced by the usual methods. The titer of antibodies in the gamma globulin is levelled to standard gamma globulin, that is, it contains 1,000 units per ml. For the prophylaxis of treatment-encephalitis we recommend 20 units per kilogram of body weight. For therapy 100 to 200 units per body weight (see *Dostal*, 1966, 1969). When we give the primary vaccination to older children or young people, we make three controls by EEG. The first control is made on the day of vaccination. The two other 7 respectively 14 days later. Up to now we have carried out about 300 investigations of this kind *(Lorenzoni* et al. 1969). In this way we have noticed frequent changes in EEG curves. When we found abnormal curves before the vaccination the following controls 7 and 14 days later were much worse. The patient complained of headaches, light meningitis signs, sore eyes and so on. In three cases we applied 1,000 units of

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gamma-globulin and the symptoms disappeared within a few hours. But the changes in EEG curves can be seen for several months. With a different group of vaccinees, these changes became evident only after the vaccination. On the therapy of acute encephalitis we have no experience because up to now we have not had seen a case.

The next figure 3 shows the result of the success of the gamma-globulin treatment by *Marennikova* (1967). We think that a gamma globulin when containing high concentration of vaccine antibodies is not only able to protect from encephalitis but is also very important in the effective treatment of postvaccinal encephalitis.

Fig. 1
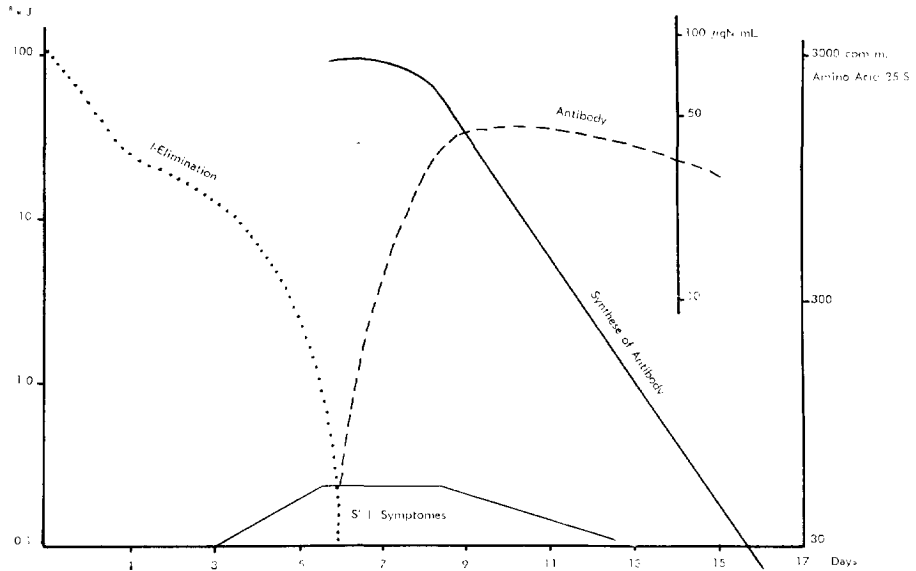
U. V. -- Absorption Vaccinevirus in density gradient

Fig. 2
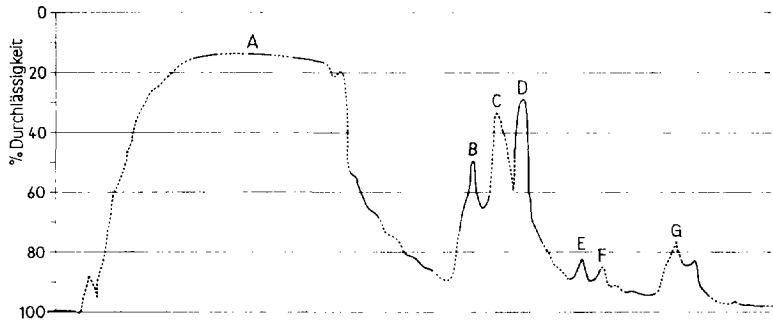
Serumillness after *Dixon, F., S. Vazquez, O. Weigle* and *C. Cohran:* Ach. Path. 65, 18 (1958)

Fig. 3

Potency of Vaccinia Gamma-Globulin
Treatment-Gamma-Globulin

Group	Treatment-Gamma-Globulin		Nr. of cases	without tretment
	without	with		
	Cortison			
patients	33	20	53	41
death	0	1	1	32
%	0	5	1,9	80

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POSTVACCINAL CONVULSIONS, AGE DISPOSITION AND PROGNOSIS

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Postvaccinal seizures of a child are most shocking impressions for the whole family. In the acute stage it is not always possible to differentiate between febrile convulsions and those following postvaccinal encephalopathy. Often only the further course of the malady enables us to make a definite diagnosis. The prognosis of febrile convulsions has to be given cautiously (Doose and Eckel, 1968; Ehrengut and Ehrengut, 1965; Millichap, 1968).

The chance to reduce the incidence of febrile convulsions following smallpox vaccination was not very great until now. According to the German Vaccination Act it is not allowed to vaccinate a child with convulsions within one year following the seizure. Whether this law is sufficient to avoid paroxysms in predisposed subjects is questionable.

Another way to reduce the frequency of febrile convulsions is to postpone vaccination of thus disposed children until the 6th year of life. At this age febrile convulsions have disappeared, whereas 6% of the predisposed may have epileptic seizures from this period onward and thus can be eliminated from vaccination (Doose and Eckel, 1968).

A further step in the prophylaxis of febrile seizures is the reasonable selection of the vaccinee. This selection is not always done with the necessary thoroughness. We feel that the so-called »child at risk« should be vaccinated only after a careful review of the anamnesis, including EEG records in particular cases (Ehrengut, 1968). Later we perform the vaccination under the cover of gammaglobulin or pre-immunize the child with inactivated vaccinia virus 8 days before (Herrlich and Ehrengut, 1965). But how to reduce the incidence of febrile convulsions in view of an unsuspected anamnesis?

In the years 1960–1964 we were not able to register in Hamburg any case of febrile convulsion among 9484 infants vaccinated within the first 6 months of age (Ehrengut and Ehrengut, 1965). In contrast we found among 43 619 children 1 to 2 years of age 67 febrile postvaccinal convulsions, in other words, a ratio of 1 convulsion in 651 vaccinees. The fact that,the very young baby seems to be relatively exempt from febrile convulsions is already known (see Friderichsen and Melchior). Millichap's compiled statistics on 7000 patients with febrile convulsions support this statement. It was the task of the present investigation to elucidate the *age disposition* of children suffering from convulsions following smallpox vaccination and also the prognosis of neural complications.

MATERIAL AND METHODS

A prerequisite of the evaluation of postvaccinal risks is a thorough collection of neural complications, which is not always possible (see Ehrengut, 1968). Since in Hamburg a good co-operation exists in this respect between the general practitioner, the pediatrician, the chidren's hospitals and the Vaccination Institute, we were able to collect almost all postvaccinal complications. Due to the fact that – as mentioned above – febrile postvaccinal convulsions are difficult to differentiate from those following encephalopathies, we have evaluated both forms of convulsions together as far as age of the patient and prognosis are concerned. We have made a survey of postvaccinal neural complications in Hamburg for the period of 1956 till 1968. Catamnestic investigations had been made with 200 patients with postvaccinal convulsions also irrespective of febrile or encephalopathic origin (Dahm, in preparation). For the cohorts of birth-years (1956–1968), the vaccination results are registered in a central bureau. For each vaccinee a record is available; the compiling of the statistics (age at the time of a successful vaccination) therefore made no difficulty.

In Hamburg claims of »postvaccinal injury« are checked by an official commission (members: a virologist, pediatrician, neurologist, Public Health Officer, Director of the Vaccination Institute). Cases accepted as »vaccinal injury« are included as »sequelae« in this study. The overwhelming majority of convulsions was seen at the acute stage by doctors in children's clinics. All cases with coma, paralysis etc. were observed in clinics. In cases with convulsions, the duration of the seizure was taken from clinical records (for details: see Frankenberg, 1969). Just in a few cases (3%), immediate convincing notifications of the parents were taken, when the doctor had been called and seizures had ceased already.

RESULTS

On the first table 235 patients (0 to 3 years of age) with »postvaccinal convulsions« see above) occuring between 4 to 20 days after primary smallpox vaccination are tabulated *according to age.* We found only 2 cases in 0–6 months' old children. The majority of convulsions (150) occurred in children of 1 to 2 years of age. As can be seen from table 1 there is no doubt that the *probability of succumbing to postvaccinal convulsions is highest among children from 18 to 24 months of age* (1 convulsion per 511 vaccinations).

In table 2 we have compiled the *incidence of postvaccinal encephalopathies* according to age (cases without and with convulsions, the latter being already registered on table 1). The diagnosis was based on the characteristic clinical symptoms as incubation-time (Weber and Lange), neural symptoms as drowsiness, coma, paralysis and convulsions, sometimes abnormal spinal fluid (s. Ehrengut 1966). No great difference between all age groups were to be seen, except the incidence during the period of 18th to 24th months of life, which seems to be rather high. The 4th half-year of life seems to be predisposed for all kinds of neural complications (compare table 1 and 2). A further study in still wider material is forthcoming.

As far as the *fatality of postvaccinal encephalopathy* is concerned, one can see on table 2 that there was 1 death among 30 811 vaccinees during the first six months of life, and 2 deaths in the group of 12–18 months of life (1 case in 35 521 vaccinees).

Of great importance is the *prognosis* of our 243 patients with postvaccinal neural complications. There were 5 cases with permanent sequelae among patients with febrile convulsions (s. table 3). If one includes also the 30 cases of postvaccinal encephalophathy, the *danger of permanent injury is also highest in the second year of life* (see table 3).

DISCUSSION

The pros and cons of smallpox vaccination before or after one year of age are the following:

1) Parents are more open minded towards prophylactic measures in infants than in children (Neff and Lane, 1968).

2) Infants vaccinated within the first 6 months of life may have a *milder local and general reaction.* We have seen that the local reactions of young babies in a population which has been revaccinated at 12 years of age are often accelerated as in revaccinees (larger area, scab already forming on the seventh day).

3) Due to the fact that children may sometimes touch the vaccination spot, *secondary vaccinia* more often occur in the older ones than in infants.

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4) *Under the cover of maternal antibodies* tiny babies *rarely* suffer from measles or other *infectious diseases*. Double infections may be followed by postvaccinal encephalopathy (Herrlich, Ehrengut and Schleussing, 1965).

5) There is a definite *age disposition towards postvaccinal febrile convulsions* (Ehrengut and Ehrengut, 1965). Frankenberg (1969) found in an analysis of 516 vaccinal febrile convulsions notified in the Federal Republic of Germany (1956–1968) identical results (60,8% of the cases in the second year of life). The data of Ruziczka (among 144 febrile convulsions of various origins only 3 cases in infants 0 to 6 months of life), of Friderichsen and Melchior as well as those of Millichap (7000 cases in literature) are in complete harmony with our observation. It is of interest that by including also paroxysms of encephalitic origin the *risk for a child to suffer from a »postvaccinal seizure« is highest in the second year*, mainly in the 18 to 24 months' period (see table 1).

Studying statistics of neural complications, one may see that the second year of life is not exempt. Only the data of Griffiths make a unique exception (just 1 case of encephalitis among over half a million vaccinees 1–4 years of age in Great Britain). The reliability of the British figures has been questioned by Dixon. Data on postvaccinal complications seems to be an illusion (see later).

The main points against smallpox vaccination in the first year of life are as follows:

1) The *take rate may be smaller*, if the vaccination technique is poor.

2) *Mortality* due to different causes *in the first year of life* is approximately three times higher than in the second year (Ehrengut, Mai and v. Mutzenbecher). Unjustified claims of a causal relationship between vaccinations and intercurrent fatal diseases may arise (see Ehrengut and Ehrengut-Lange 1968, 1969). We were able to show (1969) that such death-rates occurred with the same frequency after oral polio vaccination than after primary smallpox vaccination in Bavaria (1962–1964), only to be explained by the expected death-rate in the respective age groups.

3) Cases of *generalized vaccinia* occur more often in the first six months of life (Conybeare). They are usually of minor importance, just a few satellite pustules arise and heal later on (Ehrengut, 1963).

4) Some doctors believe that the *»child at risk« will be more easily recognized in the second year of life*. A study of the anamnesis of 778 German cases of post-vaccination encephalopathy respectively encephalitis for the years 1939–1965 (Ehrengut and Thiyagarajan, in preparation) did not support this argumentation. There were just 23 infants (6% of the patients) with previous neural persistent injuries in the case history, vaccinated in the first year of life, versus 43 corresponding cases (= 13,4% of the patients), vaccinated in the second year of life. Since the percentage of children vaccinated compulsorily in the first 2 years of

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life is practically the same in Germany (Ehrengut and Ehrengut-Lange, 1968), the *chance to omit vaccination of such an injured child in the second year of life is evidently not better than in the first one.*

5) There is no evidence that *postvaccinal encephalopathy* occurs more often in the first than in the second year of life, as seen in table 2 of our study (see also Berger and Puntigam with similar figures).

6) Another important aspect is the *prognosis of postvaccinal neural complications.* Catamnestic studies made by Dahm and us (in preparation) on 200 patients suffering from »postvaccinal convulsions« in Hamburg (1956–1968) have shown that among 178 patients with »febrile convulsions« there were 5 definitely injured (mental retardation, seizures). The prognosis is still doubtful in some young children. We are therefore of the opinion that »postvaccinal febrile convulsions« should not be considered as harmless as some doctors seem to do.

In literature the *frequency of epilepsy following »febrile convulsions«* varies according to the authors between 2,8 to 40,0% (Keith). Doose and Eckel, who compiled all »postvaccinal convulsions« (including cases of postvaccinal encephalopathy), found a poor prognosis in 19% of their cases. In this connection it is of interest to note that the majority of all neural complications with poor prognosis was found in the second year of life (1 case in 7742 vaccinees, see table 3). Seelemann, who reviewed the period of 1939 to 1958 in Hamburg, found an identical incidence.

Since seizures are, in 96% of our material, the dominant feature of postvaccinal neural complacitaons, our observation that the first 6 months of life are significantly exempt from convulsions in contrast to the second year of life, gives a first hint to reduce such complications.

Though a few cases may not have been registered in our material, it is not probable that this was the case only in *one* surveyed age group. In the first six months of life, infants are definitely better observed and looked after than in older age periods due to frequent feeding and diapering. Therefore it is improbable that, in this younger group, a more potential bias has existed than in the older ones.

Neff and Lane recently outlined that this age period has not been investigated in this respect. We could show (Ehrengut, 1969) that the number of »postvaccinal convulsions« has dropped markely in Hamburg (period 1960–1967) with the increase of early vaccinations (4–6 months) from 2,2% in 1960 to 25,1% in 1967. Neither the use of different vaccine strains nor a change in the titer of the lymph has influenced the incidence of convulsions.

In view of the high incidence of »postvaccinal seizures« with corresponding permanent injury in the second year of life, the recommendation of the American Academy of Pediatrics to vaccinate »between the ages of one to two« seems therefore to be problematic.

In this connection we want to state that a young baby should be vaccinated only, if the doctor is quite sure of the well-being of the infant. In all doubtful cases, the inoculation should be deferred.

A comparison of the incidence of postvaccinal neural complications in different countries reveals enormous differences Berger and Puntigam (Austria, 1954); Herrlich et al. (Federal Republic of Germany, 1956); Griffith (Great Britain, 1959); Neff et al. (USA, 1967). Larbre et al. (1964) coined in a discussion of the above problem the data of Griffith (1959) »the climax of paradox«. If the co-operation between doctors and the reviewer of complications is as good as can be seen from the study of Spillane and Wells (1964), than also the British figures concerning neural vaccinal complications are not too different from the Austrian and our data. Spillane and Wells found among about 800 000 primary and revaccinees 39 neural complications. Surprisingly, there were no young infants in their material. The authors seriously critize the notification system of postvaccinal complications in Britain (»There is no clear directive to the general practitioner to notify cases of post-vaccinal encephalitis or any other complication involving the nervous system«) as well as the »shortage of precise information« what the optimal vaccination period is concerned.

Triau et al. think that the higher complication rate in Austria and Germany might be due to the vaccine strains used in these countries, but their thesis is not supported by the necessary data. As already outlined, the use of two different vaccine strains in Hamburg over longer periods did not bring any change in the frequency of complications (Ehrengut, 1969). This was not expectable anyway, since the use of the recommended British strain Elstree gave practically analogous figures, what postvaccinal encephalopathies (see Spillane and Wells) are concerned. It may be feasible that vaccines followed by lower general reactions do also reduce the incidence of »febrile« convulsions. In Hamburg, a trial is under way, to check this problem.

According to De Vries vaccination »may accelerate or cause convulsions, increase their intensity and sensitize the nervous tissue, as a result of which histopathologically diffuse cellular damage and lesions of the vascular wall with haemorrhages may occur. In a number of cases idiocy and oligophrenia following convulsions no encephalitis has probably existed; instead, the convulsion is the cause of the severe cerebral damage«. Spillane and Wells suppose too that in vaccinia infection followed by seizues there is a toxic factor with secondary hypoxemia and oedema of restricted areas for the brain, giving rise to focal symptoms. They group seizures under the heading of »post-vaccinial encephalopathies«. We ourselves are of the opinion that this problem should be carefully looked after anyway. The differential diagnosis (encephalitis: febrile convulsion) is only possible after a longer observation of the patient, though also here misinterpretations are possible, since cases of encephalitis with good prognosis will be grouped later under the category of »febrile« convulsion.

Table 1

Postvaccinal convulsions including postvaccination encephalopathies within 4 to 20 days after smallpox vaccination in 0 to 3 years old children
(Hamburg: cohorts of the birth-years 1956–1968)

months of life	number sucessfully vaccinated	No. of postvaniccial convulsions	1 convulsion per ... vaccinees
1 – 5	30 811	2*	15 405
6 – 11	101 035	65	1 554
12 – 17	71 042	92	772
18 – 23	29 677	58	511
24 – 35	14 082	18	782
Total	246 647	235	1 049

* including 1 questionable case of postvaccinal encephalopathy

Table 2

Postvaccinal encephalopathies with and without convulsions and death – rate in relation to age
(Hamburg: 1956–1968)

Age (months)	0–6	6–12	12–18	18–24	24–36
Without convulsions	—	1	4	3	—
With convulsions	2	11	4	4	1
Cases / deaths	2/1	12/0	8/2	7/0	1/0
1 death per ...... vaccinees	30 811		35 521		
1 encepalophaty per ...... vaccinees	15 405	8 419	8 880	4 239	14 082

Table 3

Permanent injury after neural complications among 246 647 vaccinees
(Hamburg: 1956–1968)

Age (months)	0–6	6–12	12–24	24–36
Postvaccinal »febrile convulsions«*	—	1	4	—
Postvaccinal encephalopathy	—	2	9	1
Prognosis still doubtful	—	1	2	—
Permanent sequelae	0	3	13	1
No. of vaccinees	30 811	101 035	100 719	14 082
1 sequela per ...... vaccinees		33 679***	7 742***	14 082

* Result of catamnestic studies in 1968 on 200 patients.
*** The difference between the frequency of permanent injuries after smallpox vaccination in the first six month of life and the second year of life is significant at the 1 percent level of probability (calculated according to Pän (1967) and Vogel (1959).

SUMMARY

243 neural complications following primary smallpox vaccination in (Hamburg 1956–1968) among 246 647 vaccinees 0–3 years of age are reported. There were 235 »postvaccinal convulsions« (of febrile and encephalopathic origins). Only 8 encephalitic patients (out of 30 cases) did not show any convulsions. The risk of acquiring postvaccinal paroxysms was the least in the first six months of life (1 case in 15 405 vaccinees) and the hgihest in the second year of life, especially within 18 to 24 months of life (1 convulsion in 511 vaccinees). In view of catamnestic studies on 200 patients with postvaccinal convulsive disorders, it was also possible to evaluate the number of injured patients in the various age groups. The prognosis in respect to permanent sequelae was poorest within 18 to 24 months of life (1 sequel per 7742 vaccinees). By consistent propagation of an early vaccination of infants (up to 25%), the number of postvaccinal convulsions has dropped. A plea is made to perform vaccinations preferably between 4 to 6 months of life.

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FREE THEMES

STUDIES ON MECHANISM OF DELAYED (CELLULAR) TYPE OF HYPERSENSITIVITY

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This communication represents a general survey of studies performed in our department of experimental immunology during the last 10 years. It contains also some pertinent data obtained in other laboratories. For detailed description of experimental techniques and results see the original papers.

1. *General considerations*

Delayed (cellular) type of hypersensitivity constitues a part and at the same time the experimental prototype of broad field of immunology called now usually Cell mediated immune responses (WHO 1969).

It plays an important role in resistance to and in pathogenesis of some microbiol diseases, in side-effects of vaccination, in transplantation and antitumor immunity and in autoimmune diseases. The basic characteristics of delayed type of hypersensitivity is that it can be transferred passively to normal recipients by living lymphocytes but not by serum of sensitized organism. Serum antibodies may be present but are not essential. Hypersensitive lymphocytes are the responsible cells evoking by a trigger effect changes leading to delayed hypersentivity manifestations.

For characterization of Cell mediated immune response it may be quoted from the introduction of the above mentioned WHO scientific group (1969):

»The immune response is a complex phenomenon involving the differentiation of cells in at least two directions. One of these leads to the production of cells specializing in the synthesis and secretion of humoral antibodies of the various immunoglobulin classes. The other leads to the production of specifically sensitized cells, accepted as being lymphocytes, which are responsible for intiating the events generally recognized as constituting cell-mediated immunity. The term cell-mediated immu-

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nity is used there in the broad sense, encompassing not only protective immune function and hypersensitivity phenomena but also the other cell-mediated reactions described later. Cell-mediated immunity is increasingly thought to play a crucial role in a variety of defence mechanism and disease processes of great clinical importance. Among these are transplant rejection, defence against neoplastic growth, resistance to certain types of bacterial, viral, mycotic, and parasitic agents, and autoimmune processes.

In the last few years the mechanism underlying cell-mediated immunity have been clarified by increased understanding of clinical and laboratory models of the phenomena involved. The following topics are therefore discussed in some details: (1) the type and source of immunocompetent cells responsible for the reactions; (2) delayed hypersensitivity of the tuberculin type and related hypersensitivity reactions; (3) the cytotoxic activity of sensitized and non-sensitized activated lymphocytes; (4) the relation of specific immunoglobulins to the phenomena; and (5) methods currently available for controlling or suppressing cell-mediated immunity. Understanding of cell-mediated immunity requires definition of the separate events involved as well as a precise analysis of their pathogenesis. An attempt is therefore made to summarize present knowledge of the phenomena of cell-mediated immunity, delineate the areas of uncertainty, and point out the problems to be solved. Another aim is to demonstrate the important role of cell-mediated immunity in health and disease in man.«

It should be stressed that before 15–20 years these problems were not known and delayed hypersensitivity studies were limited to diagnostic skin testing in some infectious diseases. The decisive role in changing the general opinion after the year 1950 is to be atributed mainly to studies of transplantation and antitumor immunity in United Kingdom, of autoimmune reactions and disease in USA, etc. The pioneering work in studies of delayed hypersensitivity by Chase (1945, 1965), Gell et all (1951, 1961) Waksman (1958, 1961) and others must also be mentioned.

2. Present state of the hypothesis in »mediated« mechanism of delayed (cellular) type of hypersensitivity (DH) reactions

Lymphoid cells from DH organism form and release after both in vivo and in vitro contact with specific antigen biologically active substances which are responsible (by a trigger effect) for different DH reactions; in the proper manifestation of DH mostly non-hypersensitive host celis are invalved. Besides the activity resembling the lymph node permeability factor (LNPF), two further factors were demonstrated by incubation of DH lymphoid cells with the specific antigen in vitro.

a) A substance with direct migration inhibiting activity to normal, non-hypersensitive cells obtained by incubation of DH cells with a large antigen dose, having also a pharmacological affect (typical delayed inflammation).

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b) An antibody-like (antigen dependent) substance; it does not influence migration activity of normal cells itself, but evokes migration inhibition after binding to these cells and adding a further antigen dose to the test system.

Both substances are not present preformed in lymphoid cells of DH organism (with some possible exception in early stages of sensitization), are formed inside the cells and subsequently released in the supernatant fluid of DH cells culture with antigen during a 6–24 hours period. Under similar conditions (reintroduction of antigen) analogical substance can be detected in vivo, i . e. in serum of the respective DH organism.

Biological manifestations of DH reaction both in vivo and in vitro can be supposed to proceed as follows. Small number of actually DH cells react with the specific antigen introduced. Formation and release of both types (or may be also of other types) of biologically active substances takes place. The former substances might influence directly (pharmacologically) the function of other non-hypersensitive cells; the latter substances may change these cells (by an analogy passive sensitization) and enable them to react specifically with the antigen. The altered behaviour of host's cells is supposed to be the basis of the proper DH manifestations. Some differences in various experimental systems (skin inflammation, in vitro cell migration inhibition, etc.) could be explained by changes of proportion and activity of the both substances.

This general scheme could be extended hypothetically also to other states (autoimmunization, transplantation immunity, etc.) more or less related to the DH mechanism.

3. Experimental background for the hypothesis (short survey)

a) Sistemic fever reaction:

During 1958–1962 a number of papers was published concerning formation of s. c. hypersensitive pyrogen by incubation of lymph node and spleen cells or their extract with the respective antigen in vitro (Johanovský 1959, 1960). These experiments were not confirmed by Atkins et all (1965) and also in our laboratory (Castrová et all 1966 a, b) and cannot be therefore considered as perfectly demonstrated in spite of a confirmative paper by Allen (1965) and some similarities in the respective conclusions to recent results by Atkins et all (1967) and to those obtained in different laboratories by the tissue culture technique.

Some analytical work was made by Castrová et all. on conditions necessary for systemic sensitization and for characterization of antigens involved (Castrová et all 1966a, b.), the role of attenuated Mycobacteria (BCG) in ovalbumin sensitization (Castrová et all 1967) and on in vitro formation of leukocytar pyrogen by hypersensitive polymorphonucleare cells (Castrová et all 1968). In addition, Kolín et all (1968a) demonstrated profound pathological organ changes following i. v. BCG sensitization.

In respect to these findings and to the known difficulty of passive cell transfer of DH sensitization to systemic fever reaction the possibility is

to be considered that this reaction requires a special and not yet characterized physiological state of the organism.

It is known that during the development of delayed type of hypersensitivity changes in RES activity and endotoxin susceptibility can be demonstrated (see WHO 1969). Our studies in this respect concerned the quantitation and time dynamics of endotoxin susceptibility changes and other related phenomena (Johanovský et all 1961, Vejbora et all 1961, Kolín et all 1968a, Pekárek et all 1966, 1968). A hypothesis has been formulated considering these processes as a consequence of interaction of hypersensitive cell with the specific antigen persisting or introduced in the sensitized organism.

b) Tissue culture studies (first part).

A standard method was developed and used for a number of studies (Švejcar et all 1961a, b). Observation of several authors was confirmed, i. e. a change in general behaviour and sensitivity of cells from DH organism, differing however basically from the proper DH cellular reaction to specific antigen (Švejcar et all 1967a). After confirming (Švejcar et all 1961 c) Waksman's statements on stimulation occuring in DH reaction in vitro under some conditions (Waksman et all 1958), the subsequent studies extended this observation to the migration test, too, both in respect to the time sequence and to the dosis of antigen used (Švejcar et all 1963, 1965, 1966c).

The tissue culture technique was used also successfully for studies of transplantation immunity (Hašková et all 1967) and antitumor immunity, i. e. demonstration in a special arrangement (cell monolayer as a source of antigen) of DH to SV 40-Adeno 7 hybrid transformed cells (Pekárek et all 1968).

c) Tissue culture study (mediated mechanism).

First approach to this problem was made by the technique of simultaneous cultivation of two (hypersensitive and normal) spleen fragments in the same cultivation chambers; the migration of normal cells was influenced due to the reaction of hypersensitive fragment cells with the antigen (Johanovský, 1963, Švejcar et all. 1963, 1967 b). Subsequently, an attempt was made at producing biologically active substances in vitro by incubation of hypersensitive spleen cells with the antigen (Švejcar et all 1967 e); distincly positive results were obtained, however, only by using lymph node cells (Švejcar et all 1967 f).

In the same experiments it was demonstrated that the active substances can be found after exposure to antigen both inside the cells and in supernatant fluid. Moreover, the activity of the migration inhibiting substances was enhanced by further addition of specific antigen (Švejcar et all 1967 f, 1968 a, 1969 a). The subsequent paper described the quantitation of production of these substances and demonstrated that they were formed primarily inside the cells and then gradually released in the supernatant (Švejcar et all 1968 b). The presence of antigen during cultivation of hypersensitive cells was essential; these substances could not be demonstrated in cell extracts or supernatants without incu-

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bation with the antigen. Control experiments using normal non-hyper-sensitive cells yielded always negative results.

Two types of active substances were demonstrated: one having a direct migration inhibitory activity and also some pharmacological properties (evoking delayed inflammation – Krejčí 1969) obtained by cultivation of cells in presence of a large antigen dose. This substance is in its effect species non-specific (Švejcar et all 1967, Johanovský et all 1969). Another factor evokes early skin anemization; this substance is formed by proteolytic splitting of serum components of cultivation medium (Pekárek et all 1969). The other substance obtained by cultivation with a small antigen dose is itself without effect and requires an additional dose of antigen in the test system and has therefore the antibody-like nature; its effect is immunologically specific (Švejcar et all 1967 f, 1968 a and 1969 a). This immunologically active substance can be removed from the supernatant fluid by adsorption to spleen cells or macrophages and renders them by this procedure to be able to react in presence of specific antigen by inhibition of migration. (Johanovský et all 1969, Švejcar et all 1969 b). So far the nature of the pharmacological substances and the relation of the immunological one to the known types of immunoglobulins are not known. The biochemical properties of migration inhibitory factor (MIF) indicate that it is a relativelly small – molecular substance (Johanovský et all 1969).

The above results are basically in conformity with similar experiments and conclusions by Bloom and collegues (Bennet et all 1967, 1968, Bloom et all 1966), David (1966, 1967), Dumonde (1967) and others.

Under similar experimental conditions a specific stimulation of erythrocytes phagocytosis by macrophages due to the release of the immunological factor could be demonstrated (Barnet et all 1968, 1969).

d) Histological studies of skin reactions.

During analysis of DH skin reaction the principal findings on the type of cellular infiltration of Gell et all (1951), Waksman (1960), Spector and collegues (Boughton et all 1963) and others (Dienes et all 1932), were confirmed. Besides that a new observation was made, i. e. the early (in first hours) tissue damage manifested in fat tissue and muscle fibers was demonstrated (Kolín et all 1965a, b). These changes occured before massive cellular infiltration and seemed therefore to be rather the cause and not the consequence thereof. The reaction was specific, was confirmed by passive transfer experiments (Kolín et all 1966) and by analysis of other types of immunological and toxic inflammation (Kolín et all 1968 b).

On the basis of these experiments a hypothesis was formulated on biphasic course of the skin reaction. In the first stage due to the interaction of few actually hypersentive cells and the antigen some influencing of surrounding tissue takes place, being followed by a stereotypic inflammatory reaction to demaged cells and tissue.

e) Vascular permeability studies:

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A characteristic vascular permeability reaction is one of the manifestation of DH (Voisin et all 1960, 1964). After standardization the technique (Krejčí et all 1968 a) the role of preceding antigen administration was studied; the reaction was changed and accelerated (Krejčí et all 1968 b, c). After such treatment of DH guinea-pigs depending on the dose of antigen administered substances in circulation (in the serum) were demonstrated, capable to influence directly or indirectly (after further addition of antigen) migration of cells from normal non-hypersensitive spleen fragments (Krejčí et all 1968 d). This in vivo finding is parallel to experiments using in vitro cultivation of DH lymphoid cells with the antigen.

When products of the in vitro cultivation of DH lymphoid cells with the antigen were tested by permeability reaction, a substance resembling the LNPF (Spector et all 1968, Willoughby et all 1962) was found in most supernatants and extracts from both hypersensitive and normal cells. On the contrary, in supernatants obtained by cultivation of DH cells with a large dose of antigen (see point 3 c) a factor was demonstrated evoking several hours lasting skin inflammatory reaction resembling macroscopically and histologically the DH skin reaction. In contrast to the in vitro experiments similar effect could not be produced by mixing supernatants having the antibody-like activity with a further antigen dose (Krejčí et all 1969).

4. Conclusions and perspectives

The basic »mediated« mechanism of delayed type hypersensitivity has been already demonstrated. Of course, the analysis of events on cellular and further on molecular level, the relation of the newly described antigen-dependent (antibody-like) substance to known immunoglobulins, etc. will require still a number of studies.

The recent progress enables, however, various medical applications of the experimental results obtained (see WHO 1969). Among them are for example:

a) Diagnostic use of in vitro techniques for demontrating delayed hypersensitivity, avoiding the possible hazard of skin testing.

b) Testing of new immunosuppressive agents by a standard in vitro procedure.

c) Research of substances antagonistic to the action of pharmacological mediators of delayed hypersensitivity.

d) Research of interference phenomena between cell mediated immune response and antibodies or antibody forming mechanism for possible influencing of delayed hypersensitivity reactions.

e) Consideration of relation of some recent discoveries in cell mediated immune responses to various clinico-pathological findings in man.

In conclusion I would like to stress again the great and increasing importance of delayed hypersensitivity and cell mediated immune responses in bology and medicine and the urgent need for intensification of the studies on their mechanisms and significance.

SUMMARY

Delayed type of hypersensitivity forms a part of a broad field of immunology called now usually »Cell mediated immune responses«.

The development of a general hypothesis on the mechanism of delayed hypersensitivity has been outlined. It is now generally recognized that a relatively limited number of actual hypersensitive lymphoid cells form and release under the influence of the specific antigen humoral substances which influence the behaviour of other principally not hypersensitive cells. Most of the work in last years has been done by a tissue culture technique.

One of the decisive biologically active substances seems to have a pharmacological character. It influences directly the migration of normal cells, evokes blast transformation of lymphocytes and initiates inflammation when injected intradermally to normal recipients. The other substance is antigen-depended (antibody-like); it changes the behaviour of other cells to the antigen by an analogy of passive sensitization.

The biological role and beochemical characteristics of these substances are not yet sufficiently known. The discovery of the »mediated mechanism« of delayed hypersensitivity reaction enables, however, the use of in vitro techniques for diagnostic purposes in both the infectious and non-infectious immunology and the studying of possible ways how these reactions could be influenced and controlled in practical medicine.

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DOCUMENT

PROBLEMS AND PERSPECTIVES

Although an effective smallpox vaccine has been available for several decades, variola has continued to flourish unchecked in many countries of the world. However, the dramatic fall in the global incidence of the disease since 1967, portrayed in the epidemiological reports of the world Health Organization, reflects the effectiveness of the WHO smallpox eradication program. The progress to date augurs well for the future of the program, which deserves all possible support both from endemic and nonendemic countries.

The availability of a stable, potent freeze-dried vaccine is fundamental to the success of the sheme. The selection of suitable vaccinia virus strains for the production of such a vaccine was one of the most important of the numerous technical aspects of vaccine manufacture discussed at the Symposium. An ideal vaccine strain should confer protection against smallpox while inducing the fewest possible signs and symptoms. A number of vaccinia virus strains approach this ideal.

Man is the only host in whom the immunogenicity and pathogenicity of these strains can be adequately evaluated. Further comparative studies of vaccinia strains are required. Results of these tests in man should be carefully correlated with various laboratory tests, including virulence testing in animals, to determine if one or more laboratory tests could be established which would serve to indicate the probable degree of immunogenicity and virulence of various candidate strains in the human host. In view of the large number of strains employed in vaccine manufacture and because of the magnitude of the task, it would be necessary in such studies to restrict the number of candidate strains to be examined ini-

tially to those believed, on the basis of current experience, to be least pathogenic (while acceptably immunogenic). The Lister and EM-63 strains, which are today the most widely employed and among the most carefully studied are particularly good candidates for further evaluation.

Possible substrates for the propagation of vaccinia virus in manufacture fall into three main groups: animal skin, eggs and cell culture. At present, the majority of smallpox vaccines are prepared in animal skin. Considering the difficulties encountered in preparing an animal skin derived vaccine which is bacteriologically sterile and free from adventitous agents and considering the progress in recent years in production of vaccines in eggs and tissue culture, it would be most desirable to pursue studies directed toward the development of economical methods for the production of potent, stable vaccine in eggs of tissue culture. Both of these substrates can be much more rigorously examined to exclude adventitous agents. Eggs, for example, are now commercially available from leucosis free flocks that have been examined to exclude avian encephalomyelitis, CELO virus, fowlpox, infectious bronchitis, infectious laryngotrachitis, Newcastle disease virus, Mycoplasma gallisepticum and Salmonella pullorum. The requirements for the manufacture of measles vaccine recommended by WHO exemplify the regulations that could be formulated by national authorities for the control of smallpox vaccine prepared in cell cultures and embryonated eggs. As discussed at the Symposium, however, a number of problems in production have been encountered which require further study. In addition to determining the most suitable strains, as noted above, methods must be worked out to assure greater vaccine stability, particularly in freeze--dried vaccines produced in tissue culture; results obtained by titration in various assay systems must be carefully evaluated in terms of human immunogenicity and protection against variola virus itself, and further work is required to assure improved virus yield to permit commercial production.

With the progressive decline in the incidence of variola, the mortality and morbidity following vaccination are thrown into prominence. Several possible future developments in smallpox vaccine production were discussed at the Symposium. Morbidity following vaccination could be further reduced by any of four approaches: administration of a less pathogenic vaccine strain; simultaneous administration of current vaccines with hyperimmune globulin; sequential administration of an inactivated vaccine followed by administration of one of the currently available strains; sequential administration of a less pathogenic and less immunogenic strain such as the CV-1 or CV-2 strains followed by administration of a currently available strain. No work pertaining to the first approach was presented and no new information pertaining to the second ap-

proach was made available. The last two approaches show promise for the future. Both approaches are based on the observation that complications of vaccination are infrequent among revaccinees (i. e. porsons with some previous immunity). By providing first some minimal level of immunity through administration of inactivated vaccine or a weakly immunogenic strain, currently available strains could probably be administered without inducing complications as frequently as in the past and without apparently compromising the level of protection conferred. Further studies are required, however, in which due attention is paid to careful selection of control groups, determination of neutralizing antibody levels and close surveillance for possible complications.

SYMPOSIUM ON ACUTE

RESPIRATORY DISEASES

1 & 2 OCTOBER 1969.

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INTRODUCTION TO SYMPOSIUM
ON ACUTE RESPIRATORY DISEASES

WELCOMING SPEECH

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On behalf of the Yugoslav Academy of Sciences and Arts and in the absence of its president it is my honour to open this Symposium on Acute Respiratory Diseases.

The Internationl significance of this meeting in which side by side with Yugoslavs specialists from France, Holland, the Federal Republic of Germany, the United States of America, the Socialist Republic of Poland, the Socialist Republic of Bulgaria, the Soviet Union, the United Kingdom and a representative of the World Health Organization are taking part – is not only a guarantee for an adequate solution of problems to be discussed at this gathering; by its character it is in full harmony with tendencies which the Yugoslav Academy is drawing attention to everywhere and at all times, that is – the closest collaboration of peoples all over the world in the field of culture and sciences on an equal footing.

Acute respiratory diseases are nowadays a very important problem, both medical and economic. This especially applies to influenza, which comes in epidemics, spreads from one continent to another threatening to seize the whole world. The interest in finding some way to control respiratory diseases is easily understood, for they attack, within a short time, great numbers of people, paralyse the activities of many institutions and substantially increase the morbidity of the population.

Among these diseases acute respiratory infectious diseases of the respiratory tract are the most important. The era of antibiotics has meant that the importance of diseases caused by bacteria has markedly diminished while diseases caused by viruses are just as frequent as they were.

With the advancement of cell culture techniques many causative agents of respiratory diseases have been newly discovered, the most important if which being influenza and parainfluenza viruses, adenoviruses, respiratory, syncithial virus and rhinovirus. To this group of diseases we may also add atypical pneumonia, although its causative agent is not a virus but a Mycoplasma.

Among many attempts to fight these infections three methods have proved to be worth further study. These are: vaccine, chemotherapeutics and increase of resistance in an organism by stimulating the formation of interferon.

We clinicians, conscious of the consequences that can be caused by respiratory infections, especially in sensitive groups of the population, consider that the prevention of such infections is the most ideal solution. In this precise and complicated laboratory work on immunological investigations must be connected with clinical and epidemiological studies. There is still a necessity to better define the clinical and epidemiological problem of acute respiratory diseases and to establish what is the purpose of immunization and who it is meant for. Although we are still far from the goal we hope that one day the results of this work will be possible to apply to the benefit of human health, which is the ultimate aim of all scientific aspirations.

There is no doubt that this is the right moment to discuss this matter, The Yugoslav Academy will see that your discussion and the results of your work are made available to wide medical circles in the form of a book.

I wish you all every success in your work and I invite you to visit the Yugoslav Academy. We shall be looking forward to seeing you there.

ON THE SIGNIFICANCE OF THE SYMPOSIUM ON ACUTE RESPIRATORY DISEASES AND THE RESULTS EXPECTED FROM IT

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Acute respiratory virus diseases are a great and still unsolved problem, although knowledge of these diseases has been greatly expanded during the past twenty years. They are still the most widespread infections throughout the world, occupying top place in the tables of diseases. Mortality from them is also high. It is more than 30 years since the virus of influenza was identified in the laboratory but even to-day influenza remains a disease which is not controlled. Our weapons are still inadequate and we do not seem to be using them as they should be used against acute respiratory diseases. Sometimes we are completely disarmed.

Influenza was known as far back as antiquity. Clinical symptoms are characteristic and all descriptions, even those from the older eras, are very alike. In character influenza is unique. It comes in epidemics which break out suddenly and spread at great speed. Epidemics vary in intensity, from minor, local ones to pandemics, which take place at irregular intervals and spread rapidly all over the world. Between pandemics there are major or minor epidemics.

The incidence of this disease is high, 20 per cent or more. Incidence is highest in the age group 5–14 years, lower in the group 15–25 years, higher again among those 25–34 years old and it lowers among those over 40.

Our biggest problem concerns new variants of group A influenza virus. The nature and the basis of antigenic variations, i. e. whether these represent a fundamental cyclic phenomenon or a progressive evolution, are still open questions. An ever greater number of viruses from group A, which we encounter in animals – birds, pigs, horses and perhaps in

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other species, are in this connection of paramount importance. Although we have no proof that animal strains can be transferred to man, we must consider the possibility that animal reservoirs of human influenza exist and that new strains can appear as a result of hybridization of human and animal virus strains in nature.

Other respiratory virus diseases are of greater or lesser general significance or are important for certain groups of people. Thus parainfluenza virus types 1, 2 and 3 have been found very important in diseases of the upper and lower respiratory tracts of children. The respiratory cyncithial virus is the most important causative agent of respiratory diseases in young infants and it very often attacks the lower parts of the respiratory tract. Adenoviruses are also important, especially in the first two years of life. Although Mycoplasma pneumoniae is not a virus, because of the disease it causes it has been included in the group of acute respiratory viruses. It is also important for some groups of people. Rhinoviruses, which cause colds, are of many different serological types without antigenical interrelationship. To express an opinion on all these questions in one single symposium is impossible, which explains why another symposium dedicated to acute respiratory diseases is taking place in Atlanta in only two weeks.

The mechanism of immunity and the patophysiology of acute respiratory diseases is a complex phenomenon influenced by many factors, the relative importance of which is unknown. Although there is a good correlation between the level of circulating antibodies in the blood and resistance to infection, this correlation is indirect. A direct correlation, however, has been established with the level of antibodies on the respiratory surface. The endogenous resources of interferon in the tissue of the respiratory tract are considered also to play some role along with some other unspecific factors. The local resistance of the surface of the respiratory tract represents the first barrier for virus invasion.

Difficulties which we encounter in developing an effective live vaccine against respiratory diseases arise mainly from the fact that during its passage in the laboratory a virus can become overattenuated and thus lose its immunogenicity. Besides, we lack reliable markers by which to determine which of the candidate strains are suitable for vaccine production. What we want are attenuated strains that would not cause reactions, with immunogenic properties separated from the pathogenic ones, highly effective, strains that could be characterized as such in the laboratory.

Some from our group consider that the inactivated killed vaccines against respiratory diseases should be given priority.

The preparation of purified vaccines and vaccines prepared from isolated subunits of virus in order to obtain a vaccine free of nucleic acids, adventitious proteins and oncogenic and toxic products, along with further work on adjuvants – this would be the way to reduce the morbidity from acute respiratory diseases.

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The Symposium also includes problems of unspecific resistance and chemoprophylaxis.

The detection of interferon in many various virus infections, either natural or caused by the inoculation of live causative agent, shows that interferon plays an active role in prevention of the spreading of respiratory infections. Interferon can be, in theory, applied as a prophylactic against virus infections, either as an exogenous product or as a stimulus for the endogenous formation in one's own organism. In the first case the application of sufficient concentrations at the right place in an organism might cause many problems. The induction of endogenous interferon in man has not yet been much studied. Work on interferon should be speeded up.

The study of antiviral substances is under way. Amantadine, with all its drawbacks, is a specific chemoprophylactic against influenza A_2 virus.

EPIDEMIOLOGY AND ANTIGENIC
VARIABILITY OF INFLUENZA

THE EPIDEMIC VARIANT OF INFLUENZA A2/HONG KONG 1/68 VIRUS AND ITS ANTIGENIC RELATIONS TO INFLUENZA VIRUSES OF ANIMALS

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When 10 years expired after the last influenza pandemic, virologists were expecting that a next new epidemic A strain would occur, because such variants regularly appear every 10 to 12 years. But though the occurrence of a new variety of influenza virus was not unexpected, no one could predict what it would be. One aspect was not doubted, however, namely, that it would be such a big and resolute step in variation that this new variant, differing from all previously known strains, could be designated as influenza A3. As it is known, these expectations have been proved not in full.

The new epidemic variant was discovered in July 1968 in Hong Kong, but, still, it could not be excluded from the family of A2 viruses and called A3. In the USSR the new variants appeared only in December and in all their properties were similar to the reference strain A2/Hong Kong 1/68 (Table 1).

From data shown in this Table it can be seen that new strains did not exhibit close antigenic relation to older influenza viruses. The inhibition of hemagglutinating activity of the new variants with antisera prepared to previously known A2 viruses constituted only 1/32nd–1/128th portion of the homologous serum titer. But occurrence of this relation made it necessary to consider the Hong Kong variety as an additional member of the A2 virus group.

At the same time, its distinctions are so great that it would be desirable to reflect this in strain's designation. Taking into consideration the antigenic changes, which are observed in the A2 Subtype, Prof. L. Y. Zakstelskaya has suggested to subdivide varieties of this serologic subtype into sub-subtypes in the following way: it is suggested to designate

Table 1

Stages in variation of influenza A2 viruses

Serum Antigen	A2 (Singapor) 1/57	A2 (England) 12/64	A2 (Gorki) 62/65	A2 1/68 Kong) (Hong	A2 (USSR) 052/68	Sub- subtype
A2/Singapore 1/57	1	1/8	1/8	1/16	1/32	A2/1
A2/England 12/64	1/8	1	1/4	1/32	1/64	A2/2
A2/Gorki 62/65	1/8	1/4	1	1/32	0	
A2/Hong Kong 1/68	1/128	1/32	1/64	1	1	A2/3
A2/USSR 052/68	0	1/32	0	1	1	
A2/USSR 046/68	0	0	1/32	1	1	
A2/USSR 069/68	0	1/32	1/32	1/2	1/2	

the original pandemic variety as A2(1) Singapor 1/57; the second variety – as A2(2) England 12/64; and the third variety – as A2(3) Hong Kong 1/68.

By designating strains with additional indexes the necessity is eliminated of explanations, as to which reference strain this or that isolate corresponds. The expediency of this suggestion can be well demonstrated in strains of 1968, since in that year, during its first half, strains were isolated with well known old antigenic structure, while by the end of the year the Hong Kong variety became widespread. Both varieties should be designated as A2/68, and in order to indicate which of the A2 variants is meant, additional remarks are required. But by the use of indexes every strain is at once characterized as belonging to this or that variant. Thus, all strains isolated in the USSR in the spring of 1968 would be classed as belonging to Sub-subtype A2/2, while strains of Hong Kong variety, isolated in December of 1968, – as A2/3.

Clear differentiation in nomenclature is necessitated not only by distinctions in antigenic structure, but also by independent development of epidemic processes determined by spreading of the varieties and, what is especially important, by the necessity of timely adjustment of prophylactic and diagnostic preparations.

The circulation of A2 strains at present is characterized by general widespread occurrence of Hong Kong varieties. And it is certain that in the nearest few years strains of this or very similar kind will continue to occupy the dominant position. This explains why such great attention is being paid to investigation of this strain.

In its biological properties the new epidemic strain is in many respects similar to other representatives of A2 viruses. Morphologically, Hong Kong variants were characterized by presence of numerous filamentous forms with diameter equal to that of spherical forms, that is approximately 90 mμ. The isolates possessed high sensitivity to non-specific serum inhibitors, found in guinea-pig, rabbit; rat, and horse serum. No inhibitors were found in the sera of roosters and white mice.

Just like with previously known A2 viruses, it was possible from the new strains to obtain inhibitor-resistant forms, using passages of the virus mixed with a serum, containing the inhibitor in high titer, for instance with the guinea-pig serum. There were reports about isolation of inhibitor-resistant variants from patients (3 strains out of 455). Nevertheless, sensitivity to the inhibitor is a characteristic feature of the overwhelming majority of strains from the epidemic of 1968–1969, just like it had been true for all A2 strains in recent years.

As a rule, the new strains were apathogenic to white mice and 2-day-old chicks. With chick embryoes, frequent isolation was noted, which is characteristic of epidemic strains in general. During the epidemic period (December–February) the All-Union Influenza Center of the USSR studied 466 virus strains isolated in 41 city of the country. From these, 352 isolates were typed as A2/Hong Kong/68 (See the Review of the All-Union Influenza Center for the first quarter of 1969).

In most instances, virus became isolated from naso-pharyngal washings already in the first passage and possessed sufficiently high hemagglutination titers. When hemagglutinating activity of newly isolated strains was studied towards different erythrocytes (hen's, guinea-pig, human) the highest results were obtained with hen's erythrocytes, the titers being 1 : 2048; 1 : 512; and 1 : 128, respectively.

Elimination of serum inhibitors to the new virus was most successfully achieved by the use of Vibrio cholera culture filtrat (RDE), but it should be mentioned that complete elimination of the inhibitor was very difficult in case of guinea-pig and horse sera.

With the appearence of the new Hong Kong virus more numerous have become findings pointing to antigenic relations of human Type A viruses with influenza viruses isolated from mammals and birds. It has been shown in several laboratories that the new A2 variant exhibits some relations with strains of Type 2 equine influenza (Equi 2-Maiami/63). There were numerous reports earlier on detection of antibodies to the equine virus in sera of aged persons.

In our laboratory, another interesting fact was observed bearing on antigenic relations of the new variant. Comparative analysis of its antigenic structure in HA-inhibition tests, presented in Table 2, has shown that influenza viruses of ducks, isolated in the USSR as early as in 1960–1963, also possess cross-relations with the new Kong Kong variety. This finding has been supported by results of neutralization tests in chick embryo (Table 3).

Table 2

HA – Inhibition tests with influenza viruses of ducks

	A	A1	A2	A2	A2	A2			A – Duck					
	PRS	Pan	Singapore 57	Gorki 65	Hong Kong 68	Sverdlovsk 68	England 56	England 62	Czecho-slov. 62	Yalta 60	Borki 63	BV 1	1891 67	3362 67
A – PRS	2560*	10	10	0	0	0	0	0	0	0	0	0	0	0
A1 – Pan	0	1280	0	0	0	0	0	0	0	0	0	0	0	0
A2/Singapore 1/57	10	0	2560	160	40	0	0	0	0	0	0	0	0	0
A2/Gorki 62/65	0	0	80	320	10	0	0	0	0	0	0	0	0	0
A2/Hong Kong 1/68	0	0	80	0	1280	0	0	0	0	0	0	160	0	0
A2/Sverdlovsk/68	10	0	80	160	80	160	0	0	0	0	0	0	0	0
A–Duck/England/56	0	0	0	0	0	0	320	0	0	0	0	0	0	0
England/62	0	0	0	0	0	0	0	160	0	0	0	0	0	0
Czech./62	0	0	0	0	0	0	0	0	160	320	0	0	0	0
Yalta/60	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Borki/60	0	0	0	0	0	0	0	0	160	0	160	0	0	160
BV-1/63	0	0	0	0	0	0	0	0	0	320	40	640	0	0
Poland 1891/67	0	0	0	0	0	0	0	0	0	0	0	160	1280	0
Poland 3362/67	0	0	0	0	0	0	320	80	40	0	0	320	0	640

* Reciprocals of HA-inhibition titers

Table 3

*Neutralization tests in chick embryoes with
A2/Hong Kong 1/68 virus and influenza viruses of ducks*

Virus	Serum 1 : 20	Virus dilution							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Hong Kong		$\pm$	$+$	$-$	$-$	$-$	$-$	$-$	$-$
B V – 1	B V – 1	$-$	$+$	$-$	$-$	$-$	$-$	$-$	$-$
Equi–2		$+$	$+$	$+$	$+$	$+$	$-$	$-$	$-$
Hong Kong	Borki	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$
Borki		$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$
Hong Kong		$+$	$+$	$+$	$\pm$	$+$	$+$	$+$	$+$
B V – 1	Normal rat	$+$	$+$	$+$	$+$	$+$	$+$	$+$	$+$
Equi–2		$+$	$+$	$+$	$+$	$+$	$+$	$+$	$-$

In the HA-inhibition tests we used in parallel 8 duck strains which were at out disposal: 2 strains isolated in England, 1 in Czechoslovakia, 2 in Poland, and 3 in our country. Of these, only two strains – Borki/60 and BV1/63 – reacted serologically with the Kong Kong virus and its antiserum.

We did not undertake large scale studies of sera from aged persons with duck strain antigens. However, a number of sera were examined for presence of antibodies to strain Borki in HA-inhibition test. In 4 pools, out of the total number of 68, the antibodies were revealed in titers 1 : 10 to 1 : 40. In two of these cases, antibodies to the Hong Kong strain, which was also tested, were absent, i. e. no correlation was observed between occurrence of antibodies to one and the other antigen in human sera.

The observed cross-relations between duck and human strains of influenza viruses make us to recall findings of Dr. Pereira and Dr. Tumova, on the existence of some cross-relation between the human strain, A2/Sigapor/57, and the Turkey strain, A/Turkey-Massachusetts/65. The authors observed these relations with hyperimmune rat sera and noted with regret that the Turkey strain had been isolated many years after the pandemic, which raised some doubts regarding purity of the strain. They had suggested that if A/Turkey–Massachusetts/65 virus were isolated before 1957, it would be permissible to think of birds as a possible source of the human A2/57 strain.

The situation with the Hong Kong virus seems to be more lucky. Strains A/Duck–USSR–Borki/60 and A/Duck–USSR–BV1/63 were both isolated long before the Hong Kong variety appeared. We cannot state for how long they have circulated on the whole before the year of their isolation, since for birds' strains this cannot be determined so far.

But there is hardly any possibility to think that this variant had been transmitted to birds from human population. Human viruses are being isolated regularly, the occurrence of new variants is accompanied by epidemics, and existence of such a variety could not have passed unobserved. The data may be explained in the following way. Either the origin of A2/3 variant is in some way linked to birds' strains which seem to be widely circulating in nature for a long time (suffice it to mention foul plaque, which agent belongs to influenza A viruses). Or all these related strains possesed common ancestors, which are not known to us so far.

It is noteworthy, that it has not been possible to reveal relation of duck viruses to any other human strain except Hong Kong. Likewise, strain A/Turkey–Massachusetts/65 appeared interrelated with the pandemic variant A2/Singapor/57, only, and exhibited no relations to other A2 strains. Thus, some narrow strain-specific relation is evident, precisely to these epidemic strains, which fact cannot be readily explained at present.

It seems interesting to us to compare findings on antigenic relation of two highly epidemic (pandemic) strains of human influenza viruses with influenza viruses isolated from birds. It can be seen from Table-Diagram 4, that antigenic cross-relation of human and avian influenza viruses is being proved already by three cases instead of one. The antigenic relation of Hong Kong virus is more pronounced than that of Singapor

Table 4

The antigenic relation of influenza viruses of man and birds

A2 (Singapor) 1/57   A (Turkey – Massachusetts) 65

A2 (Hong Kong) 1/68   - · · –   A (Duck – USSR) 60–63

Serum / Antigen	Singapore	Turkey Macsach.	Serum / Antigen	Hong Kong	Borki	B V – 1
Singapore	1	1/16	Hong Kong	1	1/2	1/2
Turkey Macsach.	1/32	1	Borki	1/8	1	1/2
			B V – 1	1/16	1/2	1

Note: Homologous serum titer is taken as 1, and fractions indicate portions of the titer.

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virus. The antisera prepared to avian viruses are more active in neutralizing the antigens from human strains than the antisera to human viruses are with respect to the avian antigens. This may indicate that avian viruses are more active biologically, and if so, that would make them still more important.

Some support to this suggestion has already been given in the report of Mitchell and Guerin (1968) of Canada, who showed that stability of avian viruses in airsols is much higher than that of human viruses. Obviously, those strains which maintain their viability in airsols for longer periods constitute greater potential danger.

Considering all that has been set forth above, one cannot exclude the possibility that the origin of highly epidemic strain may be connected in some way with circulation of influenza virus of animals in nature. It may be supposed also that, prior to the occurrence of pandemic and epidemic variants, there pre-exist in nature some parent strains, which by recombination or due to some other conditions provide origin to many different variants, inter-related in more or less degree.

As far as the origin of the new A2/3 variant is concerned, we are of the opinion, already expressed by us (1969), that its evolution has not been the consiquence of direct changes in the influenza A2/65–A2/67 virus varieties, but rather the result of repeated variation of the initial pandemic virus with possible participation in the process of avian influenza viruses.

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COMPARATIVE STUDIES OF ANTIGENIC STRUCTURE AND OTHER BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS TYPE A2 HONG KONG/68

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At the begining of 1969 an epidemic of influenza A2 Hong Kong/68 spread over the territory of U.S.S.R. In February 1969 we isolated from sick people in Moscow and studied 53 A2 strains. The virus difered from its predecessors in its antigenic structure, a high sensitivity to the inhibitors in normal human and animal sera and an unusual frequency of isolation from the nasopharynx of sick men.

Positive serological findings confirmed the etiological role of isolated viruses in 87% cases (Table 1).

Table 1

Mean geometric Titres of antibodies to the A-2 influenza virus

Year	Strains	Total number of sera tested	Of them number of those with diagnostic increase of antibodies		Meam geometric titres (log₂)	
			abc	%	Serum 1	Serum 2
1957	A–2 Sangapore 1	72	40	55	3.9	5.3
1968	A–2 Moscow 1	72	51	68	1.6	6.1
1969	A–2 Moscow 76	72	63	87	1.0	5.4
1966	B Moscow 1	72	5	6	5.1	5.2

MATERIALS AND METHODS

In this study 72 strains of influenza viruses A and B were used. Ten of these were isolated in December 1968, before the epidemic, 53 strains at the time of epidemic and 9 were the old strains from the collection of the Department of Virology. All strains were isolated by the method of inoculation of nasopharyngeal washings of patients into the amniotic cavity of 10 days old chick embryos. For the comparative study of the antigenic structure of A2/69, the strains Nos 35, 38, 36, 42, 44, 59, 55, 63, 67, 68, 70, 72, 75, 76, 79, 93, 94 and 96, which had not passed more than two passages in the allantoic cavity of chick embryos, were used.

Haemagglutinating activity was determined by the haemagglutination test which was performed at room temperature in the final volume of 1 ml with 1% of chicken erythrocyte suspension.

Infectivity was assessed by inoculating 0.1 ml of 10-fold dilutions of the material under study into the allantoic cavity of chick embryos. Four to five embryos were infected by each dilution. The results were calculated accoring to Reed and Muench.

The antigenic structure of influenza viruses was studied by the hemagglutination inhibition test and neutralization test in chick embryos. For this purpose freshly prepared immune rat sera were used which were heated for 45 minutes at 56°C and treated with potassium periodate (KIO_4).

The haemagglutination inhibition test was performed in plexiglass plates with 1 ml containing 4 HA units of virus plus 1% suspension of chicken erythrocytes.

Sera taken from human patients were treated with potassium periodate. The potassium periodate solution 0.05 M with distilled water was prepared on the day of the test. For this purpose 1.5 g KIO_4 was dissolved in 100 ml distilled water with heating in water bath (70–80°C) for 2–5 minutes until a clear solution was obtained. One volume of periodate solution was added to one volume of undiluted serum previously heated at 56°C during 30 minutes. The mixture was allowed to stand at room temperature for 2 hours, whereafter 2 volumes of 5% glucose solution were added for the neutralization of periodate. All these admixtures were taken into consideration when calculating the initial serum dilution.

The objects of the study were, in addition to the determination of the antigenic structure of viruses, also the susceptibility of viruses to the inhibitors of normal animal sera, the rate of agglutination of the chicken, horse and rat erythrocytes, thermoresistance of viruses, enzymatic activity, multiplication in the chick embryos, in the mouse lungs, in primary tripsinized cells and in cell lines.

The assessment of neuraminidase activity was carried out by N. Orlova (Virology Department, Microbiological Institute N. F. Gamaleia), using the incubation medium containing 0.6 mg ovomucin (as substrate), 0.6 ml of 0.4 M phosphate buffer at pH 6.0. The amount of 0.6 ml of

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Table 2

Antigenic structure of Influenza A-2 virus strains isolated in 1969

No.	Year	Strains	Rat sera													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1957	Singapore 1	512	16	64	8	8	8	8	8	8	8	8	8	8	8
2	1965	Moscow 21	64	512	512	128	512	8	8	8	8	8	8	8	8	8
3	1968	Moscow 1	8	32	512	256	256	16	8	8	8	8	8	8	8	128
4	1968	Moscow 20	32	32	256	512	256	16	8	8	8	8	8	8	8	128
5	1968	Moscow 22	32	32	256	512	512	16	8	8	8	8	8	8	8	128
6	1968	Moscow 1	8	8	64	8	8	512	256	256	256	256	512	256	256	256
7	1969	Moscow 76	8	8	16	16	16	128	512	256	256	512	256	512	256	256
8	1969	Moscow 79	8	8	16	16	16	256	512	512	256	256	512	512	512	512
9	1969	Moscow 44	8	8	32	16	32	128	256	256	256	256	512	512	512	512
10	1969	Moscow 63	8	8	32	16	32	128	256	256	256	512	256	512	512	512
11	1969	Moscow 68	8	8	16	16	32	128	128	128	128	256	256	256	256	256
12	1969	Moscow 94	8	16	32	32	32	256	256	512	512	512	512	512	512	512
13	1969	Moscow 59	8	16	16	16	32	256	256	512	512	512	512	512	512	512
14	1969	Moscow 49	8	16	256	128	256	32	8	16	16	8	32	16	16	16

Significant: Figures show the reciprocals of antibody titre in RHI test

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virus suspension had been diluted five-fold. Data characterizing the activity of the ferment were given for 1 ml volume of undiluted virus suspension. Neuraminic acid split off by neuraminidase was determined with thiobarbituric accid according to Warren (1959).

RESULTS

Table 1 shows the results of the haemagglutination inhibition test with 72 paired sera taken from influenza patients during the peak of the epidemic.

As can be seen from the table the increase of antibodies against the new A2 strains was 68–87%.

The antigenic structure was studied in the cross haemagglutination inhibition test and neutralization test in chick embryos with rat antisera and the corresponding strains (Table 2).

Table 2 shows the results of the haemagglutination inhibition tests with the representatives of type A viruses and the corresponding sera sorted out in chronological order. For this purpose we used the inhibitor resistant viruses. A 100% inhibition of haemagglutination by a homologous serum was taken as unit for comparison.

The analysis of the antigenic structure shows a poor neutralizing activity of the A2 Singapore/57 and A2 Moscow 21/65 antisera against the newly isolated strains. This is of great importance as it indicates a high variability of the antigenic structure within one type.

Special attention should be paid to A2 strains isolated before the beginning of the epidemic because of their possessing a unilateral resemblance. The immune A2 1968 antisera of a high homologous titre showed a limited relation to the A2 1969 strains but they retained the relation to the old strains.

We can follow the antigenic variability of the A2 strains more closely if we take one antiserum and a set of strains year by year, or one virus and a set of antisera in chronological order (Figs 1, 2, 3, 4 and 5). The unit for comparison was again a 100% inhibition of homologous sera shown as columns. Part of this unit corresponds to the ration of the titre of heterologous serum to the homologous one which is shown as column.

Fig. 1 (left part) shows one A2 Singapore 1/57 antiserum (along the abscissa) and a set of strains (taken year by year) along the ordinate. As can be seen from the figure the neutralizing activity of the A2 Singapore 1/57 antiserum towards A2 strains collected year by year gradually dicreases and is completely lost when interacting with the A2 Moscow 76/69.

Figure 1
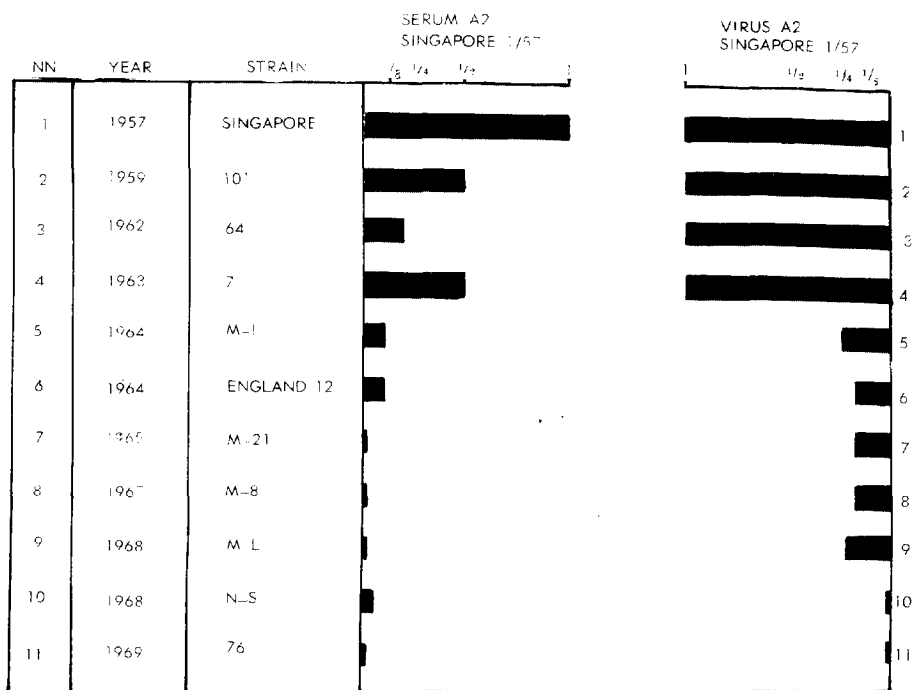
Absciss, left – A – Singapore 1/57 serum; right – A2 – Singapore 1/57 virus
Ordinate, left – strains of influenza A and A2 virus; right – Antisera to the respective
  strains

Figure 2
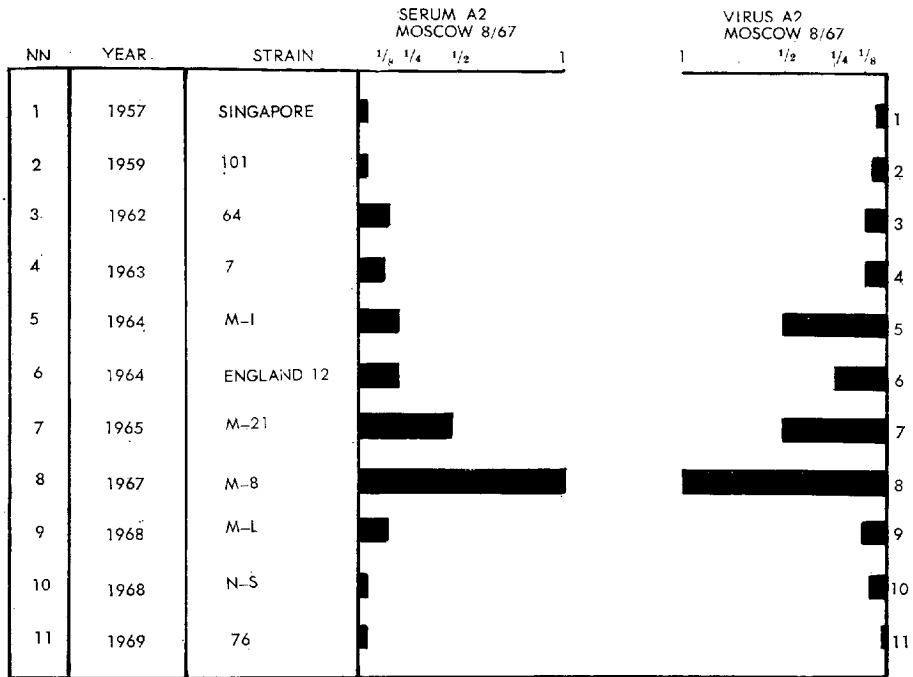
Absciss, left – A2 Moscow 8/67 serum; right – A2 Moscow 8/67 virus
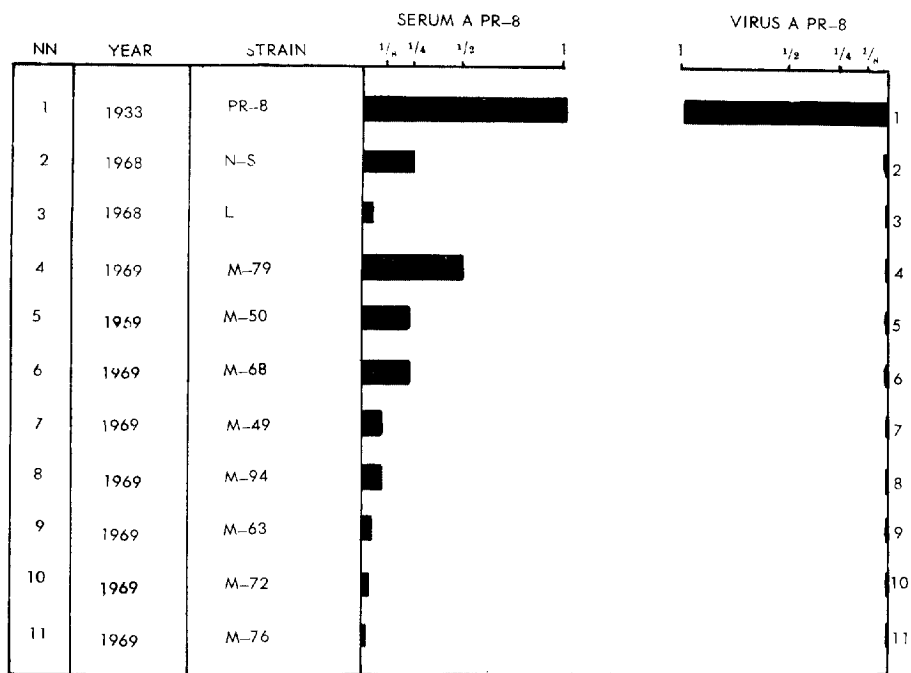Ordinate, left – strains A2 virus; right Antisera to the respective strains

Figure 3

SERUM A2 MOSCOW 76/69

VIRUS A2 MOSCOW 76/69

NN	YEAR	STRAIN
1	1933	PR–8
2	1949	M–2
3	1957	SINGAPORE
4	1959	101
5	1962	64
6	1963	7
7	1964	ENGLAND 12
8	1965	M–21
9	1967	M–8
10	1968	M–L
11	1968	N–S
12	1969	76
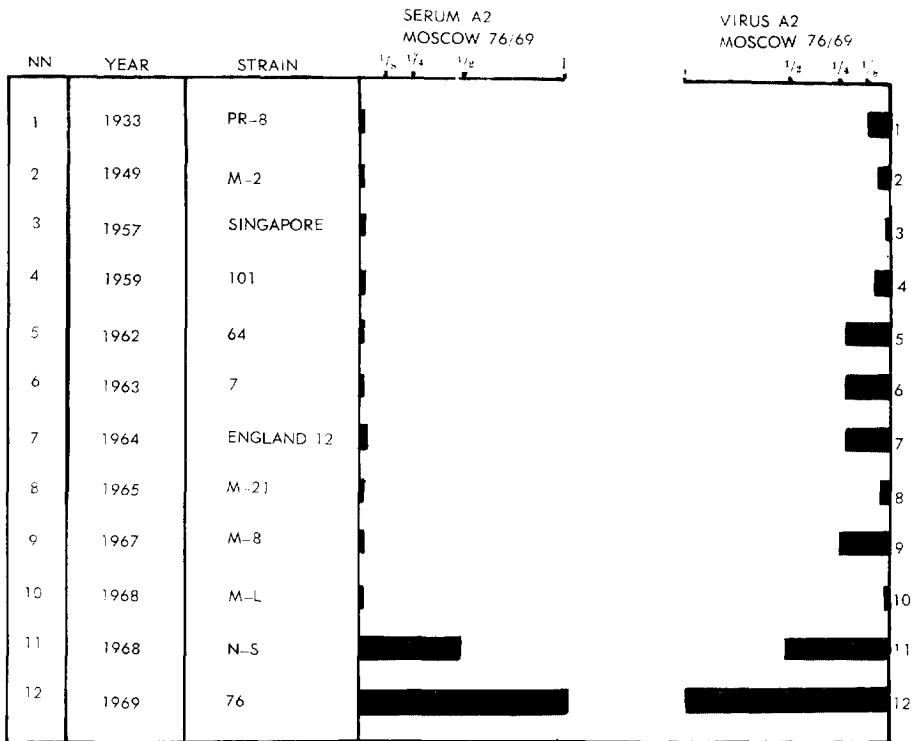
Absciss, left A2 Moscow 76/69 serum; right A2 Moscow 76/69 virus
Ordinate, left – strains of A2 influenza virus; right – Antisera

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Figure 4

NN	YEAR	STRAIN	SERUM A PR 8	VIRUS A PR 8	
1	1933	PR-8			1
2	1957	SINGAPORE			2
3	1959	101			3
4	1962	64			4
5	1963	7			5
6	1964	ENGLAND 12			6
7	1965	M-21			7
8	1967	M-8			8
9	1968	M-L			9
10	1968	N-S			10
11	1969	76			11
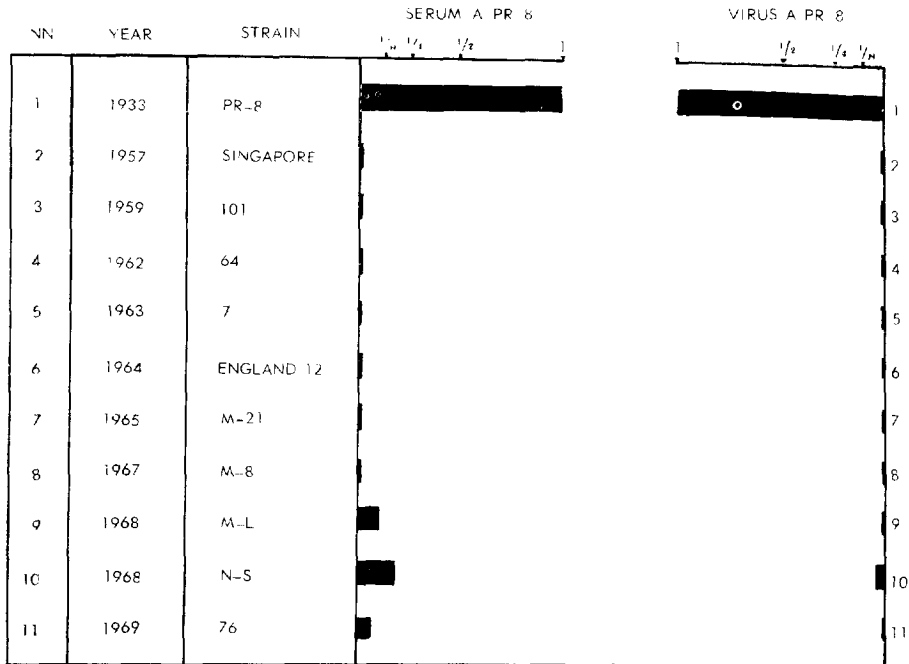
Absciss, left A–PR8 serum; right A–PR8 virus
Ardinate, left strains of A and A2 influenza virus; right respective antisera

Figure 5

Absciss, left A–PR8 antiserum; right A–PR8 virus
Ordinate, left strains of A and A2 1968 and 1969 influenza virus
    right – respective antisera

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If we take one A2 Singapore 1/57 virus (along the abscissa) and a set of antisera year by year (along the ordinate) we can see that antisera neutralize the strains isolated till 1964 and then their activity is lost, and that the antisera from 1968 and 1969, even with a high homologous titre, do not neutralize the A2 1/57 Singapore strain.

If we take one A2 Moscow 8/67 antiserum (Fig. 2) and a set of strains year by year we can see that the neutralizing effect is gradually decreasing towards the older strains and gradually increasing towards the A2 1965–67 strains.

And lastly, the A2 Moscow 76/69 antiserum completely loses the neutralizing activity towards the previously isolated strains (Fig. 3) while the antisera of previous years partially maintain the capacity to neutralize the new A2 Moscow 67/69 strains.

The A-PR-8 antiserum did not show neutralizing activity towards A2 strains of previous years (Fig. 4) and partially neutralized the A2/69 strains. The neutralizing activity of this serum towards the set of strains A2 1968–69 showed some differences (Fig. 5). Some strains were neutralized by this serum up to 1/2 holomogous titre, the others only up to 1/16. On the other hand, the A-PR-8 virus itself was not neutralized by antisera A2 1968–69.

Data obtained with rat sera and corresponding strains in the cross haemagglutination inhibition test show a distinct antigenic difference of the A2 Moscow/69, from the pandemic A2 Singapore 1/57 and the epidemic A2 Moscow/65 strains. This permits us to recommend to treat them as independent variants and denominate them by the year of isolation, namely A2 Singapore 1/57, A2 Moscow/65 and A2 Hong Kong/68.

The peculiarity of the antigenic structure of the A2 epidemic strains is confirmed by the results of serological investigations in different age groups in Moscow, Kiev and Volgograd. Persons up to 60 years of age did not posses antibodies to the A2 Hong Kong/68 virus and among persons over 60 antibodies were found in as much as 74 per cent. In Table 3 are presented the mean geometrical titres of antibodies to type A2 viruses. It follows that in person between 17 and 59 years there were no antibodies to the epidemic strain A2 Moscow/69, while in persons over 60 years the mean geometric titre of antibodies to this virus was 26. This induced us, as well as some other investigators, to believe that there exists immunological relationship between the Hong Kong influenza and its early predecessors. Sera of people born before 1900, that never had had Hong Kong influenza, contained antibodies to Hong Kong influenza. On the basis of serological tests we can also assume that an influenza virus similar to the Hong Kong one was the cause of the pandemia in 1889–1890 and it dominated towards the end of the last century.

Table 3

Geometric mean titre of antibodies to influenza viruses A-2 in humans of different ages

Viruses	Sera taken from men					
	17–59 years old			older than 60 years		
	Total of examined sera	M	Geometric mean titre of antibodies $\log_2$	Total of examined sera	M	Geometric mean titre of antibodies $\log_2$
A–RJ8	210	9.8	3.5	60	20	4.3
A–2 Singapore 1/57	210	30.8	4.7	60	30	4.9
A–2 Moscow 21/65	210	35.7	4.9	60	29	4.0
A–2 Moscow 76/69	210	< 2	—	60	26	4.6
B Moscow 1/66	210	25	4.6	60	106	6.7

When we observed the variability of the antigenic structure of the A2/69 strains we also became interested in other biological properties of the virus.

In search of certain laws of the variability of influenza virus, as well as in the attempt to select vaccinal strains, we have systematically tried, beginning with 1960, to establish correlation between pathogenic properties of these strains for man and other properties (2, 3, 4).

It has been observed that inhibitor sensitivity is a rather constant property related with pathogenicity of viruses. This was also confirmed by a gradual increase of the prevalence of inhibitor sensitive strains in the course of the epidemic process. Taking into consideration these data from the beginning we have tested al the 53 strains of A2/69 by the haemagglutination inhibition test using normal rat, mouse, guinea-pig, rabbit and horse sera. In rat and mouse sera no thermostable inhibitors were observed. In guinea-pig, rabbit and horse sera there were inhibitors in high and low titres. One only strain (No. 49) out of all strains under study was inhibitor resistant, as it was explained later; by its antigenic structure it belonged to the strains of the previous period.

Analyzing data obtained we found it appropriate to consider two groups of strains within one serological subtype of A2 taking into account the properties of haemagglutinins. The first group consisted of strains with a high sensitivity to inhibitors in normal animal sera (1:160 – 1–2560) and the second of strains with a low sensitivity to the inhibitors (1:10 – 1:80) (Table 4).

Table 4

The effect of normal serum inhibitors on A–2/1969 influenza viruses

| Year | Strain No. | Normal animal sera | | | | |
		Mice	Rats	Rabbits	Guinea-pigs	Horses
1969	1	0	0	160	2,560	640
1969	9	0	0	160	2,560	320
1969	13	0	0	80	1,280	640
1969	17	0	0	80	1,280	320
1969	19	0	0	160	640	320
1969	23	0	0	80	1,280	640
1969	31	0	0	80	1,280	640
1969	35	0	0	80	1,280	320
1969	36	0	0	80	1,280	320
1969	42	0	0	160	160	640
1969	44	0	0	40	1,280	320
1969	75	0	0	320	640	640
1969	76	0	0	80	1,280	160
1969	79	0	0	40	1,280	320
1969	86	0	0	40	1,280	320
1969	91	0	0	160	320	160
1969	93 Hong Kong	0	0	160	320	160
1968 Hong Kong (result)		0	0	160	1,280	320
1969	2	0	0	20	40	80
1969	15	0	0	20	80	80
1969	24	0	0	10	160	80
1969	50	0	0	20	40	160
1969	63	0	0	40	40	20
1969	84	0	0	40	80	40
1969	95	0	0	20	40	20
1969	49	0	0	0	0	0
	(selection)	0	0	0	0	10
1969	76	0	0	0		
1968	Hong Kong 1 (selection)	0	0	0	0	0

Legend: Figures show the reciprocals of antibody titres in IH test

The strains of the two groups of viruses did not differ in their activity to chick embryos. They were similar in their multiplication rate in the allantoic cavity and on the 4th day they killed part of them. The infection index for chick embryos was high, $10^{10} – 10^{12}$ $1D_{50}$ (0.1 ml). Neither of these groups caused primary pneumonia in white mice but the isolations of virus from the lungs of infected animals showed a difference in the intensity of virus multiplication. The viruses differed in thermorestistance of haemagglutinins and the rate of elution from the erythrocyte surface.

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The strains of the second group retained their haemagglutinating activity after one hour's heating (56⁰C), the strains of the first group lost it in the course of the first 5–10 minutes and they also slowly eluated in physiological saline solution.

The strains differed in their sialidase activity (both in the rate and in the total quantity of N-acethylneuraminic acid). The strains of the first group had low indices of sialidase activity (2–10 μg/ml/min.) and strains of the second group had a high sialidase activity (10–23 μg/ml/min.) (Table 5).

Table 5

Average figures of solidase activity of A–2 strains, 1968–1969

Year of isolation	Strain Nos	Sensitivity to thermostable inhibitors	Capacity to produce interferon in white mice	average figures of solidase activity (γ/μg/ml/min)
1969	1	high	passive	6,75
1969	9	high	passive	2,3
1969	13	high	passive	6,4
1969	17	high	passive	6,1
1969	19	high	passive	1,7
1969	23	high	passive	6,7
1969	31	high	passive	4,6
1969	35	high	passive	4,4
1969	36	high	passive	4,05
1969	42	high	passive	6,2
1969	44	high	passive	10,0
1969	75	high	passive	7,5
1969	76	high	passive	9,25
1969	79	high	passive	7,9
1969	86	high	passive	7,1
1969	91	high	passive	6,1
1969	93	high	passive	6,5
1968	Hong Kong (result)	high	non tested	10,0
1969	2	low	active	12,3
1969	15	low	active	11,4
1969	24	low	active	12,9
1969	50	low	active	20,6
1969	63	low	active	11,2
1969	84	low	active	11,6
1969	95	low	active	15,4
1969	49	non-sensitive	active	18,7
1968	Moscow 1	non-sensitive	not tested	23.0
1968	Hong Kong 1 (selection)	non-sensitive	not tested	21,0

We have tried to establish a relationship between the structure of viruses and their biological properties using electron microscopy. No significant differences in the structure of virions between the groups were observed (Bocharov, 1969) (Figs 6 and 7).

Figure 6 and 7
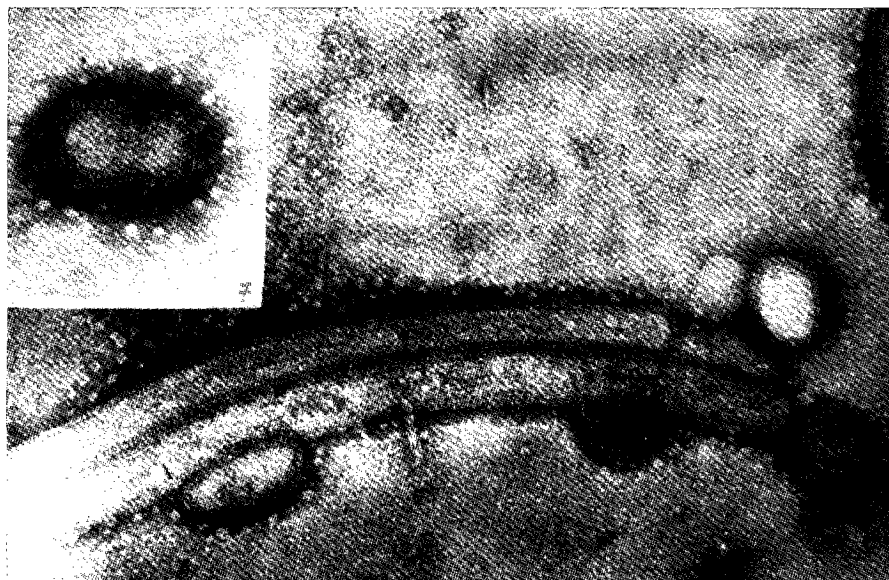
Virious of A2 1969 influenza virus (magnification 150.000)

A recently studied property of influenza virus is its interferon producing capacity shown in experiments in mice. According to the results obtained by N. R. Gutman, the interferon producing capacity in the A2 influenza viruses is not always the same. The interferon-passive strains belonged to the first group and the interferon-active ones to the second.

Pathogenicity for men was studied in representatives of these groups of viruses (strains 1 and 2) and in selected variants (76S$^+$, 76S$^-$). Preliminary investigations showed the strains of the first group to be more pathogenic.

On the basis of the investigations performed we have come to the conclusion that among the inhibitor sensitive strains of the A2 Hong Kong/68 there were strains with more or less pronounced properties of reactogenicity for men. Table 6 presents summarized data on some biological characteristics of these two groups of viruses of the A2 Hong Kong/68 type.

The strains of the first group proved to be highly susceptible to the normal animal serum inhibitors, to have low indices of sialidase activity and a low rate of elution in physiological saline solution, to be less re-

Table 6

Certain biological characteristics of A–2 1969 influenza viruses

S. Nos	Biological characteristics	Symbols	Strains 1st group 1, 9, 13, 17, 19, 23, 31, 35, 36, 42, 44, 75, 76S+ Hong Kong /68S+	Strains 2nd group 2, 15, 24, 49, 50, 63, 18, 84, 95, 76S—, Hong Kong /68S—
1	Antigenic structure	A	A–2	A–2
2	Sinsitivities to the inhibitors	Si	High (160–2560)	Low (10–80)
3	Patogenicity for chick embryos	Vm	+	+
4	Thermostability of haemagglutinin	Tv	—	+
5	Thermostability of virus	Th	—	+
6	Solidase activity	n	Low (2–10 γ μg/ml)	High (10–30 γ μg/ml)
7	Interferon	J	passive	active
8	Pathogenicity for humans	Vh	+	--

sistant to temperature and to be passive inducers of interferon production and more pathogenic for man.

The strains of the second group showed a low susceptibility to the normal animal serum inhibitors, had a high sialidase activity, a high rate of elution from the erythrocyte surface, better stability to temperature, and they were actively inducing the production of interferon in mice and were less pathogenic for man.

Our earlier opinion on the correlation between the reactogenicity of the A2 strains and other laboratory properties was not only confirmed by this study; we have also rendered more concrete the possibility of separating the inhibitors-sensitive strains of A2 Hong Kong/68 into two genetically heterologous groups, differing in their pathogenicity for man. We recommend the use of these laboratory markers tentatively for the selection of vaccinal strains and to check their reliability on a larger scale in volunteers.

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SUMMARY

Fifty-three strains were isolated from sick humans during the pandemic of A–2/69 influenza and further studied. These A2 strains differed from those isolated before in the peculiarity of their antigenic structure, high sensitivity to inhibitors in normal animal and human sera extraordinary frequency of isolation from the nasopharynx of the sick people.

The present study contains the comparative characteristics of the antigenic structure of A2/69 strains in cross inhibiton haemagglutination and neutralizing tests in chick embryos.

Etiological role of newly isolated viruses was confirmed in 87% by serological investigations of sera from the sick persons.

The analysis of antigenic structure showed the high degree of variability of A2 viruses within one type. The strains isolated before the epidemic preserve only unilateral relationship with epidemic strains and were referred by us to transitional strains.

Besides antigenic structure we also stidied some other biological properties.

Analysing the data obtained we noticed that all the viruses can be divided into 2 groups: the 1st group – the strains with high sensitivity to inhibitors in normal animal sera (1:160, 1:2560) the 2nd group – the strains with low sensitivity to inhibitors (1:10, 1:88).

Both groups are identical in their entigenic structure, but differ in their pathogenicity for man.

The correlation of pathogenicity of strains with other biological properties was found. Interferon production appeared a new property. A2 strains with low pathogenicity are active inducers of interferon production under experimental conditions.

Thus, the possibility of dividing the inhibitor sensitive A2 strains into 2 genetically different groups are shown. These groups differ in their pathogenicity for man. These indications may be recommended for preliminary selection of apathogenic strains for production of vaccine.

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THE EPIDEMIOLOGY OF INFLUENZA

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Information about the influenza epidemic of 1968/1969 was provided with unprecedented exactness and promptness, so that all those concerned were well-informed. We owe this to the WHO and its co-operators. The public health services of the individual countries were enabled by these informations to take preparative measures for the things to come and to pass on their informations to the physicians and hospitals as well as to public undertakings. In many cases vaccinations could be given just at the right moment. Vaccines containing the Hong Kong strain were available four months after the outbreak of the epidemic in Hong Kong. The published virologic-serological observations served for a correction of the method of serological testing which increased the reliability of the tests.

In the Federal Republic of Germany a network of investigation and information extending over all Laender was organized by the Federal Health Office (Anders in press). The scheme of this organization is shown in table 1. This enabled quick dissemination of weekly reports. This type or organization proved of great value because of its uncomplicatendness and was more successful than any regularized duty of notification. This kind of surveillance covering approximately 2% of the population gives a true picture of the degree of the spread of influenza. Special importance was given to the epidemiological reports of the neighbouring countries. Of decisive importance and indispensable for the assessment of the situation is the continuous control of the immune

state of the population, so that data of the pre-epidemic period are at hand for comparison purposes by help of which interpretation of the results obtained during serological investigations will be possible. On this I shall enter into more detail.

We must take great efforts to search for influenza viruses during the interepidemic periods. Sporadic clinical influenza cases should be investigated virologically. The very interesting and important question as to the whereabouts of influenza viruses during the interepidemic periods up to now has remained open. And the difficult diagnosis of influenza out of epidemic periods can be made in the individual case only by laboratory tests.

I do not intend to speak about the epidemics of 1968/1969 caused by influenza virus Type B. We have become more and more conscious of the special peculiarities of this type. New experimental results of Cuadra (Cuadra 1970) seem to me to be of some importance since they bear the possibility of a fundamental explanation. Cuadra was successful in demonstrating how the behaviour of influenza virus Type B strains differs in the cells of the cloriallantoic membrane from that of influenza virus strains Type A. The cytopathogenic process takes a different course as to the time, quality and quantity. A subacute phase may be observed over still a long period of time. This could possibly indicate that influenza virus Type B persists longer in the cells, which could be of some importance for immunity. You cannot help being under the impression that by these experiments properties of influenza virus Type B came to light that hear importance also for both the epidemiologly and the clinic.

The influenza epidemic of 1968 starting from Hong Kong raised more virological and epidemiological problems than it gave answers to so far unsolved questions. It demonstrated again the special position of influenza viruses Type A as the cause of epidemics among all other agents of infections. This may be due also to the pronounced individuality of influenza virus strains. Number and duration of passages through man and animals, possibly also the installation of host substance are influencing and partaking factors. In the development of a new variant the immune state of the host plays at least a selective role.

The antigenicity which is represented by substances on the surface of the virion has a large spectrum of variation within a genetical frame given for each type and subtype. From the hazard of mutations and varations subtypes and variant strains may develop. Not only serological but also biological properties, virulence, infectivity, the behaviour towards the various serum inhibitors, the so-called mucinotropism (Ger-

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bunova 1964), and the behaviour of the neuraminidase antigen, alone or in combination, are influencing the chance of a new variant to prevail, after these special properties accumulated in the population of a strain. The exclusive way of infection, droplet infection, means a decrease in the spread of virus. The amount of virus, the so-called starting dose, which is necessary for the taking of the infection will depend upon various circumstances in the environment and in the recipient. The general disposition of the·latter and the sensitivity of the mucous membranes of the nose, the pharynx and the conjunctivae play an important part. Specific antibodies and inhibitors (Mascoli et al. 1965, Waldman et al. 1968), possibly also interferon, contained in the secretions of the mucous membranes are decisive for the possibility of influenza viruses to settle. The amount of virus spread in the environment by droplet aerosol demands heavy multiplication in the mucous membranes of the patient and sneezing and coughing as symptoms. Thus the connection between illness and spread of the agent is given. The host is under the influence of the climatic condition and the quickly changing weather fronts which may influence the spread of influenza and play a trigger part. Influenza is a seasonal illness, so that viruses of sporadic cases or of strongly isolated foci will not spread at an inappropriate moment or will at least not induce a wave of cases.

The course and spread of the influenza epidemic of 1968/69 gave again in many cases evidence of the connection between the spread of the epidemic and the tracks of the virus spreading human beings. Here influenza appears to be a civilization illness, in which case crowded people and frequent contacts connected with industrialization play an essential part.

In which other infectious disease do exist such a plenty of stable and changing factors, due both to the virus and the individual, that play such an important role in the taking of the infection, its promotion and inhibition, as in the case of influenza? And in which other infective agents are included so many chances of direct or indirect participation of the host in the development of mutants, e. g. by formation of hybrides (Kilbourne et al. 1967, Kilbourne 1968), and variants, as in the case of influenza virus (Hoyle 1968)?

The character and behaviour of influenza virus A2/Hong Kong/68 and the course of the epidemics induced by this virus have set up many a riddle to all of us. Can we speak of a pandemic? Principally yes, since the chains of infection spread from one region in all directions and extended over all countries. The Hong Kong virus appeared as pandemic virus. The previously unknown Hong Kong strain was found everywhere

in temporal sequence. Only the morbidity showed differences in the individual populations. There arose epidemics, or only increases in the incidence of cases were noted, or there occured only sporadic cases. The unequal distribution of the cases shows that the Hong Kong strain represents, it is true, an extreme relative of the subtype A2, however, it cannot be regarded as a strain of a new subtype, otherwise epidemics should have occured everywhere. As a consequence of the proceeding epidemics caused by A2 variants, an immune state had developed in the populations, which, depending on the degree of the relationship with the A2 variants, promoted or inhibited the spread of the Hong Kong strain.

When comparing the antigenicity of the A2 strains isolated since 1957 with the antigenicity of Hong Kong strains (table 2), it can be noted that some strains have no or only little antigenic relationship to one another and that some of them come antigenically nearer to the Hong Kong strains. As examples for this may serve A2/Japan/305/57, A2/Tokyo/3/67, A2/Texas/2/68, as well as A2/Georgia/1/67. (Coleman et al., 1968, Archetti et all., 1950, Zhdanov et al. 1966 and 1967, Pereira 1969).

The similarity coefficients (WHO Int. Infl. Center Americas 1968, Archetti et al. 1950, U. S. Dept. Health, Education, and Welfare 1967, Coleman et al. 1968) listed in table 3 for the Hong Kong strains and the previously isolated A2 strains demonstrate the high dissimilarity. However, they also demonstrate that Hong Kong strains are not fully identical with one another (WHO Int. Infl. Center Americas 1968). Furthermore, from this follows that if the serological tests will not be carried out with the Hong Kong strain, it will also not be possible to diagnose cases caused by the Hong Kong virus by means of the other variant strains of A2. Persons without previous contact with the Hong Kong virus do principally not have homologus antibodies to this strain. However, some exceptions were found in persons above 60 years of age (Masurel et al., 1966). Additionally, an antigenic relationship to A/equine/2 was noted and also confirmed. Horses have high antibody titers to the Hong Kong strain after an A/equine/2 infection (Coleman 1968). This is an additional sign of the pecularity of this strain of the subtype A2. In theory it would be imaginable that after the epidemic of 1889, which is said to be caused by a relative of the subtype A2, the variants of this strains developed in direction of a strain similar to the Hong Kong strain. The antigen component influenza A/equine is said to have played certain part in the epidemiology around the turn of the century. (Masurel et al. 1966).

The infection with the Hong Kong strain stimulates antibody production to previous A2 variants, whereas the antibodies of these previous A2 variants do not, or only to a limited extend, overlap those of the Hong Kong strain (Köhler in press, Coleman et al. 1968, Stuart-Harris 1965 – Antoniadis et al. in press).

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That's the reason why the appearance of the Hong Kong strain lead to total elimination of the previously widely spread A2 variants. The booster effect induced by the Hong Kong strain blocked the way for the locally present A2 variants so that the infection chains were interrupted. Additionally can be suggested that the A2 variants were exhausted in their infectivity in consequence of frequent passages through partly immune persons. The respective A2 variant could no more be demonstrated nor isolated. Only in the case that antibodies were stimulated which were directed against a variant nearly related to the Hong Kong strain, infection by the Hong Kong strain was also inhibited.

These circumstances are likely to explain the unequal morbidity among the populations of the different countries. If A2/Tokyo/3/67 or A2/Texas/2/68 were epidemically spread among a given population they did not leave immunity against Hong Kong because they were antigenically not related, therefore the Hong Kong strain could cause an epidemic. If many other variants, e. g. A2/England/12/64 or A2/Berlin/68 preceded the spread of the Hong Kong strain, certain degree of immunity sufficient to inhibit the infection by the Hong Kong strain could be expected, because these strains were antigenically nearer to the latter. Therefore, the Hong Kong strain was not able to cause an epidemic. The specific antibodies to the previous A2 variants were probably enhanced by booster reactions in the secretions of the mucous membranes, so that multiplication of the Hong Kong strain could not occur or influenza could not develop. In these cases, however, specific antibodies to Hong Kong can be demonstrated (Köhler in press). Such observations can be made only in persons with previous contact with viruses of the Type 2. From the observations made in Berlin West where a Hong Kong epidemic did not occur, although the strain could be demonstrated directly and indirectly can be concluded on the grounds of serological comparison that A/2Berlin/2/68 is antigenically relatively similar to the Hong Kong strain. The serological similarity of A2/Berlin/2/68 to A2/England/12/64 was confirmed by investigations carried out in London. (Farnik et al., 1966, Möbest et al., 1969).

Perhaps for the first time it has become possible to demonstrate that the variants of a subtype do not only differ from each other in their antigenic structure but also by their biological properties, e. g. the sensitivity to inhibitors which possibly does also play a part in the defense of the organism and may also represent peculiarities of the variants. Initial difficulties in the hemagglutination inhibition test for the demonstration of antibodies to Hong Kong virus could only be overcome after it had been noted that strong RDE-treatment of the serum was indispensable. (Antoniadis et al. 1968, Zhdanov et al. 1966.)

It can be regarded as a surprising fact that Hong Kong virus may be cultivated directly in the allantoic cavity and not only in the amnionic cavity, and that already the titers of the first passage show high values. (U. S. Dept. Health, Education, and Welfare 1967, Paniker et al. 1969.)

Since 1964 investigations on the immune state of the population have been carried out in the Robert Koch Institute (Köhler in press) which allow comparisons and prognostic forecasts. The tests are performed on 100 sera each of blood donors by means of the hemagglutination test. This number has proved sufficient for the above test. The antigens used in the serological tests were exchanged during the course of these five years in competitive comparisons to improve the rate of success of the test. The strain A2/Berlin/12/1960 proved its specificity practically up to the moment of the appearance of the Hong Kong strain.

The immune state expressed by the antibody titer may be calculated by the geometrical mean value. These investigations yielded the following results (Köhler):

1. During the years 1960 to 1969 an increase in the antibody titers was observed and a decrease in the percentage of persons who had no measurable antibody titers or only low antibody titers. These data are presented in table 4. Thus the population of Berlin West entered into the autumn of 1968 with a good immune state and Hong Kong influenza could not spread. Table 5 presents the immune state of the population of some other towns in the F. R. Germany in 1967.

2. In a comparison with sera from other countries – as shown in table 6 – differences were observed which may be explained by the different epidemiological situation. Striking were the high titers of sera coming from Warsaw and Helsinki. In both cases influenza epidemics are said to have occured, whereas in Paris, where only low antibody titers were found in the samples, there was no history of an influenza epidemic. On the basis of these experiences high antibody titers of approximately 1:200 and more may hint to acute influenza.

3. If serum tests are set up with the antigens of different variants of the A2 subtype isolated at one and the same place, here, Berlin West, at different intervals, antigenic shifts are to be observed as can be seen in table 4. Similar observations were made by others (U. S. Dept. Health, Education, and Welfare 1967, Pereira 1969, Zhdanov et al. 1966, Zhdanov et al. 1967).

4. A gradual increase of the antibody titers proves that during the years 1960–1969 there occured no influenza epidemic in Berlin West (table 4).

5. From the percentage of persons with antibody titers lower than 1:56 one can conclude to the extend of the lack of immunity to the virus strains A2 (table 4).

6. The presence of the Hong Kong virus was evidenced by hemagglutination-inhibiting antibodies which were markedly increased in March 1969 in a higher percentage of the population (table 4).

Influenza Surveillance Federal Republic of Germany

Sources of Information

General Morbidity (Absenteeism) reported by Insurances, Administration Bodies, and Industries (4% of population age 15–65)	Virological findings by the 4 National Influenza Centres	Immunity Register	Reports on Observation and Measures by Health Authorities	WHO Records and personal Informations from abroad (East *and* West)

Wkly (Daily); *Influenza Express Information* (Leaving Berlin by Telex every Tuesday)

Strains	Feret anti sera				
	A2/Sing	A2/Engl/ 12/64	A2/Engl/ 10/67	A2/Tok	A2/HoKo/1
A2/Singapore/1/57	1280	160	480	120	160
A2/England/12/64	160	3840	1920	480	80
A2/England/10/67	120	480	2560	120	40
A2/Tokyo/3/67	20	30	80	480	<20
A2/Hong Kong/1/68	40	30	80	<20	2560
A2/Hong Kong/8/68	120	30	120	<20	3840
A2/Hong Kong/19/68	80	30	120	<20	2560

(WHO)

Strain Relationships of Type A2 Influenza Viruses with 1968 Hong Kong Isolates

	A2/Jap	A2/Georgia	A2/Tok	A2/Tex	A2/HoKo
A2/Japan/305/57	1.0				
A2/Gergia/1/67	4.0	1.0			
A2/Tokyo/3/67	16.0	5.7	1.0		
A2/Texas/2/68	22.6	2.8	4.0	1.0	
A2/Hong Kong/8/68	?	22.6	?	64.0	1.0

Similarity coefficients according to formula of *Archetti* and *Horsfall*

(WHO Int. Infl. Center
for the Americas)

7. In table 7 are presented the results of a study of antibodies to influenza viruses of Type B carried out in March 1969. The findings suggests that he population of Berlin West would face an influenza B epidemic practically without any protection. As contra-example may serve data from the USSR which prove the good immune state against influenza Type B.

The fanning-out of a subtype into a number of serologically differentiable variants represents a phenomenon characteristic of influenza virus Type A, which, for example could also be observed on subtype A1. But

in subtype A1, no variant has become known so far that had such a pe-
netrating force to spread all over the world. The variants of A1 (Stuart
Harris 1965), e. g. Scandinavia, Liverpool, Dutch, caused epidemics of
limited range, although the strain Liverpool spread to South Africa,
Australia, and the USA and Canada. Nevertheless, they remained epi-
sode strains. The variant Liverpool must have had special biological
properties since the epidemics caused by it were characterized by stri-
kingly high lethality. The mentioned variants and all other members
of the A1 subtype did not suppress or did suppress only temporarily the
otherwise spread virus strains of the subtype A1. The pecularity of the
A2 Hong Kong strain becomes evident also in this respect.

Distribution (%) of hah antibodies against influenza A₂
Berlin-West 1960–1969 (Köhler 1969)

Month/Year	Strains	ANTIBODY TITER (recip. val.)			GEO-METRIC MEANS
		<56	56–224	>224	
1960	A2/Sing/1/57	84	12	4	8.5
II /1962	A2/Berl/12/60	79	21	—	12
III/1965	,,	31	59	10	60
III/1966	,,	52	45	3	28
IV/1967	,,	31	55	14	60
II /1968	A2/Berl/2/65	30	60	10	60
III/1968	,,	21	52	27	137.5
		<40–40	80–320	>320	
III/1969	A2/Berl/2/65	2	71	27	247

Hah antibodies (recip. val.) against influenza A₂ in healthy persons
January 1967 (Köhler 1969)

CITIES	DISTRIBUTION (%) OF ANTIBODY TITERS			
	<56	56–224	>224	GEOMETRIC MEANS
Berlin-West	24	72	4	59.7
Frankfurt/M.	23	72	6	72
Dortmund	39	58	3	46
Hamburg	51	45	4	29
München	42	44	14	46
Stuttgart	36	57	7	49.5
Rotenburg/Hann.	24	67	9	77

Although it is audacious, it is the more fascinating to forecast the further development of influenza epidemics. The influenza Type B epidemics will reappear in the so far experienced manner always then when the lack of immunity to this virus type has reached certain extend. This condition will be reached temporarily and regionally differently in the individual countries. Vaccinations against influenza Type B will be advantageous and, in all probability, also effective.

Completely different are the circumstances referring to influenza Type A. Only a new subtype A3 will cause a true pandemic. If there would be followed a rule, this subtype A3 should be similar in its main component to the subtype of the pandemic of 1918. It would meet with a population that would in more than 60% not possess antibodies to the imagined subtype. The A2-variant Hong Kong will reach during the next few years those regions where it has not yet come to and in those regions where it had already induced epidemics it will glean. In places where it could not prevail, a big lack of influenza cases is likely to arise. Only a new A2-variant of a special character could cause here an influenza epidemic. Virological investigations to be carried out during the forthcoming months could give us certain answers.

Let's solve this task together!

Hah antibodies (recip. val.) against Influenza A_2 in healthy persons Spring 1967 (Köhler 1969)

| CITIES | DISTRIBUTION (%) OF ANTIBODY TITERS | | | |
	<56	56–224	>224	GEOMETRIC MEANS
Helsinki	17	67	16	102
Warschau	3	61	36	192
Innsbruck	19	56	35	125
Paris	39	49	12	49
Berlin	31	55	14	60

Hah antibodies (recip. val.) against Influenza B in healthy Persons (Köhler 1969)

| CITIES | DISTRIBUTION (%) OF ANTIBODY TITERS | | | |
	<40–40	80–320	>320	GEOMETRIC MEANS
USSR, 4th quarter 1968	16.4	70.3	13.3	136
Berlin-West, Oct. 1968	85	15	—	8
Berlin-West, March 1969	84	11	5	9.5

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SUMMARY

The influenza pandemic of 1968/69 is characterized by its special pecularities which are above all due to the fact that the agent, the Hong Kong strain, does not belong to a new subtype of influenza A but represents an extreme variant of the influenza subtype A2. Although the strain could be isolated in all countries of the world, a commensurate all over increase in clinical influenza cases has so far not occured. The reason for this is to be sought in the relationship between the Hong Kong strain and the A2 strains spread in the different countries during the months before the appearance of the Hong Kong strain. If there is any relationship, the Hong Kong strain will induce a booster effect. The antibodies to preceding A2 strains will increase and, beyond this, production of antibodies to the Hong Kong strain will be induced without the individual to become sick. The well-known phenomenon that a new influenza strain inhibits the spread of the preceding one appeared also during the Hong Kong epidemic. – It has proved of great value for the assessment of the epidemiological situation that since 1964 current investigations into the immune state of the population have been carried out at the Robert Koch Institute. The serological findings enabled clear determination of the moment of appearance of the Hong Kong strain and specific assessment of the immune state to this strain. The populations of certain areas have proved highly susceptible to influenza virus Type B. However, in all probability an epidemic caused by the Hong Kong strain or its variants is not very likely to occur.

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INFLUENZA IN POLAND IN THE PAST DECADE

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Epidemiological pattern of influenza to a great extent is determined by the most essential character of the virus – its enormous variability. The variation cycle is known to cover the period of roughly 10 to 12 years and at such intervals large epidemics (or pandemics) occur, as a rule associated with the appearance of a new virus variant.

Of course, variation is a continuous process and virus strains isolated even at one-year intervals may differ antigenically, in spite that they may belong to one and the same type or subtype. The greater are the differences, the higher is as a rule the number of cases in a given region. Such a correlation is clear considering the fact that immunity of a population – both its level and the range of antigenic variants covered by it – is the second important factor by which epidemiology of influenza is determined.

The report on epidemiological situation of influenza in Poland should begin from 1957, the year in which A2 variant of influenza virus appeared. However, a systematic serological survey of the whole country is performed since 1965; therefore, the preceding years will be discussed only in short. The situation at the turn of 1968 and 69 and at the beginning of 69 will be discussed in more detail, for the recent epidemic as well as the appearance of A2 Hong Kong variant may be supposed to open a new period in the epidemiology and variation of influenza.

Since 1957, when A2 pandemic first occurred, in almost each year outbreaks were observed variable in extent and intensity. All they were preceded by a higher incidence of illness in November and December, and occurred in the first quarter with peak usually in February. The situation in the individual years is illustrated by data in Table 1.

Table 1

Influenza in the years 1957–69

Year	No of cases	Virus isolation
1957	3,101,166	A₁, A₂
1958	145,640	—
1959	1,969,234	A₂
1960	230,425	—
1961	580,224	A₂
1962	3,131,027	B
1963	299,729	A₂
1964	776,663	A₂ partly B
1965	894,028	A₂
1966	645,170	B
1967	1,535.965	A₂
1968	142,811	—
1969	4,470,072	A₂

It is evident that large epidemics with highest numbers of cases occurred in 1957, 59, 62, 65, 67, and 69. Morbidity rate also was highest in these years (Table 2).

Table 2

Influenza epidemics in recent years

Year	Morbidity per 10^5 inh.	Fatal cases
1959	388.0	—
1962	801,0	1,308
1965	148.0	827
1967	295.0	1,088
1969 (1–VI)	345.0	740

The yearly seasonal increase were observed only in regions with concentrated population, while the above-mentioned larger epidemics occupied large areas including rural regions.

Since 1965 serological survey of healthy population is performed in the whole country. The results are shown in Fig. 1, in which mean titers of A2 and B antibodies as well as morbidity rate are graphed.

It is striking that the level of A2 antibodies was low during the whole year 1968 in which (in winter season 67/68) no outbreak was observed

and the number of cases as well as morbidity were lowest for the recent several years. This might result in extinction of the population immunity what in turn might be a factor predisposing to epidemic in 1969.

Virological results in the period under discussion were in general concordant with those reported from other countries. Strains isolated in Poland were as a rule shown to be related or identical with strains accepted as standards at a given time.

The recent epidemic was preceded by outbreak in 1967 which involved the whole country. A total of 1,535,965 cases were recorded in January and February 1967. The average morbidity rate was 297 per 10^5 in January, the highest indexes being noted in Wrocław (1707) and Warsaw (1516). Seven virus strains were isolated from the specimens collected. The isolation was difficult, many isolates could neither be adapted to chorioallantois nor grown in the amnion of chick embryos.

As shown by cross HI test, all the strains were homogenous and related to A2 England 12/64 strain. Antigenic relation was also demonstrated with strain A2 Moscow EBT/67 isolated in Moscow at that time. These results were confirmed by tests with paired sera (Fig. 2). Remarkable increase in antibody titer was detected with A2 England 64 antigen only.

Therefore, the 67 epidemic was induced by A2 virus related to variant that occurred in other countries in 64/65.

Strains isolated in 1967 were characterized by high sensitivity to serum inhibitors and by low enzymatic activity.

In 1968 influenza epidemic did not occur. Seasonal influenza-like illnesses showed the lowest indexes for several recent years (8.1 in January and 11.1 in February), and mean antibody level in the population, as found by serological survey, was unusually low for A2 virus. As shown in Fig. 1, such situation was observed for the whole year 1968.

In October the new A2 Hong Kong 68 antigen was introduced into the serological survey in Warsaw city. As soon as at the end of October and in November antibodies for this new antigen began to appear. They were detected first in age group below 14, in titers 1:20 to 40, then both the titers and percentage of positive sera gradually increased.

The first strain identical with A2 Hong-Kong 68 standard was isolated from a sporadic case at the beginning of December.

The epidemic began in the 2nd decade of January in Warsaw and Łódź, then it spread in the whole country. In the individual regions high number of cases was noted until the end of March, peak being observed in the 1st decade of February. The situation developed as illustrated by data in Table 3.

The highest number of cases was recorded in Warsaw (568,443) and in Katowice province (498,873). This was the largest outbreak in the past 10 years; some 13 percent of the whole population was affected, of what some 26 percent were children up to 15 years.

Table 3

Influenza epidemic 1969

Month	No of cases	Morbidity per 10^5 inh.
January	1,124,178	345.0
February	3,082,565	950.6
March	264,911	81.7
April	14,809	4.6
May	4,215	1.3
June	2,636	0.8
Total	4,493,314	

The disease was generally mild. Complications (pneumonia and bronchitis) were observed in about one percent of cases; they were major cause to death. Elderly persons (over 60) constituted vast majority of fatal cases recorded in the first quarter 69.

As a result of diagnostic procedures a total of 218 virus strains were isolated; they were identified as identical with A2 Hong-Kong 68 standard strain. The isolation was easy, almost all strains were isolated in the first chick embryo passage. A part of the specimens were parellelly tested in primary monkey kidney cultures; however, isolation frequency was 3 to 4 times lower in cultures than in chick embryos.

Serological tests with paired sera confirmed identification of the epidemic strains. Nevertheless, in a rather high percent of cases diagnostic rise of antibody titers was also observed with A2 England 64 antigen, thus suggesting some relation of the epidemic virus to the virus which occurred in the population previously (in 1967). This supposition was confirmed by the results of cross antigenic analysis. The diagnostic procedures are illustrated by data in Table 4.

Antibody rise between the acute and convalescent period as tested with A2 England 64, A2 Hong-Kong 68, A2 homologous (epidemic strain), and B antigens, is presented in Fig. 3.

Strains representative for the individual regions were selected and used for cross antigenic analysis. Major part of them showed low immunogenicity and adjuvant had to be used for preparing rat immune sera. In spite of low immunogenicity, the isolated strains were good antibody detectors.

The results of antigenic analysis are presented in Fig. 4 which is a graphic representation of antigenic relations of the isolated strains and standard ones.

Table 4

Diagnostic procedures in influenza epidemic 1969

Province	Specimens tested (total)	Throat washings	Other (swabs, cerebro-spinal fluid)	Strains isolated	Paired sera tested	A_2 Hong Hong 1/68			A_2 England 12/64		
						rise 4×	rise 2×	decrease	rise 4×	rise 2×	decrease
Warszawa (S.-E.)	51	32	20	19	49	24	8	12	—	—	—
Warszawa (PZH)	19	19	—	6	95	57	11	7	48	14	3
Kraków	40	40	—	16	29	20	—	11	—	—	—
Wrocław	23	8	—	4	28	15	2	—	17	3	8
Gdańsk	103	90	11	12	117	32	—	—	—	—	—
Katowice	102	100	2	45	87	52	5	11	not tested		—
Białystok	166	166	—	61	240	97	2	3	34	14	—
Bydgoszcz	63	63	—	0	63	36	8	—	2	2	1
Szczecin	43	43	—	11	5	5	—	—	7	—	—
Lublin	74	48	—	23	64	26	—	4	115	30	31
Rzeszów	10	7	3	6	253	55	40	—	—	—	—
Olsztyn	11	10	1	6	4	4	—	—	—	—	—
Opole	22	10	—	4	61	31	3	—	28	5	9
Poznań	26	21	5	3	25	16	1	—	14	—	—
Kielce	14	6	3	2	13	4	1	5	2	1	1
Total	767	663	45	218	1133	474	81	53	267	69	53

Antibodies for antigen:

Fig. I
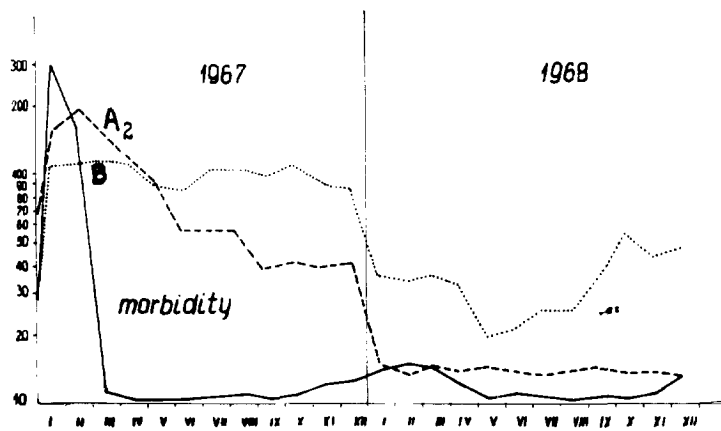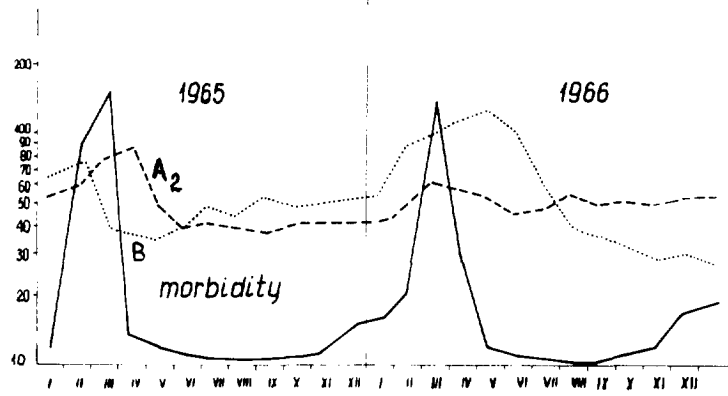
Serological survey of healthy population in the years 1965–68
(After F. Przesmycki and M. Wiśniewski)

Fig. 2
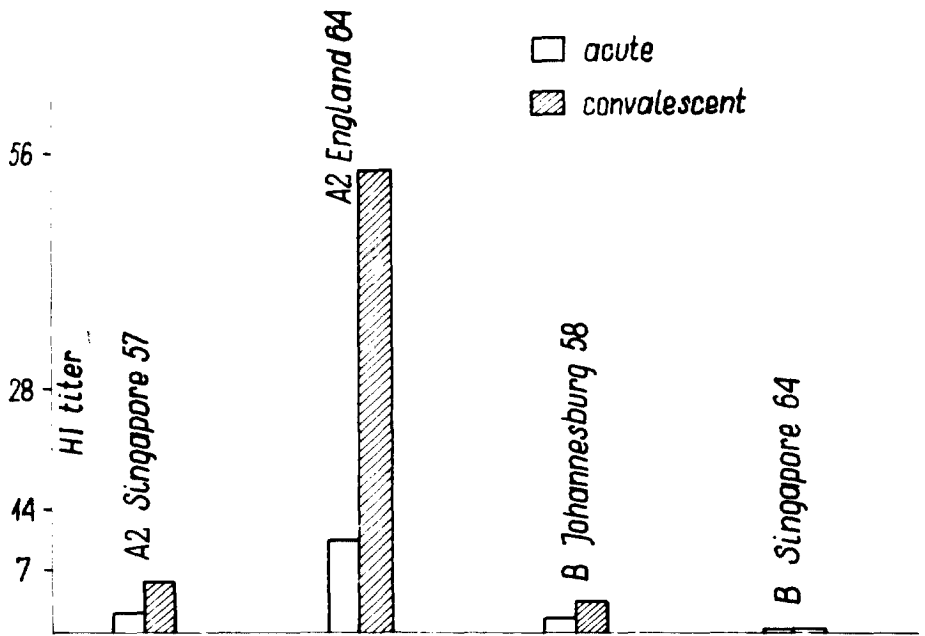
HI test with pareid sera collected from cases in 1967 epidemic

Fig. 3
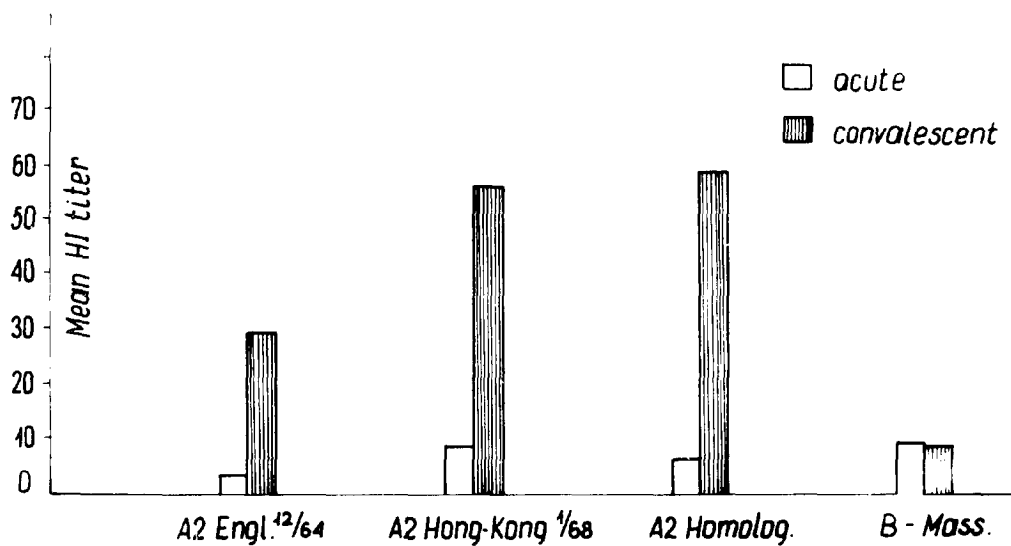
HI test with pareid sera collected from cases in 1969 epidemic

Fig. 4
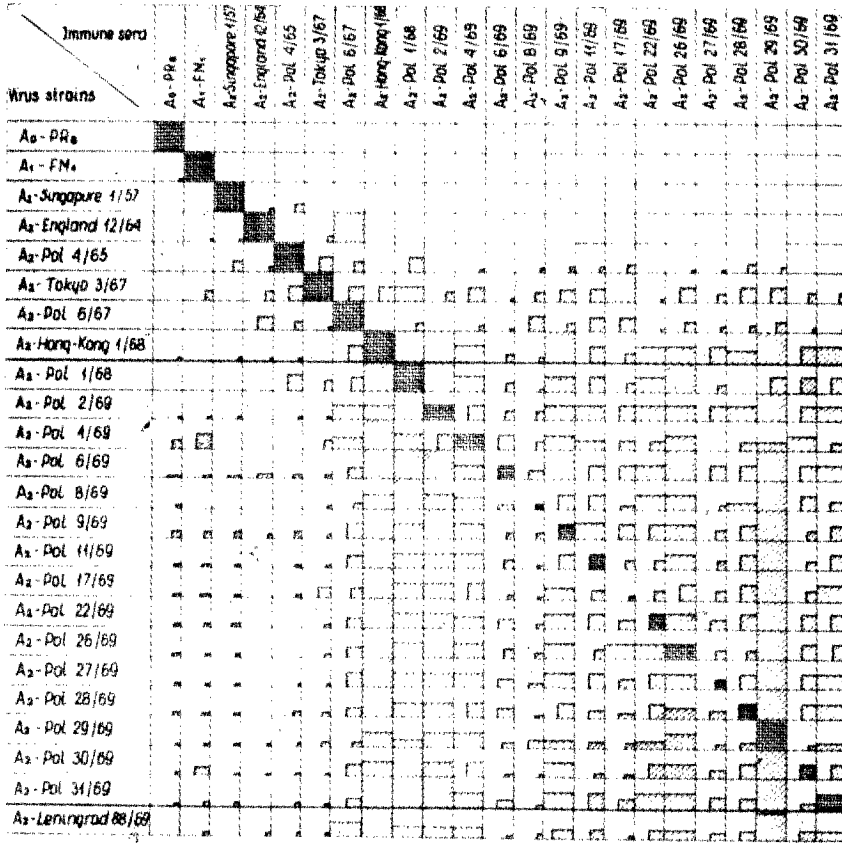
Fig. 4

The results of antigenic analysis (cross HI tests) of strains isolated in 1969 and standard strains

Fig. 5
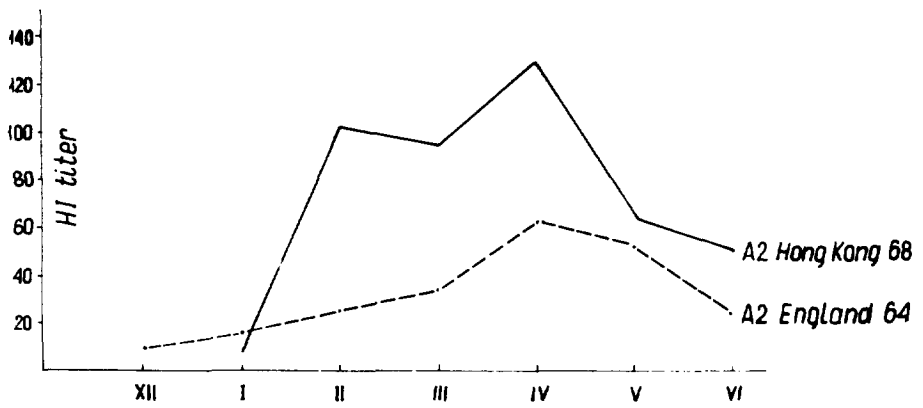
Serological survey in Wroclav province in XII. 68 and I–VI. 69.

Almost all strains tested reacted to the highest titer with A2 Hong Kong antiserum, and showed higher or lower relation among themselves. Good reaction of all strains with A2 Pol 29/69 antiserum is worth mentioning. It is also striking that all strains were related to strain A2 Pol 6/67 isolated in 1967.

Among all the strains tested, A2 Pol 29/69 was distinguished by relatively high immunogenicity and stable enzymatic properties; it was recommended for vaccine production.

Beside diagnostic procedures, serological survey with A2 Hong Kong and A2 England 64 antigens was performed in the first half of 1969. The dynamics of antibodies in the individual months is illustrated by curves in Fig. 5, in which the results obtained in Wrocław province are exemplarily presented.

The increase in A2 Hong Kong antibodies was accompanied by not as high but roughly parallel increase in A2 England antibodies. The results in other provinces were similar, except Gdańsk province where no increase in A2 England antibodies was observed.

The results presented suggest that virus which induced 1969 epidemic in Poland, though corresponded to A2 Hong Kong standard, contained a component related to virus that appeared in the population in 1967. Such a conclusion can be drawn from the results of antigenic analysis, although it cannot be excluded that the rise of antibodies against the 67 virus might be a kind of the anamnestic reaction. In any case, it seems to be proper to conclude that vaccine possibly applied in this country should contain one of the recent strains which may be expected to induce in part immunity to the 67 virus. On the other hand, selection of the actual strain is to be discussed. The results of antigenic analysis enzymatic properties, and immunogenicity point to strain A2 Pol 29/69 as the most suitable one.

Serological survey of influenza seems to give a good insight into the population immunity and to permit some prognostic conclusions. There are two essential elements of such investigation.

The antigen to be used in a given region should be selected in a proper way, as is the case with strain selection for vaccine production at a given time. Taking into account high strain specificity of HI test, which is routinely used for such investigation, the antigen should be continually actualized and virus variation and the appearance of new variants should be followed. This is possible only with an efficient system of international information such as the WHO system which worked very efficiently during recent pandemic.

It is known that, when a new virus variant appears, children up to 14 years constitute the most sensitive age group. Therefore, special attention should be paid to the results of serological investigation of this group.

SUMMARY

The development of the epidemiological situation of influenza in Poland in the past ten years is briefly discused, with special reference to the recent outbreaks in 1967 and 1969.

As shown by epidemiological survey as well as by laboratory virological and serological findings, the A 2 virus responsible for 1969 epidemic in this country was to some extent related to virus isolated in 1967.

Implications of these findings as to the selection of strains for epidemiological surveillance and for vaccine production are briefly discussed.

CONTINUOUS SURVEILLANCE OF INFLUENZA WITH TYPE SPECIFIC COMPLEMENT FIXATION TEST

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The quick recognition of the first cases of Influenza like syndrome announcing an epidemic is one of the most important measures to be sure to control this disease.

First of all, it is essential to know whether these cases are actually due to *M. influenzae,* what type A or B is involved, taking into account quite different evolution and severity of outbreaks. Then, the antigenic characters ought to be known: sub-type or variant in subtype.

But, unfortunately, the material, apparatus and reagents needed for isolation and identification of viruses are not always immediately available in the laboratories responsible for that work, likewise, good connections are not always established with hospitals or Public Health Services.

In this case, it seems possible to use complement fixation test (CF) performed with »soluble« (ribonucleoprotein) type specific antigen. We tried and used this CF test in order to ascertain its reliability for:

-- rapid information concerning the beginning of an outbreak and its evolution,
-- detection of later-epidemic cases, particularly during the summer months.

The complement fixation test was chosen for the following reasons:

-- it is able to detect the atypical or typical influenza whatever the virus types or sub-types may be;
-- the antibodies detected by this test appear quickly in the course or the decrease of the disease and the titre become below 8 after two or three months;
-- it is not expensive, not time consuming, and so, it is very easy to carry out this test in all laboratories familiar with serological reactions.

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MATERIAL AND METHODS

All sera sent to our laboratory for diagnosis of acute respiratory diseases were tested one or two times a week with type specific (S) antigen A and B.

The complement fixation test was performed using an antigen and microtechnic previously described (Sohier, 1964), (Sohier et al., 1956).

The interpretation of the results is as follows:

A significant increase ($\times$ 4) of antibodies confirms the evolution of an infection due to *M. influenza* A or B. A titre 64 is taken as a recent infection (from 8 until 60 days at the most).

Isolation of influenza virus was performed as soon as possible from samples collected in hospitals or communities or institutions where influenza appeared, using primary monkey kidney and embryonated eggs.

RESULTS

Evolution of epidemics – All epidemics were detected by positive CF test (significative increase of antibodies or titre $\geqslant$ 64).

Beginning of epidemics – Positive CF test appeared at the same time as physicians observed some typical clinical cases of Influenza, or some days after, never more than two weeks.

Comparing the results of isolation of the first strain with the first positive CF test, we obtained the following results:

In all epidemics, but one, positive CF tests appeared in the course of the continuous survey one or two weaks before the first isolation. It is convenient to remind that our laboratory (probably as many others) obtains samples in two ways:

– from general practitioners observing an increase of Influenza like disease in the country and who ask our laboratory to confirm the diagnosis.

In these cases, the samples are delayed because of the time necessary for the shipment of the material.

-- from doctors in the hospital who are sending, for all viral supposed acute respiratory diseases, samples for isolation and more often serological tests. Even, in these cases, it appears that CF test give positive results before the isolation.

End of epidemics – It is indicate by the progressive reduction, then, the disappearance of positive CF tests.

Inter-epidemics period – In our temperate climates, epidemics occured only in Winter and Spring. It would be useful to know if typical, atypical or abortive cases of Influenza occur in Summer.

If one admits that a titre of antibodies 64 reveals a recent infection, one observes very few cases during inter-epidemic period and very rarely during Summer except if the outbreak is extented until May. In 12 years, no cases were detected in July and in September.

Fig. 1
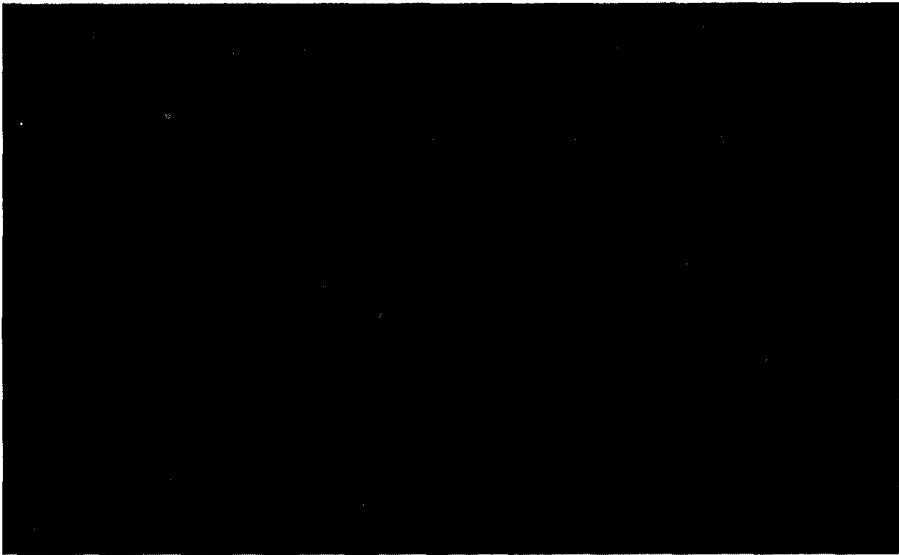
Two inter-epidemic periods draw our attention:

In 1960: the epidemic ended in April and the next appeared in January 1962. For 18 months, the continuous survey detected only 5 positive cases (64) respectively in January, March, June and October.

In 1963: the epidemic ended in March and the next began in February 1965. For 24 months, our survey detected 5 positive cases, respectively in June, October, March, April and August.

SUMMARY

A continuous serological survey, using »soluble« type specific antigen for all sera collected in cases of acute respiratory diseases and sent to our laboratory, made possible continuous knowledge of the evolution of influenza.

Taking in account the 19 303 sera tested in 12 years (from 1957 to 1968) it appears that the beginning of 10 epidemics has been detected, using this test and, in all but one of them, one or two weeks before the first positive isolation.

Inter-epidemics cases were rarely detected and never in July and September.

Since it is necessary to have the strain involved in an epidemic to perform the others serological tests and, since CF test can detect the greatest part of influenza (except in babies), a continuous survey using this test can be – provided that a sufficient number of sera are tested every week – very useful for the surveillance of Influenza.

Completed with the rapid isolation of the strain and others serological tests, it permits to have all precisions necessary to set up an effective control of the disease.

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INTERNATIONAL AND NATIONAL SURVEILLANCE
OF INFLUENZA

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I. INTRODUCTION

Among the most important epidemiological characteristics of influenza are the rapidity with which it spreads from country to country and the considerable antigenic variation of influenza viruses, occuring at intervals and producing new strains which differ radically in their antigenic composition from their immediate prodeccessors. The former requires rapid collection and dissemination of epidemiological information and the latter rapid isolation of strains and their speedy final characterization, particularly at the beginning of epidemics, with a view to making available to research and vaccine-producing laboratories cultures of the new strain within the shortest possible time.

In view of the above and the ever-increasing volume and rapidity of international travel, which even further facilitates the rapid spread of influenza throughout the world, the international and national surveillance of influenza are of utmost importance in order to guide effective and rational preventive and control measures, both internationally and within individual countries. A national influenza surveillance programme could not be developed adequately without international surveillance, since a knowledge of observation in other countries is necessary so that the public health administrator can evaluate the risk for his own country and decide on appropriate measures. These measures might entail the introduction of new strains into vaccines, vaccination of a particular strata of population of certain public services, preparation of a stock of antibiotics and other medicaments necessary to treat complications, and preparation to meet increased demand for hospitalization.

2. SURVEILLANCE OF COMMUNICABLE DISEASES IN GENERAL

Epidemiological surveillance represents all the activities that are necessary in order to acquire the knowledge on which sound preventive and control action of communicable diseases must be based. In its widest sense, as now being applied to communicable diseases, it means:

(a) a continuing watch over the distribution and trend of incidence of such diseases through the systematic collection, consolidation and evaluation of morbidity and mortality reports, including the laboratory diagnostic information supplementary to the notification of infectious diseases;

(b) the collection, consolidation and evaluation of information on the nature, prevalance and distribution of infective agents in the population and its total environment;

(c) the collection of information on the availability and extent of utilization of control measures;

(d) the assessment of the relative effectiveness of control measures;

(e) the regular dissemination of the consolidated data and interpretations to all who have contributed and to all others who have responsibilities for prevention and control of communicable diseases.

Surveillance should, therefore, not be restricted to cases of disease alone, since even in the absence of obvious cases, important information can come from studies of the occurence of the causal agent in symptomless carriers or vectors or of the distribution of antibodies in the population. Immunological surveys, carried out by adequate sampling procedures and laboratory techniques, represent a type of surveillance activity which may not only help the ecological studies of diseases, but may also provide an analysis of the epidemiological situation in an area. Further studies are indicated as more information is needed on the value of serological surveys for estimating the susceptibility of human population of infection and the usefulness of these surveys in providing data on groups most in need of vaccination. An effective surveillance programme requires, therefore, a multi-disciplinary, collective approach and close co-operation between epidemiologists, clinicians, laboratory experts, health statisticians, ecologists, public health administrators (and others, depending on the disease).

3. INTERNATIONAL INFLUENZA SURVEILLANCE PROGRAMME

For international influenza surveillance the World Health Organization has since 1947 gradually developed a network of 85 National Influenza Reference Centres in 55 countries in collaboration with two international centres: the World Influenza Centre in the National Institute for Medical Research, Mill Hill, London and the International Influenza Center for the Americas at the Communicable Disease Center,

United States Public Health Services, Atlanta, Georgia. These two centres maintain very close contact with each other, with the Virus Unit of WHO Headquarters, Geneva, and with the national centres. The functions of the two centres are:

(a) to obtain, fully characterize and preserve representative strains from outbreaks in different parts of the world and distribute them to research and production laboratories;

(b) to advise on the strains which should be included in influenza vaccines;

(c) to arrange for the training of research workers in specialized techniques;

(d) in collaboration with the Virus Unit at other units at WHO, Geneva, to collect and distribute information about the types of influenza virus prevalent in different parts of the world;

(e) to assist the national centres in their technical problems.

The usefulness of the WHO international influenza surveillance programme was clearly demonstrated in the 1957 and 1968 epidemics when it provided a continuous picture of the spread of the disease and, even more important, enabled early identification of the new strain and arrangements to be made for producing and administering vaccines against it before its further spread, thus protecting strata of the population in which influenza is often fatal (old people, debilitated or ill persons, pregnant women) and therefore achieving one of the main objectives of influenza surveillance. However, in contrast to the exact information obtained about the viruses, the quality and quantity of the epidemiological information varies partly because different countries use different methods of assessment and partly because in many countries schemes for the regular collection of information on a uniform basis do not yet exist.

In view of the above, the Twenty-second World Health Assembly, held in Boston Mass., USA, in July 1969, adopted a resolution (WHA 22.47) on diseases under surveillance in which »considering that the risk of the occurence of an epidemic of viral influenza is always present and that a knowledge of the frequently changing antigenic characteristics of the causal virus is necessary for the preparation of an effective vaccine; and taking into account the success of the WHO influenza programme since its inception in 1947« and »recognizing that an epidemiological surveillance programme based on speedy notification and, in the case of influenza, rapid identification of the virus strain involed, can be of immense benefit in giving early warning of impending outbreaks«, it requested health administrations of Member States to inform the Organization promptly by telegram or telex of the occurence of any outbreak of viral influenza in any areas of its territory, and to supplement these reports, as soon as possible, by information on the source and type of the disease and the number of cases and deaths.

In the same resolution the Assembly requested the Director-General of WHO:

»(i) to send to health administrations, when necessary by means appropriate to the urgency of the situation, the information received in accordance with paragraph 1 of this resolution;

(ii) to publish such information in the Weekly Epidemiological Record and to dispatch the Record by airmail;

(iii) to publish annually an epidemiological study of the incidence and trends of these diseases;

(iv) to publish information, whenever appropriate, on changes in these trends; and

(v) to develop as soon as possible a manual on international surveillance of selected communicable diseases and to assist Member States in utilizing their existing services to perform epidemiological surveillance most effectivelly.«

The Organization has been carrying out the activities mentioned under (i) – (iv); the development of a manual on international surveillance of influenza is now in hand and WHO will continue upon request its assistance to the Member States to develop or expand their national influenza surveillance programmes.

It is evident, however, that the World Health Organization and the two international influenza centres would find it impossible to carry out an effective international surveillance programme with the continuous collaboration of the national health administrations and the assistance of national influenza centres.

Further details on the functioning of the WHO influenza programme such as: collection and dissemination of information, collection and transport of specimens for laboratory examination, provision of diagnostic reagents, may be obtained direct from the Virus Unit, WHO Headquarters, Geneva.

4. NATIONAL INFLUENZA SURVEILLANCE PROGRAMME

In planning a national influenza surveillance programme provision may be made for its phased development as the increased availability of resources in manpower, material and other facilities makes this feasible. Such a programme should be considered a normal part of health service practice, and, as it requires a team approach, surveillance activities should be *directed and co-ordinated by the national health authorities.* The programme should use the existing resources and promote improved co-operation between the various services involved. The struc-

ture chosen will depend on such factors as the size of the country, the ease of communication and transport and the administrative set-up of the health services, but is likely to comprise the following three levels:

(a) national (central or federal),

(b) intermediate (State or provincial),

(c) local.

It is the responsibility of each national health administration to allocate specific duties and delegate responsibility at each level, and also to define in detail the functions and procedures for each service (epidemiology, clinic, laboratory, health statistics and others).

Though a national influenza surveillance programme requires a team apporoach, an efficient organization and facilities, the importance of the *essential role played by the national influenza centres* cannot be over-emphasized. National influenza centres are designated by the national health authorities and are brought into the WHO programme by being »recognized« by WHO. Recognition is given to laboratories which can meet the following requirements:

(a) ability to isolate influenza viruses from specimens from patients;

(b) ability to make a reliable serological diagnosis of influenza and facilities to take part in serological surveys of selected population groups in interepidemic periods;

(c) facilities and willingness to send freshly isolated representative strains from each outbreak very quickly to one of the international centres;

(d) provision of virological and, as far as possible, epidemiological information to the Virus Unit, WHO Headquarters, Geneva, and the international centres.

For the past two years, national influenza centres have been invited to send, during their influenza season, weekly reports to the WHO Virus Unit, Geneva, on a special form (Annex II). This system of reporting has been well received by the centres and it is to be continued.

More than one laboratory may be recognized in a country. The number depends in general on the size of the country, on the desinty of the population and on the number of competent virus laboratories in existence. Whatever the number of influenza centres in a country, for an efficient surveillance programme it is of particular importance that:

(a) the whole territory of a country be »allocated« to the influenza centre(s), leaving no »blind« (unserved) areas;

(b) the centre(s) be actively supported by the health authorities (who should provide the necessary resources and facilities) and their work continuously co-ordinated with other services;

(c) the centre(s) maintains direct contact with epidemiologists, clinicians and also with the hospital and public health laboratories in the area(s) allocated to it.

The above system would ensure early detection of influenza epidemics and facilitate regular serological surveys in inter-epidemic periods. National centres encounter many difficulties in fulfilling their functions, particularly at the beginning of epidemics when specimens may be few in number, epidemiological information lacking and technical difficulties encountered in isolating new strains which often grow very slowly.

The epidemiological characteristics of influenza are different in epidemic and inter-epidemic periods and consequently the emphasis of the programme and the type of activities to be carried out differ in these two periods:

(a) *In an epidemic period*, clinical data are less important than epidemiological data, the typical pattern being rapid diffusion with short intervals between cases, much absenteeism and high mortality, mainly among old people and sometimes young children.

The laboratory plays an essential role in co-ordination with the epidemiologist. Owing to the lability of influenza viruses, their isolation is best carried out either at the bedside of the patient ou when he enters hospital, and specimens should be transported at low temperatures.

An influenza surveillance programme should imply continuous search for the infection in certain groups of persons. This may be obtained by arranging that material for taking specimens is constantly ready in paediatric hospital wards, including tissue cultures which will enable isolation of respiratory viruses other than influenza. The first influenza virus isolated must be sent immediately to the World Influenza Centre for accurate determination of its antigenic characteristics in relation to previous variants, in order that other countries may be quickly informed.

It is important to gather serological information for diagnostic purposes, preferably by the complement fixation test which will always detect an influenza virus, even if it belongs to a new variant. When the strain involed has been isolated, haemagglutination-inhibition tests can be used to determine the antibody titres to this strain. It is necessary to obtain paired sera, which is not always easy, the patients often being hospitalized too late to obtain the »acute serum«. High titres in the convalescent serum allow a presumptive diagnosis of recent infection by the complement fixation test, owing to the decrease of antibody titres in the three following months. Research efforts are being made to try to diagnose recent infections more accurately, according to the class of immunoglobulin found in the serum.

(b) *In inter-epidemic periods* cross-sectional or longitudinal surveys may be made, arranging for a sufficient number of samples, for representative sampling sources, and for good representation of the important age-groups Cross-sectional surveys will use haemagglutination-inhibition tests with strains previously isolated whereas longitudinal studies

should preferably use complement fixation tests to detect influenza activity at an early stage. Attempts to isolate strains from influenza-like cases in paediatric and other wards should also be continued.

Such investigations will show the immunological profile of a population and should detect influenza activity, as was recently achieved with the A2/HgKg/68 strain in some European countries.

National influenza surveillance was discussed at the European Seminar on Methods of Surveillance of Communicable Diseases, held in May 1969 in The Hague. The participants agreed that it was necessary for countries to send information and strains very quickly within the framework of reciprocal international exchanges through WHO. Bilateral exchanges of information between neighbouring countries were also common in some instances.

Various epidemiological indices used were also discussed, for example, morbidity from influenza-like diseases and pneumonia (by age-groups), mortality from influenza and pneumonia, medical practices doing voluntary reporting, school and work absenteeism, request for hospitalization, requests for sickness benefit. Alarm levels had been fixed by health administrations in some countries. Many of these indices had influenza virus in an outbreak of influenza-like disease. It was also essential for all epidemiological and laboratory data to be subjected, prior to dissemination, to narrative interpretation by a national epidemiologist; this was the case in the weekly epidemiological bulletins disseminated by several countries. It was difficult to formulate indices which could be readily applied to all countries. Each country should therefore adopt the method which it finds most practicable. Probably the most useful index was sickness absenteeism combined with laboratory data on the viruses prevalent in the area concerned, and it would be a distinct help in the international surveillance programme if as many countries as possible would adopt this method of assessment.

The importance of *narrative analysis* was made still more evident by the fact that, during the last influenza season, the indices did not react according to expectations, and showed variations in the usual chronological order of their appearance.

It was appreciated that close working relations between laboratory and epidemiologists were necessary. Too many laboratories carry out influenza diagnosis without informing the epidemiological services, although, in a number of instances, the first alarm has to come from the laboratory, as was seen during the last influenza season in a number of countries who were already invaded by virus A2/HgKg/68 although absenteeism and mortality indices were still at a low level. Influenza is not a noticeable disease in many countries; moreover in some of these countries in which it is notifiable the value of the information is extremely limited because it often consists merely of a number of cases of undifferentiated respiratory disease unaccompanied by any information on etiology.

Several countries have established an influenza surveillance network including a central institute with laboratory and epidemiological services, and peripheral stations with laboratories and epidemiologists. These stations carry out serological enquiries in inter-epidemic periods and supply epidemiological and virological information in epidemic periods.

Contacts between laboratories and clinicians are also very important. In several countries laboratory specialists have secured the co-operation of a network of selected »spotter physicians« who send specimens from acute respiratory illnesses. This has proved particularly useful in cases of respiratory virus infections other than influenza. Examples were described of one scheme with encouragingly high virus isolation rates from specimens sent by »spotters« in a simple transport medium through the ordinary post, and another scheme, whereby a mobile laboratory could visit an outbreak and inoculate specimens directly from patients.

It was mentioned that electronic computers could be successfully used for the quick weekly analysis of morbidity and absenteeism data from the main age-groups, possibly with the production of a cartogram showing their geographical distribution. Its use would facilitate the preparation of a weekly epidemiological record which could be distributed quickly to health administrators in the various regions of a country and to WHO, though it can be done very well also without computers. A narrative analysis, however, remains essential for interpreting the results, owing to inaccuracy in the epidemiological data fed to the computer.

5. CONCLUSION

Influenza provides an outstanding example of the many public health problems which do not begin or end at national boundaries and which require continuous international co-operation between all countries throughout the world in their own interest. Experience gained over the past twenty years in the WHO influenza programme has proved the value of and the need for influenza surveillance and has shown the way to effective and rational preventive and control measures. Although the programme has attained its main objectives – the early isolation, characterization and distribution of the strains – there is scope for improvement in the epidemiological part of the surveillance, both nationally and internationally. This can best be achieved through a collective approach and close collaboration of the representatives of the various disciplines involved, with co-ordination of efforts by the national health authorities and the World Health Ouganization respectively.

ABSTRACT

The facility with which influenza spreads from country to country and the considerable antigenic variation of influenza viruses, occuring at intervals and producing new strains which differ radically in their antigenic composition from their immediate predecessors, are among the most important epidemiological characteristics of the disease. Rapid collection and disseminantion of epidemiological information and rapid isolation of strains and ther speedy final characterization, particularly at the beginning of epidemics, with a view to making available to research and vaccine producing laboratories cultures of the new strain within the shortest possible time, are, therefore, among the most important and objectives of the international and national influenza surveillance programme.

Over the past 20 years the World Health Organization has gradually developed a network of 85 National Influenza Reference centres in 55 countries and two International Centres and these centres represent the backbone of influenza surveillance view of the essential role played by laboratories. As the surveillance of influenza requires the collective approach of epidemiologists, microbiologists, clinicians, statisticians and others, the co-ordinating role of the national health administration on a national scale of WHO internationally are of the utmost importance.

Experience gained so far has proved the value of, and the need for, influenza surveillance and has shown the way to effective and rational preventive and control measures. Further efforts are, however, indicated to improve the epidemiological part of survellance, both nationally and internationally.

LIST OF WHO REFERENCE CENTRES FOR INFLUENZA

INTERNATIONAL REFERENCE CENTRES

World Influenza Centre
National Institute for Medical Research
The Ridgeway
Mill Hill
London N. W. 7.
England

International Influenza Center for the Americas
National Communicable Disease Center
Atlanta
Georgia 30333
USA

NATIONAL INFLUENZA CENTRES

AFRICAN REGION

Institut Pasteur de Bangui
Boîte postale 923
Bangui
Central African Republic

Institut Pasteur de Brazzaville
Boîte postale 120
Brazzaville
Congo (Brazzaville)

Department of Medical Microbiology
University of Ibadan
Ibadan
Nigeria

Institut Pasteur
36 Avenue Pasteur
Boîte postale 220
Dakar
Senegal

Virus Research Unit
The Medical School
Observatory
Cape Town
South Africa

The South African Institute for Medical
  Research
P. O. Box 1038
Johannesburg
South Africa

Central Pathology Laboratory
P. O. Box 9073
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THE WHO INFLUENZA PROGRAMME

Weekly Reports on Influenza to the Virus Unit, WHO, Geneva

Report made by .... for week ending / /   (a) Nil to report ☐   (b) cases/outbreaks[1]
(name of laboratory)   as under:

Geographical locations(s)[1] and Epidemiological pattern[2]	Approx. date of		Approx. No. of cases and attacks rates by age groups, (0–4, 5–14, 15–24, 25–29, 60+yr)	Laboratory findings – virus isolations and serological results & dates strains sent to International Centre
	Start	Peak	End		

Notes and comments: e. g. special epidemiological features, laboratory observations of special interest, unusual clinical syndromes and fatality.) Use reserve side of necessary.

[1] Make separate entries for separate incidents.
[2] Describe as e. g. »Outbreak in a primary school or an old people's home or a military camp« etc. or »Local outbreaks in general population«, or »Regional outbreak«, or »Covering eastern half of the country« etc.

RECENT ACHIEVEMENTS IN THE FIELD
OF INACTIVATED INFLUENZA VACCINE

FIELD TRIAL WITH INFLUENZA VACCINE IN CHILDREN; PROTECTION AND ANTIBODY TITERS

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Two types of investigations are being used to demonstrate the efficacy of influenza vaccine

1. Analysis of H. 1. or neutralizing antibodies in the blood
2. Assessment of the degree of protection against influenza infections. This can be done with the C. F. test or with the sickness rate as parameter for infection.

The subject of today are some aspects of two serological studies, carried out in an out-patient department in the Hague on several hundreds of children. Their age varied from 2 to 15 year and they were all asthmatic patients.

FIRST EXPERIMENT

The first serological investigation, from Nov. 64 till Sept. 66 was done with the aim to study the H. I. antibody titres after vaccination with an adjuvant vaccine or with aqueous vaccine of different strengths.

Both types of vaccines are inactivated and contained the strains A_2 Engl'63 and B Joh'58.

Part of the children had been vaccinated also in 1963. Blood samples were taken at regular intervals for the titration of H. 1. antibodies. For the evaluation of vaccination results in 1964 we have taken the absolute figures and the fold-increase of the initial titre. Not knowing what influence the vaccination in 1963 would have on the level of antibodies, we have calculated the mean values separately for the in 1963 vaccinated and non vaccinated subjects.

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Table 1 shows the results of our adjuvant vaccine, Influvac Depot. All children received the same dose of 0.25 ml containing 75 CCA A_2 Eng '63 and 50 CCA B Joh.'58.

It appeared that the mean initial A titre in the group, vaccinated in 1963 is significantly higher than in the non-vaccinated group.

Table 1

Effect Influvac Depot on H. I. titer A_2 1963 (with 95% confidence limits)

| | | | Vaccinated in 1963 | |
			yes	no
Absolute figures	Prevacc.			
	Nov.	'64 (1)	N=15 126 (57~ 228)	N=48 65 (45~ 93)
	Jan.	'65 (2)	N=17 2177 (1315~3605)	N=60 1639 (1137~2362)
	April	'65 (3)	N=17 1905 (1313~2764)	N=54 1903 (1297~2792)
	Nov.	'65 (4)	N=14 1117 (688~1812)	N=47 688 (445~1063)
Fold increase	2 – 1		15 (7 ~31)	23 (16~33)
	3 – 1		13 (6 ~26)	30 (21~42)
	4 – 1		6 (2 ~16)	14 (10~18)

The absolute post-vaccination titres are about equal in both groups, but there is a considerable difference in fold-increase between the two groups.

Table 2 shows the results of the titration of the B antibodies. They point in the same direction and even more clearly. It is evident that the

Table 2

Effect Influvac Depot on H. I. titer B. Joh. burg (with 95% confidence limits)

| | | | Vaccinated in 1963 | |
			yes	no
Absolute figures	Prevacc.			
	Nov.	'64 (1)	N=15 320 (117~ 877)	N=48 57 (38~ 85)
	Jan.	'65 (2)	N=17 5470 (3438~8702)	N=60 2555 (1614~4045)
	April	'65 (3)	N=17 4024 (2457~6590)	N=54 3179 (2107~4798)
	Nov.	'65 (4)	N=14 3209 (1689~6102)	N=47 1603 (1015~2533)
Fold increase	2 – 1		14 (5~35)	36 (23~58)
	3 – 1		10 (4~26)	43 (30~63)
	4 – 1		7 (2~22)	28 (20~42)

differences in fold-increase should be reduced to the difference in initial titre.

For some authorities it is common practice to use the percentage of 4- or more fold- increase of H. I. titres after vaccination of human beings as criterion for the antigenic potency and efficacy of influenza vaccine. In the U.S.A. there is even an official requirement that a vaccination should yield in at least 75% of the subjects a 4- or more fold-increase in H. I. titre. To what confusing situation this requirement, if strictly applied, may lead is shown in table 3.

Table 3

Frequency of less than 4 – fold increase in H. I. antibody titer

Dosage	Fold increase calculated	<4–fold increase numbers	<4–fold increase percentage
300 CCA A in 1964	Jan. '65 related to Nov. '64	37/114	32%
300 CCA A in 1964 + 300 CCA A in 1965	Jan. '66 related to Nov. '65	59/85	70%
	Jan. '66 related to Nov. '64	15/61	25%

These data are also taken from the study in the Hague. A group of children were vaccinated in two subsequent years, Nov. '64 and Nov. '65, with an aqueous vaccine containing 300 CCA A_2 Eng'63 and 200 CCA B Joh'58 per ml. The dosage varied according to age; the children received 0.1 ml per year with a minimum of 0.5 ml and a maximum of 1 ml.; two injections with 4 wks interval.

These children had not been vaccinated in 1963. The percentage of less than 4-fold increase after vaccination in the first year is about normal (32%). Thesecond year this percentage increases to 70% in the same group of children with the same vaccin, if the titre of Nov.'65 is taken as the initial (prevaccination) one.

However if the fold-increase is calculated in relation to the prevaccination titre in Nov. '64 the number of unsatisfactory antibody responses is again normal (25%).

Table 4 shows similar results as to the B-titre. The seemingly discrepancy can only be ascribed to a difference in prevaccination titre in the first and in the second year.

If the average duration of antibody response of more than a year is not taken into account one could wrongly conclude that the influenza vaccine in 1965 was by far unsatisfactory, whereas it was a good one in the previous year.

This experiment has taught us again how important selection of the patient material is for a proper evaluation of the serological vaccination effect.

The fold-increase of initial antibody titre as parameter for the efficacy of an influenza vaccine is reliable only if done on subjects not vaccinated in the previous year with the same or a closely related strain.

Table 4

Frequency of less than 4 – fold increase in H. I. antibody titer

Dosage	Fold increase calculated	<4–fold increase	
		numbers	percentage
200 CCA B in 1964	Jan. '65 related to Nov. '64	43/105	40%
200 CCA B in 1964 +	Jan. '66 related to Nov. '65	25/40	63%
200 CCA B in 1965	Jan. '66 related to Nov. '64	9/36	25%

SECOND EXPERIMENT

An investigation on serum H. I. antibodies can very well be used for comparison of the antigenic potency of various influenza strains or of various vaccines of different origin, but it does not permit a conclusion as to the degree of clinical protection against influenza.

Such a protection study was undertaken two years later in the same center with a positive C. F. test ($\geq$ 4-fold increase) as proof of an influenza infection.

Three groups each of approximately 100 children were vaccinated after randomisation with a vaccine containing 300 CCA Eng'66, 300 CCA A equine$_2$ Miami or with a placebo. The dosage scheme was the same as in the first experiment.

The reason why we included the Miami strain is the following:

In the course of the past ten years the serum antibody level against the influenza A$_2$ type among the world population increased gradually and in 1967 a change in antigenic structure of the A$_2$ virus was predicted. Masurel, of the World Influenza Centre in Leyden, is already for more than 10 years engaged in a serological and virological investigation in a large number of sera collected from people of all age groups. Time does not permit me to go into details of this study.

Based on the distribution of antibodies against the various strains he worked out a theory that there are two influenza A parent strains; the A and the A$_2$ and that these strains with their derivatives occur in a specific order as epidemic influenza strains.

In this context he has predicted in 1967 that the next influenza A virus would be related to the next influenza viruses, isolated in horses in 1963, the A equi$_2$ virus. In 1967, a year in which still the A$_2$ type was circulating, Masurel vaccinated subjects with an A equi$_2$ '63 vaccine. The immunisation did not only result in a stimulation of the homologous antibody but also of the antibody against A$_2$ '57 and A$_2$66, thus demonstrating an antigenic relationship between the A equi$_2$ virus and the A$_2$ viruses. It was suggested that it could be possible with such a vaccine to immunise and protect people against a future influenza strain.

Working from the assumption that the influenza A virus could change its antigenic structure in 1967/'68 and that the new epidemic virus could be a A equi$_2$ type, we have vaccinated one third of the available children

In all children blood samples were taken in Dec.'67, Jan. '68 and April '68 and titrated for C. F. and H. I. antibody. A four-fold increase of the C. F. titre from Dec.'67 to April '68 was considered as proof of a recent influenza infection.

In the winter an epidemic occurred still of the A$_2$ type. The number of influenza infection in the three groups of children is shown on table 5.

In the control group 16% of the children caught an influenza infection, which meant that the epidemic was fairly extensive.

In the group vaccinated with A$_2$ Eng'66 and A equi$_2$ Miami the infection rates were 7% resp. 13%.

The A$_2$ vaccine yielded a protection of 58%, the Miami vaccin did not reduce significantly the infection rate.

It was not possible to correlate a positive C. F. test with a clinical influenza. Most of the children were very young and as expected nearly every child suffered from one or more A. R. D. during the observation period.

Table 5

Incidence of Influenza A_2 (serological diagnosis) in 353 children

| Vacination | N | ≥ 4-fold increase C. F. infl. A between Dec. '67 and April '68 | | protection rate |
		numbers	percentage	
300 CCA A₂ England '66	132	9	7%	58% P=0.05
300 CCA A₂ equine Miami	122	16	13%	not significant
Placebo	99	16	16%	

The reduction of the infection rate with 58% is somewhat lower than that obtained in other investigations.

This might be due to the fact that the epidemic has started already in the Hague before the day of vaccination and that probably some of the children were infected within the period of three weeks after the vaccination necessary to build up a protective antibody level.

In the tables 6 and 7 the H. I. antibody response (expressed as fold increase) against the various A strains is shown, also against A_2 Hong Kong, that appeared after the end of the trial as epidemic strain.

The vaccine containing A_2 Eng'66 stimulated the antibody against A_2 virus '63, '66 and '68 very well, against A_2 Hongkong definitely less and against A equi₂ Miami not at all. We see here again that the fold increase is much higher in those not vaccinated in 1966 with an A_2 type vaccine. For the A_2 Hongkong this difference was not observed.

Table 6

Mean fold increase of I. I. titre 3 wks after vaccination with A_2 Ergl. '66 (with 95% confidence limits) 1967–1968

| strains | vaccinated in 1966 with A₂ 63 | |
	yes N = 49	no N = 73
A₂ 1963	4.3 (3.2~5.6)	11.1 (8.5~14.5)
A₂ 1966	4.1 (3.1~5.4)	10.1 (8.0~13.0)
A₂ 1968	5.2 (4.0~6.9)	15.9 (12.3~20.5)
A₂ Hongkong	2.7 (2.1~3.3)	2.9 (2.3~ 3.6)
A equine₂ Miami	1.0 (1.0~1.0)	1.0 (1.0~ 1.0)

Table 7

Mean fold increase of H. I. titre 3 wks after vaccination with A equine₂ Miami
(with 95% confidence limits) 1967–1968

| strains | vaccinated in 1966 with A_2 63 | |
	yes N = 41	no N = 65
A_2 1963	2.0 (1.6∼2.5)	2.9 (2.3∼3.5)
A_2 1966	2.1 (1.6∼2.8)	2.7 (2.2∼3.5)
A_2 1968	2.3 (1.7∼3.1)	3.8 (3.0∼4.9)
A_2 Hongkong	2.7 (2.0∼3.9)	2.5 (2.0∼3.3)
A equine₂ Miami	1.4 (1.2∼1.6)	1.8 (1.6∼2.1)

Vaccination with the A equi₂ Miami vaccin resulted in a far less stimulation of antibody against the A_2 viruses, explaining the failure of the vaccin to protect against infection.

The antibody response against A_2 Kongkong was similar to that obtained with the A_2 Eng'66 vaccin.

The homologous antibody stimulation was surprisingly low. The fact that the A_2 Eng'66 as well as the A equi₂ Miami raised the antibody titre against A_2 Hongkong, demonstrate the antigenic relationship of three strains.

SUMMARY

In an outpatient department of a childrens' hospital in the Hague two field trials with inactivated influenza-vaccins have been carried out.

The first one lasted from November '64 till September '66. In November '64 and November '65 the children were vaccinated with an adjuvant vaccin or with aqueous vaccins of different composition. The vaccins contained the strains A_2 1963 and B Joh. '58.

At regular intervals blood samples were taken for titration of H. I. antibodies. In this paper only one aspect is discussed, viz. absolute titres and fold increase of initial titres in children vaccinated in one year and in those vaccinated in 2 subsequent years with the same or a closely related strain.

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It appeared that the fold increase after vaccination depends on the initial titre and the latter on its turn on the vaccination status in the previous year. It is concluded that the fold increase cannot be used undiscriminately as a parameter for the efficacy of an influenza vaccin.

The vaccination status in the previous year should be taken into account.

The second field trial was done in the same hospital from November '67 till April '68. Three groups each of about 100 children were vaccinated at random with an inactivated vaccin containing 300 CCA A_2 Engl.'66, 300 CCA A equine$_2$ Miami or with a placebo. The reasoning behind the choice of the strains is explained. At regular intervals bloodsamples were taken for H. I. and C. F. antibody titration.

In January and February 1968 an influenza A_2 epidemic occurred.

Protection against influenza infection was calculated on the number of positive C. F. test ($\geq$ 4-fold increase) in the two vaccinated groups as compared to the number of positive C. F. tests in the placebo group.

A_2 Eng '66 vaccin yielded a protection of 58%; A equine$_2$ Miami vaccin did not reduce significantly the infection rate.

PURIFIED INFLUENZA VIRUS VACCINE
PREPARED WITH THE K-II ZONAL ULTRACENTRIFUGE

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The inherent reactivity of influenza virus vaccine has been a major deterrent to its use in high-risk populations. Vaccine reactivity has been attributed to two main causes. Some believe that myxoviruses are basically toxic and capable of producing malaise, chills, fever, and inflammation at the vaccine injection site. Others have presumed that the growth media, in this case egg fluids, contain varying amounts and types of soluble and particulate constituents which are probably responsible for the major part of the clinical reactivity of the vaccine. Until recently, there had not been an adequate opportunity to separate the two components of influenza vaccine since sufficiently refined techniques for purification were not available. However, a cooperative research effort on the part of the Molecular Anatomy Program of the Oak Ridge National Laboratories (Atomic Energy Commission) and the Biological Research and Development Laboratories of Eli Lilly and Company has answered this problem. The development of the K-II zonal ultracentrifuge and carefully controlled partial viral purification procedure prior to centrifugation has resulted in the availability of very highly purified influenza virus vaccines.

The techniques for production of the purified influenza vaccine have been described elsewhere.[1-4] Briefly, the process includes a virus pretreatment phase utilizing a carefully controlled barium sulfate adsorption-elution process in which partial viral purification results. This is followed by passage of the virus through the K-II continuous flow zonal ultracentrifuge utilizing a sucrose density gradient solution for banding of the virus. As the virus solution is admitted to the centrifuge, soluble and low molecular weight material, the virus fraction, and high density particulate material diffuse through the sucrose gradient and distribute

themselves according to their isopycnic point in the gradient. The selectively removed from the gradient and the amorphous, particulate, and soluble egg-derived impurities are discarded. Carefully controlled laboratory studies have demonstrated that the purified virus preparations do not contain demonstrable avian antigens.[4]

Utilizing these purification techniques, we prepared highly pure monovalent and polyvalent vaccines with which to study the problem of viral reactivity. A series of these vaccines were utilized in clinical studies to determine possible differences in the reaction potential of individual strains of influenza virus, between combinations of strains, and between varying potency levels of virus preparations. In addition, comparison of clinical and serological effects of injection of vaccines prepared by the K-II zonal centrifuge process and vaccines produced by the conventional Sharples centrifugation technique has also been accomplished utilizing a variety of population groups in which immunization against influenza is normally indicated. A review of these studies is as follows:

A series of pilot studies were performed in groups of individuals whose influenza immunization history was well documented. At least half of each group receiving zonal centrifuged vaccines had routinely experienced local reactions greater than 3×4 cm. in diameter following injection of conventional Sharples vaccine. None of the recipients of the conventional vaccine had ever received influenza vaccine before. Zonal centrifuged vaccines were purposely administered at CCA potency levels 50 to 100% greater than the conventional vaccine (Table 1). None

Table 1

Reactivity of Influenza Virus Vaccines
Preliminary Clinical Trial

Type Vaccine		Dose (CCA/ml)	# Subj.	Reaction Rate*	
				Local	Systemic
Zonal					
Monovalent	A/PR-8	1000	10	0	0
	A₁/AA	1000	10	0	0
	A₂/Jap 170	1000	15	0	0
	A₂/Tai	952	20	0	0
	B/Md	1000	20	0	0
Bivalent †		1200	20	5	0
Quadrivalent †		1200	15	0	0
Sharples					
Bivalent †		600	20	35%	0
Quadrivalent †		600	15	50%	0

* Reaction Rate:
  Local – Readily visible erythema and/or induration $\geq 3 \times 4$ cm.
  Systemic – Malaise and/or chills and/or fever $> 100°$ F.
† Bivalent = A_2/Tai + B/Md.
  Quadrivalent = A/PR-8 + A_2/Tai + B/Md.

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of the reaction prove subjects who received zonal centrifuged vaccine developed significant local reactions, nor could we detect strain differences at these potency levels. There were no indications of increased reactivity when strains were combined into bivalent or quadrivalent preparations. The conventionally produced vaccines, as expected, produced a significant incidence of local inflammation at the injection site. We concluded from these studies that the inherent and variable reactogenicity of conventionally produced influenza vaccine was mostly due to nonviral components of the vaccine.

Definitive clinical studies were next undertaken and have been reported elsewhere.[5] These were undertaken to determine whether the administration of equipotent doses of zonal centrifuged and conventionally produced vaccines stimulated similar serologic responses, and whether the egg fluid components of conventional vaccines might be acting as adjuvant. Also, the local and constitutional reactivity of the various vaccines were compared utilizing a standard technique for determining incidence and magnitude of reactions.

Four consecutively produced lots of a bivalent zonal centrifuged vaccine were compared to a representative lot of control vaccine produced by the Sharples process. The vaccines contained 300 CCA units each of the Taiwan A_2 and Maryland B virus strains per dose. Twelve hundred and thirty-one individuals, including geriatric and pediatric subjects, and institutionalized adults, municipal, public health, and hospital employees received a single subcutaneous dose of one of the vaccines. The serologic data (Table 2) indicated that in those individuals whose pre-

Table 2

Antigenicity of Influenza Virus Vaccine
Percentage of previously unimmunized subjects with pre-imune antibody titers $\leq 1 : 80$ who responded with fourfold or greater antibody rise

Vaccine	A_2/Tai		B/Md.	
	# Subj.	% Response	# Subj.	% Response
Zonal #1	76	85%	111	62%
Zonal #2	85	79%	60	77%
Zonal #3	51	82%	50	64%
Zonal #4	85	92%	84	56%
Sharples	188	86%	190	67%
SUMMARY				
Zonal	297	85%	305	64%
Sharples	188	86%	190	67%

immunization hemagglutination-inhibition titer was relatively low, a titer of 1 : 80 or less, no significant difference existed in the antigenicity of the various vaccines tested. There was not evidence that egg components of conventional vaccine acted as adjuvant. The data also confirm observations of others that the Maryland B virus strain, used in earlier commercial vaccines, is less antigenic than the A_2 strain. Subsequent studies utilizing vaccines containing B/Mass/3–66 virus indicate that it provides a superior antigenic stimulus compared to the old Maryland B strain. The B/Mass/3–66 strain is currently utilized in commercial vaccine production in the United States.

Reaction data derived from the 1,231 subjects were analyzed for both quantitative and qualitative differences between vaccines (Table 3). No

Table 3

*Local Reactivity of Influenza Virus Vaccine
Summary of Definitive Clinical Trial Results*

		% With Reactions*	
	# Subj.	≥ 3x4 cm	≥ 5x7 cm
Zonal Vaccine	777	4.9%	0.9%
Sharples Vaccine	454	29.0%	11.0%

* Area of erythema, induration, and tenderness at injection site.

significant differences in reactogenicity were found in subjects receiving different lots of zonal vaccine. Less than 5% of the 777 recipients exhibited erythema, induration and tenderness greater than 3×4 cm. at the injection site. On the other hand, 29% of the 454 conventional vaccine recepients exhibited local reactions of this magnitude. Eleven percent of the conventional vaccine recipients developed erythema, induration, and tenderness in areas greater than 5×7 cm., whereas less than 1% of the zonal centrifuged vaccine recipients were so affected. Constitutional reactions (Table 4) following injection of Sharples vaccine occurred with greater frequency than with zonal centrifuged vaccine. The major difference appeared in the incidence of malaise and chills which occured in 18% of conventional vaccine recipients versus less than 6% of those receiving zonal centrifuged vaccine. The absence of difference in reactivity between the four zonal centrifuged vaccines is good evidence of the consistently low reactivity of this type of vaccine irrespective of population differences.

Table 4

Constitutional Reactivity of Influenza Virus Vaccine
Summary of Definitive Clinical Trial Results

	Zonal Vaccine	Sharples Vaccine
Total Subjects	777	454
Max. Temperature >100⁰ F.	0.9%	2.2%
± 1.5⁰ F. Temp. Variation	2.7%	2.6%
Malaise	5.5%	18.0%
Chills	2.5%	12.0%
Malaise and/or Chills	5.6%	18.0%

Zonal centrifuged vaccine was also utilized in studies to determine the quantitative reactivity of standard and double doses of vaccine (Table 5). We could determine no difference in incidence of magnitude of reac-

Table 5

Reactivity of Influenza Virus Vaccine
Effects of Double Dose of Zonal Centrifuged Vaccine

	600 CCA Dose	1200 CCA Dose
# Subjects	93	99
Local Reactions		
≥ 3x4 cm. size	1	0
≥ 5x7 cm. size	0	0
Constitutional Reactions		
Max. Temp. > 100⁰ F.	0	0
± 1.5⁰ F. Temp. Variation	0	0
Malaise	4	4
Chills	1	3
Malaise and/or Chills	5	4

tivity of a 1200 CCA unit dose compared to a 600 CCA unit dose of the same lot of vaccine. The results of this study indicated that the way was clear for additional studies on the use of experimental vaccines of even greater antigenic mass in an attempt to determine the most effective antigenic level of vaccine consistent with a low incidence of local and constitutional reactions. Preliminary studies have now been reported by

Dowdle, Mostow, et al.[6] They found no clinical difference in constitutional reactivity of zonal centrifuged monovalent A_2/JAP 305 vaccine given in levels of 300, 600, 1200, 2400, 3400, or 4800 CCA units per subject. Reactions to even the highest levels of vaccine were minimal, with less than 1% developing fever. Although local reactivity did increase somewhat at the higher dosage levels, it was not considered great enough to deter further work with vaccines at these potency levels. In their studies, they also found that the 4800 CCA unit vaccine produced a 35 fold increase in antibody response, without any evidence of antibody plateau effect being observed. The higher levels of vaccine stimulated antibody which was broadly reactive with recent A_2 influenza virus strains. Since these studies were performed prior to August 1968, the sera were not studied for antibody response to the most recent Hong Kong A_2 variant.

Zonal centrifuged vaccine is also being studied for its ability to immunize by the respiratory route. Data from several studies[7-9] indicate that nasal antibody is indeed produced by the intra-nasal instillation of purified inactivated monovalent vaccine. The intra-nasal immunization route also induces IgG serum antibody to develop. Evidence indicates that the nasal antibody is in the IgA class of immunoglobulins. Subcutaneous administration of the purified vaccine also induces nasal antibody, but to a lesser extent than the intranasal route of administration.

There is an increasing amount of evidence on the effects of administering zonal centrifuged vaccines to young children. Preliminary data on the reactivity of the vaccines in the 5 to 15 year age group indicates that doses of 300 to 600 CCA units produced no greater incidence of local or constitutional reactivity than do 600 CCA units of the same vaccine in adults.

The emergence of Hong Kong variant type A_2 virus in 1968 was an unexpected event. Fortunately, the virus was readily adaptable to the purification procedure and clinical research material was available for use in October of 1968, prior to the appearance of the virus in the local population. A clinical study was performed utilizing a group of diabetic subjects of various ages to assess vaccine reactively and antigenicity. Single subcutaneous doses containing 400 CCA units of influenza virus vaccine, monovalent, type A_2/Aichi/ 2–68 were administered to 79 subjects. No constitutional reactions occurred and local reactions were minimal and similar to those found in previous studies. As has been found elsewhere, a high proportion of subjects over 70 years of age were found to have pre-existing antibody to Hong Kong virus. Of the younger subjects who had no detectable antibody against Hong Kong virus, all developed at least a fourfold rise in antibody. The mean titer of initially negative subjects in th study rose to a level of 1:320, an excellent response. Only 9 of the 79 subjects in the study failed to develop a fourfold response, all of whom possessed pre-immunization antibody. Eight of the nine had a twofold increase in titer, and one remained unchanged.

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A series of reports from investigators in the United States are forthcoming on the effectiveness of the vaccine during the 1968–1969 Hong Kong epidemic.

Finally, the behaviour of the vaccine under standard conditions of medical practice is of practical importance. Highly purified vaccine manufactured by the K-II zonal centrifuge process has been distributed through commercial channels in the United States since October, 1967. To date we have received no reports of allergic reactions to the vaccine, and reports of severe local or constitutional reactions have been received at a rate of less than one per 300,000 doses distributed. In our experience this is an exceedingly low incidence of adverse reports, and is good indirect evidence that the vaccine offers a means of immunization with a very low incidence of side-effects. Furthermore, studies on the use of high potency vaccines, already in progress, should furnish good evidence on optimum potency for maximum effectiveness consistent with a low incidence of untoward side-effects.

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SUBUNIT VACCINES OF INFLUENZA VIRUS[1]

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A major goal in the development of improved vaccines is the elimination of non-essential materials from the inoculum and retention of the protective immunizing antigens. At a minimum this implies that a non-living simple virus vaccine should consist solely of a highly purified viral suspension, but in the case of the complex viruses, the aim should be the removal of all elements of the virus particle which do not contribute to the desired immunization. Such vaccines containing purified viral components of significant protective potential will be referred to as »subunit vaccines.«

Influenza virus is a complex particle, having been shown to consist of several kinds of morphological subunits or substructures on the basis of chemical, immunological and electron microscopic studies. Starting from the center, there is a ribonucleoprotein core surrounded by a lipid or lipoprotein membrane. Projecting from the surface are the spikes, containing the envelope protein responsible for hemagglutination. The enzyme neuraminidase, important in release of virus from cells, is also on the surface and is perhaps associated with the spike. Several of the viral components are candidates for elimination in the preparation of a purified influenza subunit vaccine since they may induce unnecessary sensitization of the recipient or untoward reactions. These include viral lipids, the chicken antigens present in the envelope and ribonucleopro-

tein. Only two surface elements, neuraminidase and envelope protein of the spike, possess protective capabilities and the latter appears to be much more effective.

Several derivatives of influenza virus particles have already been employed as vaccines. Fragmentation of the viral envelope with Tween--ether provided the effective ether-split hemagglutinin vaccine (Davenport et al., 1964a). Also a detergent dissociated vaccine (Laver, 1964) has been tested in human subjects in Australia (Duxbury et al., 1968).

This discussion will emphasize a new procedure based on the dissociation of envelope protein into its simplest building blocks, the chemical subunits or polypeptides, and the subsequent spontaneous reassembly of the polypeptides into serologically active reassociated proteins. As outlined in Fig. 1, the initial step is the removal of viral lipids with metha-

Fig. 1

Schematic diagram of the procedure for preparation of envelope protein from influenza virus concentrates. The methanol-chloroform treated virus residue is extracted with either 8M urea with dithiothreitol or 67% acetic acid to reduce envelope protein to polypeptides. Reassociation follows on dialysis against phosphate buffered saline and a uniform serologically active protein results from the urea treatment, while the acetic acid procedure leads to a heterogeneous protein with both specific serological and hemagglutinating activities.

nol-chloroform, leading to denaturation of viral proteins into an isoluble mass. This precipitate is extracted with dissociating reagents, either 67 percent acetic acid or 8M urea with a reducing reagent, dithiothreitol,

DTT. In both of these solvents, envelope protein is in the form of a single polypeptide with a molecular weight of approximately 40,000 but on removal of the reagents by dialysis, the polypeptide subunits combine. The final products differ depending on the dissociating reagents used. Urea extraction results in a uniform protein, apparently a union of two polypeptides to give a dimer which binds HI antibody but does not agglutinate erythrocytes (Eckert, 1966a). The protein produced by the acetic acid procedure consists of coalesced polypeptide combinations of varied dimensions, the higher molecular weight forms of which not only bind HI antibodies but also agglutinate erythrocytes (Eckert, 1966b). both procedures yield essentially quantitative recoveries on the basis of serological activity, while the HA titer of the acetic acid-extracted protein, when measured under optimal conditions, with guinea pig cells at 4^0, is of the same order of magnitude as that of the original virus concentrate (Eckert, 1967).

The retention of specific serological activity following these two procedures of dissociation and reassembly would suggest that envelope protein polypetides are organized on reassociation into structure similar if not identical to the native protein. However, such a reversible process is not characteristic of all proteins. In this particular case, about half of the total protein of the influenza virus concentrates is rendered insoluble and is readily removed by centrifugation. At the same time neuramindiase activity is lost and there is no serological evidence of nucleoprotein or chicken tissue antigen (Eckert, 1966b). The absence of these characteristics may be attributed either to insolubilization or irreversible inactivation of the respective proteins.

Several aspects of the problem still remain to be studied. For example, what is the physical state and degree of purity of isolated envelope protein? Secondly, while envelope protein retains its specific serological activity, is it an effective immunizing agent? Finally, can one take advantage of the availability of isolated protein to produce antigens in unique forms with distinctive properties? Clarification of these points would permit an evaluation of envelope protein as a possible practical vaccine.

Physical State of Envelope Protein

The first question raised is whether we are dealing with a crude extract or a well-defined protein. Physical chemical and serological evidence corroborate that a high degree of purity is attained and that the protein is uniform in some or all of its stages of preparation. Analytical centrifugation of envelope protein dissolved in 6M urea gave a single sharp peak with a corrected sedimentation constant of 2.2 (Fig. 2), demonstrating that at the stage of the single polypeptide, envelope protein is uniform and is characterized by only a single major component. Using another criterion, the density of envelope protein determined by cen-

Fig. 2
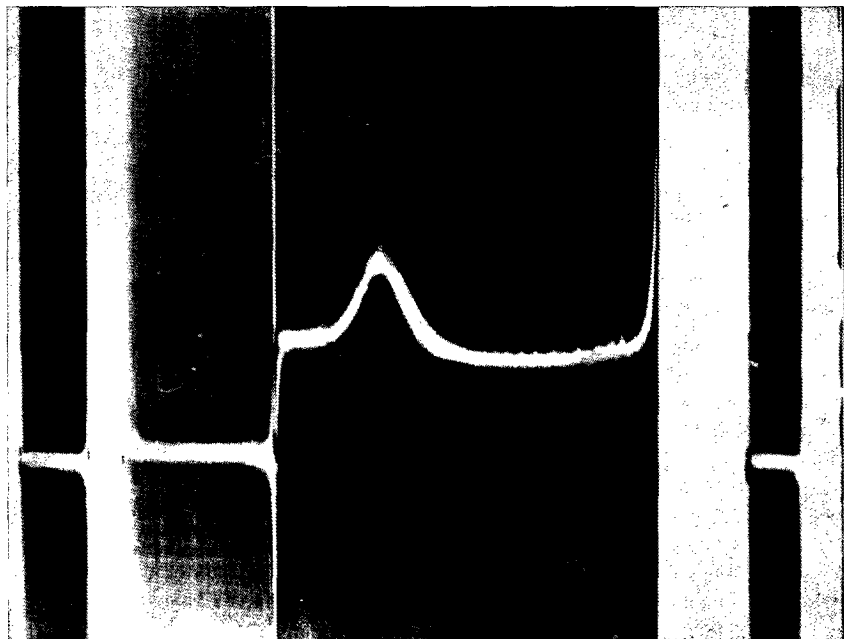
Analytical ultracentrifuge pattern of envelope protein from the PR8 strain of influenza virus dissolved in 6M urea; 256 minutes at 47,660 rpm.

trifugation to equilibrium in a cesium chloride gradient, a single peak of activity was found (Fig. 3). In the experiment illustrated, a comparison was made of urea-extracted protein, measured by its blocking antigen titer and the acetic acid-extracted protein determined by HA titers. Both were found to equilibrate at a density of 1.30, contrasting with the value of 1.17 for the less dense lipid rich influenza virus particle. These data clearly demonstrate the uniformity of envelope protein on the basis of size and density of the molecule. Chromatography on DEAE-cellulose gave a single peak of serologically active protein, establishing that the molecules are uniform on the basis of electrical charge (Eckert, 1969). The final physical evidence was obtained by acrylamide gel electrophoresis after dissociation of envelope protein preparations into polypeptides with sodium dodecylsulfate. Electrophoresis disclosed that while total viral protein was characterized by three major polypeptide bands, chromatographically purified envelope protein contained only a single kind of polypeptide (Eckert, 1969). Comparison of the electrophoretic pattern with known proteins gave an estimate of 47,000 for the molecular weight of the polypeptide. Finally, to determine its serological homogeneity, urea-extracted envelope protein was tested by immunodiffusion in Agarose gel against sera from rabbits hyperimmunized

Fig. 3
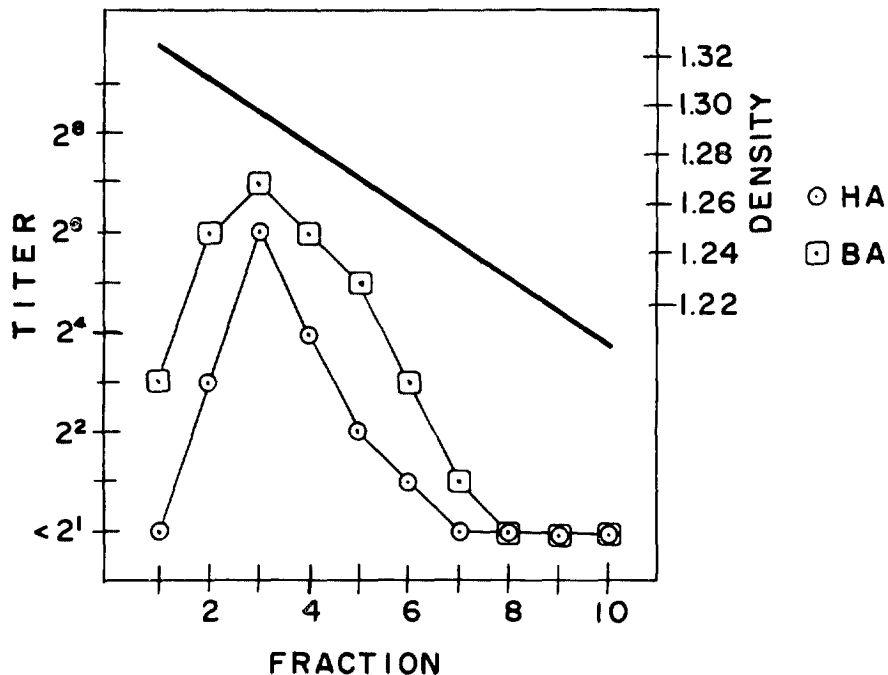
Equilibrium sedimentation in a cesium chloride gradient of envelope protein. HA values refer to an acetic prepared protein and blocking antigen (BA) titers to urea-extracted protein.

with virus concentrates. As shown in Fig. 4, a single line of precipitation developed. Thus, on the basis of physical chemical measurements of the size, density, and electrical charge of the molecule, and its polypeptide electrophoretic pattern and single immunodiffusion band, one can conclude that envelope protein is composed of a well-defined and highly purified suspension of reassociated subunits. In terms of purity the urea-extracted envelope protein is more refined since the homogeneity of the final protein product permits sharper chromatographic and centrifugal fractination.

Immunogenicity of Envelope Protein

While the isolation of envelope protein from virus entails no substantial loss of serological titer, it need not follow that immunogenicity would be retained. Since this is a major requirement in the development of a practical vaccine, the relative antigenicity of envelope protein and virus concentrates was ascertained by comparing HI antibody responses in

Fig. 4
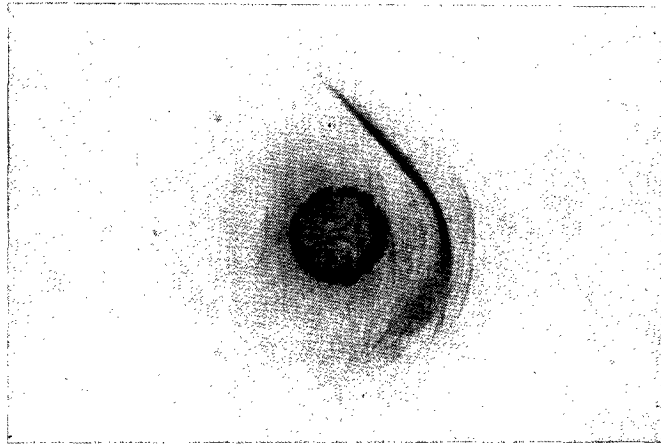
Immunodiffusion of urea-extracted PR8 envelope protein. Center well contains rabbit immune serum against strain PR8 influenza vaccine and the outer wells serial four-fold dilutions of envelope protein.

vaccinated animals (Table 1). These experiments demonstrate conclusively that formalinized virus is antigenically superior to both envelope protein preparations on a quantitative basis. They also show that under selected conditions, the purified protein can lead to excellent antibody responses. Note that the response of both rabbits and mice to protein inocula alone is minimal. However, if protein is administered with mi-

Table 1

HI titers of sera from animals inoculated with formalinized PR8 strain of influenza virus, acetic acid-extracted envelope protein, and urea-extracted envelope protein, both with and without adjuvant

	Rabbits[a]		Mice[b]	
	No Adj.	Adj.	No Adj.	Adj.
Virus	2400	4200	1400	1200
Protein (AcAc)	40	2600	40	140
Protein (Urea)	< 8	600	< 8	32

a. Titers equal average for two rabbits; Arlacel-mineral adjuvant inoculated into footpads with aqueous IV booster one month later and bleeding one week after booster; same schedule for rabbits without adjuvant, both doses IV.

b. Titers equal geometric mean values for group of six mice; IP inoculations at an interval of one week and bleeding one week after booster; aluminum phosphate adjuvant.

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neral oil adjuvant, rabbits respond weth titers approaching the values
obtained with virus vaccine. The effect of aluminum phosphate adjuvant
on the response of mice is in the same direction although the titers
achieved at this short bleeding interval were considerably lower. When
mice were bled at longer intervals (Fig. 5), higher titers were readily

Fig. 5
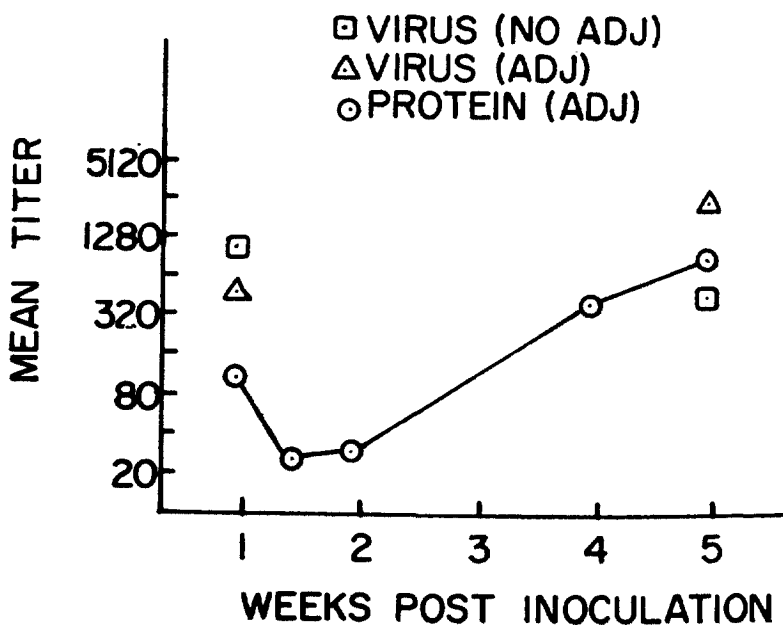
HI response of groups of mice to two inoculations at a one week interval of envelope
protein with aluminium phosphate adjuvant. Titers are compared with those of forma-
linized PR8 vaccine both with and without adjuvant at a comparable concentration
(500 HAU). Titers equal the geometric mean obtained with groups of six mice.

demonstrated. After an initial minor response, the HI titers first de-
clined and then rose until at the end of 5 weeks the titers obtained with
protein were comparable to those produced by formalinized virus.

In the above animal experiments, all comparisons were made between
equivalent amounts of antigen, that is, the protein was derived from the
same amount of virus as was contained in the parallel formalized vacci-
ne. If larger amounts of protein were inoculated with adjuvant into both
rabbits and mice, HI titers were attained which exceeded 4000, equal to
the highest induced by virus.

While the immunizing potential of protein vaccines has been demon-
strated in animals under specific circumstances, the ultimate utility will
depend on the response of the human recipient. To date there have been

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no tests in man. A suggestion that protein may be effective is derived from the results in human subjects with ether-split hemagglutinin (Davenport et al, 1964b). They found that the response was excellent without use of adjuvant even though, as is the case with envelope protein, adjuvant markedly enhanced antibody response in experimental animals (Davenport, 1968).

Synthesis of Antigens from Subunits

While subunit vaccines appear to be of potential value because of their purity, further interesting developments may arise from the availability of viral antigens in such a simple form. Simplicity permits experiments aimed at modifying the physical state of the antigen. While the conformation of the polypeptides clearly determines antigenic specificity, the macromolecular state of the antigen appears to markedly influence the immune response quantitatively as shown above. Possible mechanisms are that the physical nature of the protein may control the spectrum of cells taking up antigen, or affect the ease of entry of antigen into target cells or perhaps protect the antigen from destructive proteolysis.

While the program to produce modified antigens is in a preliminary stage, a brief projection will illustrate tle flexibility gained by utilizing proteins in subunit form. One modified antigen has been produced by mixing isolated protein with water suspensions of purified phospholipids. This leads to an immediate union of lipid and protein and the complex has given enhanced antibody responses in rabbits. Another approach is to bind subunits by covalent lingakes into packets of subunits and to determine whether there is an optimal size for maximal immunological response. Still another possibility, dependent on the foregoing, is to synthesize mixed packages constituted of subunits from several strains of influenza virus. This would be of great value if such composite proteins would promote a broader spectrum of antibody response. Further investigations of such synthesized antigens are in progress.

SUMMARY

Envelope protein, the antigen primarily responsible for hemagglutination and immunological protection, has been isolated in a highly purified state from influenza virus. The procedure is based on the dissociation of the protein into its basic polypeptide building blocks and subsequent spontaneous selfassembly of these subunits into reassociated protein macromolecules. The derived proteins have been rigorously defined in terms of their physical chemical and serological attributes. Immunological responses induced in experimental animals suggest that envelope protein is of potential value as a purified subunit vaccine for man. Isolation of the critical antigen of influenza virus in such a simple form should facilitate the synthesis of vaccines with unique characteristics by manipulation of protein subnits.

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CURRENT STATUS OF LIVE INFLUENZA VACCINE

CORRELATIONS BETWEEN THE VIRULENCE AND OTHER GENETIC CHARACTERS OF INFLUENZA VIRUSES

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The great contribution into solving of the problem of live viral vaccines was made by the study of genetic characters of virus strains and their correlation, But our information about the latter is still unsufficient in spite of growing interest to the problem.

It can be explained not only by the limits of knowledge about mechanisms of expression of characters, but also by different sources of correlative variability: polygenecity of the character, pleothropism of gene action, selection of double mutants etc.

The most interesting aspect for solution of the problem of line vaccines is the determination of the relation of the virulence with other characters of virus strain.

This paper presents results of the study on correlation between the virulence for mice with activity of reproduction at low and high temperatures, viremia, thermostability, neuraminidase activity and eluting activity in influenza virus strains.

In these experiments we used repeatedly selected AO, A2 and B strains of influenza virus with different virulence for albino mice.

Correlation of virulence to the capacity of virus strain to multiply
at low and high temperatures

Ten strains of AO and A2 virulent and avirulent for mice were studied in this respect.

Ten-day chick embryos were infected with 100 EID_{50} 0,1 ml of virus-containing allantoic fluid and incubated at different temperatures.

After the incubations the sapmles of allantoic fluids from 5 embryos were pooled in equal volumes for estimation of the titers of infectivity.

Rct- marker was determined as follows: if of titer at 40°C in comparison to that at 37°C was reduced more than by 4 logs the strain was considered as *rct 40⁻*, if the reduction was between 2 and 4 logs the strain was designated *rct 40±*. The rct 28 marker was determined by the similar way.

Table 1 presents average data of 3–5 experiments. The results show that all highly virulent strains were characterized as rtc 28⁻ and strains with low virulents and avirulent strains were rtc 28± strains.

Table 1

Activity of Reproduction at Low and high Temperatures (rct – marker)

| Strain | Viru-lence | rct 28 | | rct 40 | |
		Index of suppression of virus reproduction	rtc character	Index of suppression of virus reproduction	rtc character
A(WSN)M	+ + +	7.0	—	1.1	+
A2(Frunze)59/M33	+ + +	5.2	—	1.7	+
A2(England)12/64/M$_{40}$	+ + +	4.9	—	2.5	±
A2(Krasnodar)101/59	+	3.3	±	2.3	±
A2(Moscow)21/65	+	2.6	±	0.9	+
A2(England)12/64	—	2.5	±	1.9	+
A2(Frunze)59	—	4.5	—	1.7	+

The infectivity titers at 40°C were by 1–2 logs lower than at 37°C in all strains studied and they were determined as rtc 40⁺ strains excluding one highly virulent strain A2/England 12/64/M$_{40}$ and one low virulent A2/101/59. Both had rtc 40± character.

Interdependence of virulence and viremia

The ground for investigation of relations between virulence and viremia were the data on correlation between virulence of polyoviruses for monkeys and their capacity to cause viremia in these animals. (Bodian, 1955; Gard S. 1958; Melnick J. and Benyesh-Melnick, 1961) and absence of similar information about influenza viruses.

Fifteen strains of influenza AO, A and B possessing different virulence for albino mice were taken for study of dynamics of viremia in randomly bred albino mice and BALB and C$_{57}$B$_c$/6 mice. Mice weighing

6–8 grams were infected intranasally with 1000 E1D$_{50}$ of virus. At intervals 4 mice from each experimental groups were exsauguinated. Their bloods were pooled and hemolysed in 2 ml of destilled water (with addition of 1000 units of penicillin and streptomycine and 0,05 ml of gramicidine to 1 ml of water), that corresponded to the dilution 1:5. The presence of virus in blood was determined by inoculation it into allantoic cavity of 9–11 day chick embryos.

The concentration of virus in blood was estimated by routine titration of blood samples in chick embryos.

Results of these experiments revealed certain differences between virulent and avirulent strains in frequency and regularity of virus isolations from the blood; more frequently virus was isolated from blood of mice infected with virulent strains; in which viremia was regularly found. (Table 2). Among avirulent viruses some strains caused viremia regularly and some irregularly. As a rule the minimal dose capable to give rise to viremia was lower in virulent strains (Table 3).

Table 2

Regularity of Development of Viremia and the Frequency of the Isolation of Virus from Blood

Strain	Viru-lence	Isolation of virus from blood	Maximal titer of virus in blood log of ID$_{50}$/0,1 ml	Frequency of isolation of virus from blood (percent)
A(WSN)M	+ + +	Regular	4.5	40
A2(Frunze)59/M33	+ + +	Regular	4.3	67
A2(England)^{12}/64/M40	+ + +	Regular	4.5	47
A2(Krasnodar)101/59	+	Regular	3.2	38
A2(Moscow)^{21}/65	+	Irregular	3.0	28
A2(England)^{12}/64	—	Irregular	1.0	2,8
A2(Frunze)59	—	Regular	3.6	45
A2(Vladivostok)^{25}/67	—	Irregular	3.5	20
A2(Vladivostok)^{36}/67	—	Irregular	1.0	8
A2(Leningrad)133/65	—	Regular	2.8	21
B(Likh)59	—	Regular	1.0	17
B(Rome)63	—	Regular	2.7	22
B(Moscow)66	—	Regular	2.8	44
B(Dushanbe)66	—	Regular	1.4	22
B(Lugansk)249 66	—	Regular	1.3	25

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Table 3

Minimal Doses of Virus Giving Rise to Viremia in Mice Infected Intranasally

Strain	Viru-lence	Virus dilution						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
A(WSN)M	+ + +	+	+	+	+	+	—	—
A2(England)12/64/M_{40}	+ + +	+	+	+	+	+	—	—
A2(Frunze)59/M_{33}	+ + +	+	+	+	+	+	+	—
A2(Moscow)21/65	+	+	+	+	+	—	+	—
A2(Krasnodar)101/59	+	—	—	+	—	—	—	—
A2(England)12/64	—	+	—	—	—	—	—	—
A2(Frunze)59	—	—	—	—	—	—	—	—

At the same time there were in significant differences in the moment of appearance and the duration of viremia.

Taking into account the data described above we think viremia can't serve as a reliable criterion for distinguishing of virulent and avirulent strains although the latters as a rule showed it irregularly and had more high minimal viremic dose.

Virulence and neuraminidase and eluting activities

Ten strains studied are listed in the Table 4.

Table 4

Activity of Neuraminidase and Elution

Strain	Viru-lence	Neuraminidase activity per 1 HAU*	Eluting activity (per cent)
A(WSN)M	+ + +	0.05	50
A2(Frunze)59/M_{33}	+ + +	0.06	25
A2(England)12/64/M_{40}	+ + +	0.08	12.5
A2(Krasnodar)101/59	+	0.4	12.5
A2(England)12/64	—	0.8	12.5
A2(Frunze)59	—	0.6	12.5
B(Likh)59	—	0.21	50
B(Rumania)¹/63	—	0.03	50
B(Moscow)64	—	0.03	50
B(Dushanbe)¹/66	—	0.12	25

* Hemagglutinating unit.

Eluting activity of virus was evaluated by the speed and completeness of elution of virus adsorbed on the formalinized chick erythrocytes. After the adsorption supernatant was discarded and warmed (37°C) saline was added to the pellet in equal volume. After 30 minutes and 2 hours of incubation at 37°C hemagglutination tests were made. Then the ratio of resulting titer to original titer was calculated and expressed in percentage.

Neuraminidase activity was estimated by capacity of virus to split neuraminic acid from substrate. Thiobarbituric thechnique (Aminoff, 1059) was empolyed using M and N substances from the stroma of human erythrocytes. To 0,05 ml of eluate 0,1 ml of substrate (5 mg/ml) and 0,1 ml of 0,3 M phosphate buffer, pH-7,0 were added. The mixture was incubated for 1 hour at 37°C. The calibrating curve was constructed using the N-acetyl-neuraminic acid, supplied by Light, England. The light absorption of coloured solutions was measured in the spectro-fotometer SF-4 at wave length 549 mμ. According to the size of light absorption the quantity of splitted neuraminic acid from the substrate was calculated.

The analysis of the data obtained showed that strains of influenza virus virulent for mice possessed low neuraminidase activity. Among the avirulent strains there were both the strains with high and low activities. The association between eluting activity and virulence also was not observed.

Thermostability of infectious and hemagglutination properties and virulence

There are few publications about interrelation of virulence with thermostability of viruses and the conclusions are contradictory. Some investigators (Kolchurina et al., 1967) found that virulence correlated with resistance of influenza virus to heating, other did not observed such phenomenon (Soloviev V. D. et al., 1961).

We studied thermostability of hemagglutinating and infectious properties of 8 strains of influenza AO and A2 viruses, different in their virulence for mice.

For evaluation of thermostability virus containing material was heated in water bath (with automatic regulation). Allantoic fluid had been centrifuged at 2000 rev. per minute for 1 hour. Heated samples were tested for hemagglutination and infectivity by routine thechnique. The results were treated statistically.

The hemagglutinating titers did not fall at 50°C during 4 hours in all strains tested. There were no considerable differences in rate of inactivation of infectivity between virulent and avirulent strains.

But vaccinial strains occured to be some more sensitive to heating than the rest of them.

Thus the heating at 50°C did not reveal a distinct correlation between virulence and thermostability.

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In the next set of experiments the thermostability of virus was evaluated by resistance to heating at 60°C during 5 and 60 minutes. Virus-containing allantoic fluid was heated either undiluted or diluted 1:10 with distilled water, 2M $MgSO_4$ and 2M $MgCl_2$.

Infectivity of virus was inactivated in all variants of heating in 5 minutes. Hemagglutinins in the presence of magnesium ions became destroyed in 3 of 4 strains tested. The exclusion was vaccinial strain A2/101/59, in which hemagglutinins in the presence of $MgSO_4$ were inactivated in 60 minutes of heating.

So the results of our studies showed the existence of unilateral association between virulence and resistance of virus to heating at 60°. The thermolabile hemagglutinins belonged to highly virulent strains and among thermorestistant strains there were avirulent and virulent strains.

CONCLUSIONS

Our investigations showed unilateral association between the virulence of influenza viruses for mice and their capacity to reproduce at 28°C the virulent strains multiplied with smaller activity than did avirulent strains.

Virulent strains regularly gave rise to detectable viremie in mice, and avirulent strains did it irregularly. Virulent strains were resistant to the serum inhibitors of certain animals, while among avirulent strains there were both resistant and sensitive strains.

Virulent strains had low neuraminidase activity but of avirulent strains some had low activity and some-high activity.

Thermostability of hemagglutinins also associated with virulence. No connection was observed between the virulence of a strain and the activity of its elution from erythrocytes.

So a conclusion can be made that the association between virulence and other properties of a strain is unilateral, i. e. the characters of virulent strains can present in an avirulent strain.

In other words avirulent strains are heterogenous in respect of markers associating with virulence, some of them possess this markers and some do not.

Our results show the complicacy of investigation of interrelationship of virulence with other genetic markers. This complicacy is based on several reasons the main of which are polygenecity of virulence character, lack of information on the mechanism of expression of virulence and other characters, influence of environment on the phenotypic expression of virulence. At last it should be not forgotten that the study of genetic marker of viruses is made on the level of population, not on the level of individual as in other organisms. Differences between clones of one population of a viral strain can be rather significant. So studies on interrelationship between genetic markers must be done only on carefully selected strains.

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RECOMBINATIONS AMONG MIXOVIRUSES

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The present report summarizes results of investigaons carried out at the Laboratory of Viral Genetics during the last few years. Our work was directed towards studying the problem of intraspecies and between--species recombination of viruses.

A considerable number of publications is dealing with the problem of recombinations among viruses of man and animals both of Soviet authors Zhdanov V. M. and Stakhanova V. M., 1961; Sokolov M. I., 1962; Sokolov M. I., and Podchernyaeva R. Y., 1963; 1966; Sokolov M. I., et al., 1965, 1966a, 1966b; Stakhanova V. M., 1965; Podchernyaeva R. Y., et al., 1968.) and of foreign investigators (Burnet F. and Edney M., 1951; Burnet F. and Lind P., 1954a, 1954b, 1954c; Hirst G. K., and Gotlieb T., 1955; Gotlieb T. and Hirst G. K. 1956; Lind P. and Burnet F., 1957; Burnet F., 1959; Fraser K., 1959a, 1959b, 1959c; Kilbourne E. D., and Murphy J. S., 1960; Hirst G. K., 1962; Simpson R. W. and Hirst G. K., 1962; Kilbourne et al., 1965; Tumova B. and Pereira H. G., 1965; Sugiura Akira and Kilbourne E. D., 1966).

Viral recombination can be observed on joint cultivation of two viable viruses, of a viable and an inactivated virus, of a viable virus and the nucleic acid isolated from another virus, and, finally, as a result of joint cultivation of two nucleic acids.

In the work reported here, three last methods of recombination were used; and results are presented concerning the production of recombinants by crosses between closely related influenza viruses (A and A2), between antigenically more distant influenza viruses (A and A1), as well as between nucleic acids isolated from various influenza virus types and from an influenza virus and Newcastle disease virus.

The possibility of transmission has been studied of a number of genetic traits: hemagglutinating activity (HA); resistence (I^-) and sensitivity (I^+) to normal horse serum inhibitor; thermostability of hemagglutinins ($T_{56}HA$) and of infectivity ($T_{56}ID$) of the virus; pneumo- (Pm_{in}) and neurovirulence (Pm_{iv}) to albino mice; infectivity to chick embryoes (Pche); eluting (Erc), neuraminidase (N), cytopathogenic (TC_{km}) and plaque-forming (Pl) activities.

The following virus strains were used in the reported work; LF and WSN of influenza A; FMl of influenza Al; F_3, Lvov, Rome, 430, and Altai of influenza A2; and strain Beaudette of Newcastle disease virus. All the strains had been subjected to preliminary selection in order to obtain homogeneous clones with which all the subsequent work on recombination was done. Strains used in experiments possessed specific genetic markers which allowed their differentiation.

To prepare inactivated viruses, allantoic culture fluids were subjected to incubation for 14 days at 35^0; for 11 days at 37^0; for 2 hours at 56^0 or 60^0; in the presence of $MgCl_2$ (1 M, 0.1 M, 0.001 M) for 24 hours at 37^0.

Recombination experiments between living and inactivated viruses were carried out by joint cultivation of the viruses in allantoic cavity of 10 embryoes or in 10 tube cultures of chorioallantoic membranes (CAM).

Test fluids from each embryo or tube were collected and studied with respect to three genetic markers: antigenic (Ag) and hemagglutinating (HA) activities and sensitivity to normal horse serum inhibitor (I). The course of subsequent examination depended on the purpose of the experiment and consisted in isolation of the recombination and studying other genetic characters.

RESULTS

1. Recombination between inactivated and infectious influenza viruses

First recombination experiments were made between two closely related influenza viruses, belonging to types A and A2. 44 experiments in chick embryoes and 35 experiments in CAM cultures were made altogether between the inactivated influenza A virus (strain LF) and different living strains of A2 influenza (F_3, Rome, 430). Of the recombinants, which were obtained, 28 were studied in detail. Results of investigation of genetic markers of some of the recombination are shown in Table 1.

As can be seen from the Table, by recombination with the LF donor, possessing highly expressed hemagglutinating activity (HA^+), it was possible to transmit this marker regularly, in higher or lesser degree, to the recepients F_3 and Rome. All the recombinants acquired I^- marker, and enhanced Pche character is exhibited by 2 to 3 logarithms.

In the cross LF $\times$ F_3 two of the recombinants (R_2 and R_{10}) possessed Pm_{in} marker in the same degree as the donor strain; in R_{25} this marker was less expressed, while R_6 did not acquire this property.

Table 1

The Transmission of Genetic Characters on Recombination of an Inactivated
Influenza A Virus with Infectious Influenza A2 Viruses

Cross	Strains	Ag	HA	I	Pche	Pm_{in}	$T_{56}HA$	$T_{56}ID$	E_{re}	TC_{km}
LF x F₃	LF	A	2500	—	9.0	4.7	120	120	120	+ + + +
	F₃	A2	20	+	5.0	—	120	20	120	—
	R₂	A2	1280	—	7.2	4.7	120	20	180	+ + +
	R₆	A2	320	—	7.0	—	120	10	60	+
	R₁₀	A2	320	—	7.0	4.7	120	20	5	± +
	R₂₅	A2	160	—	7.5	1.0	120	20	120	+
LF x Rome	Rome	A2	20	+	5.0	—	10	10	120	+
	R₁	A2	160	—	7.0	1.8	120	120	120	+
LF x 430	430	A2	640	+	7.0	—	120	30	120	—
	R₁	A2	640	—	8.5	—	120	30	180	+

Abbreviations:

Ag – Antigenic properties
HA – Hemagglutinating activity
I   – Relation to normal horse serum
     inhibitor: – inhibitor-resistant
                + inhibitor-sensitive
Pche – Infectivity to chick embryoes (Lg ID_{50})
Pm_{in} – Pneumovirulence to mice (Lg LD_{50})

$T_{56}HA$ – Stability of hemagglutinins to heating at 56°
             (in min)
$T_{56}ID$ – Stability of infectivity to heating at 56°
             (in min)
E_{re} – Eluting activity from hen's erythrocytes
             (100%/min)
TC_{km} – Cytopathogenic activity in monkey kidney cells

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On crossing LF × Rome, the recombinant R_1 had the same stability to heat treatment at 56° as the donor strain. In a number of experiments with the crosses LF × F_3 and LF × 430 transmission was observed of the cytopatogenic activity (TC_{km}) in primary cultures of monkey kidney cells; the fact was supported by maintenance of the cytopatic effect in passages, by presence of the virus evidenced by HA reaction and hemadsorbtion, as well as by infectivity to chick embryoes of the 3rd passage cultural fluid.

It is of interest that on recombination of influenza A and A2 viruses, in the first progeny population, besides the recombinants possessing the antigenic properties of the A2 recepient, polyantigenic variants could be revealed, that is cultures exhibiting HA-inhibition reaction with both A and A2 antisera. The polyantigenic variant AA2, obtained after the cross LF × F_3, maintained its properties during 5 passages of end-point dilutions of the virus. Following that splitting was observed into the original strains and a monoantigenic recombinant.

Thus, on multiple infection of the cell with an infectious and an inactivated viruses, related in antigenic properties (A and A2), recombinants were obtained which combined in themselves certain genetic characters of the parent strains. According to frequency of transmission, genetic markers may be divided in two groups: 1) regularly transmitted – I^-, HA- ID characters and 2) irregularly transmitted – T_{56}, Pm_{ic}, E_{rc} and TC_{km} characters.

The purpose of subsequent experiments was to find out the possibility of transmitting genetic markers on recombination of more remote variants of viruses – influenza A and A1.

The investigation was carried out in two directions: 1. In recombination experiments between the inactivated influenza A virus (strain WSN) and the infectious influenza A1 virus (strain FM1), the possibility was studied of transmitting I^-, Pm_{ic}, N, and Pl markers.

2. In recombination experiments, where FM1 influenza was the inactivated virus, and strain WSN was the infectious one, the transmission was studied of I^- and T_{56} markers.

Recombinants R_1, R_2, and R_3, obtained in the first series of experiments between WSN × FM1 (Table 2), acquired I^-, Pl, and N markers; recombinants R_2 and R_5 possessed Pm_{ic} marker in a mild degree. The transmission of Pl marker was observed when the experiments were made in hog kidney cell cultures (ZS) and in chick embryo fibroblasts In the PS cells, the recombinants produced microplaques, observable under magnification only. When passaged in this tissue culture, the plaques were homogeneous with respect to S character. In chick embryo fibroblast cultures, infected with WSN × FMI, production of plaques could be detected only in the presence of DEAE-dextrane, though they were smaller than in the original WSN strain.

Table 2

The Transmission of Genetic Characters on Recombination of Influenza A and A1 Viruses

Cross	Strains	Ag	I	Pm_{ic}	$T_{56}HA$	E_{rc}	N	Pl_{cf}
WSN x FM1	WSN	A	—	4.0	10	25	0.05	+
	FM1	A1	+	—	60	100	0.4	—
	R_1	A1	—	—	120	100	0.05	+
	R_2	A1	—	1.0	120	100	0.05	+
	R_3	A1	—	1.0	90	...	...	+
FM1 x WSN	R_1	A	+	3.0	10	...	...	+
	R_2	A	+	2.4	10	...	...	+
	R_3	A	+	1.2	10	...	...	+
	R_4	AA1	+	1.8	10	...	...	+

A b b r e v i a t i o n s : Ag, I, $T_{56}HA$, E_{rc} – as in Table 1.

Pm_{ic} – Neurovirulence to mice (Lg LD_{50})
Pl   – Plaque-forming ability under agar overlayer
N   – Neuraminidase activity (per unit of HA)
...   – Not studied

On recombination of the inactivated FMI virus with the infectious WSN virus (Table 2), all the recombinants acquired I^+ character. Other markers (T_{56} and Pl) were those characteristic of WSN strain. In all the recombinants variations were observed in the degree of virulence to mice (Lg LD_{50} being from 1.2 to 3.0). In these experiments a recombinant R_4 was produced, which possessed polyantigenic properties and was neutralized in HA-inhibition tests by anti-WSN and anti-FMI sera in approximately the same titer (1 : 640 – 1 : 1280). The polyantigenic properties persisted for 2 passages only.

Thus, in our experiments the possibility was demonstrated of producing recombinants by crossing influenza A and A1 viruses, which are relatively remote in antigenic properties.

II. Recombination between viral RNA and influenza A1 and A2 infectious viruses

The purpose of this series of experiments was to study the transforming properties of influenza virus RNA in recombination with various infectious strains of influenza A1 and A2 viruses.

The experiments were made in chick embryoes, in CAM cultures, in chick embryo fibroblasts, and in PS cells. Cultures, infected with the infectious viruses and the RNA plus RNase, served as controls.

In this part of the report data is presented on the transforming properties of RNA which was not infectious and of RNA which possessed this property.

In the first series of experiments, RNA was used which was isolated by the cold phenol extraction procedure, according to Gierer A. and Schramm G. (1956), and possessed no infectivity. The RNA preparations had typical absorption spectra and were free of protein. Results of these experiments have been published earlier (Sokolov M. I. et al., 1965, 1967).

9 experiments were made, and 78 recombinants were obtained of which 6 were subjected to detailed study. It can be seen from Table 3, that all the recombinants acquired a number of characters (I^-, HA, TC_{cf}) peculiar to the donor strain from which the RNA was isolated. Some of the recombinants revealed enhanced reproduction ability in chick embryoes by 2 to 3 Logs (P_{che} character).

Table 3

The Transmission of Genetic Characters on Recombination between the RNA of WSN Influenza Virus and the Infectious F_3 Influenza Virus

Strains		Ag	HA	I	Pm_{in}	Pm_{ic}	Pche	$T_{56}HA$	TC_{cf}
Original	WSN	A	1280	—	3.8	4.0	8.0	10	+
	F_3	A2	20	+	—	—	5.0	120	—
Recombinants	R_1	A2	320	—	—	—	8.0	180	+
	R_2	A2	80	—	2.0	—	8.0	120	+
	R_3	A2	640	—	1.0	1.0	6.0	180	+
	R_4	A2	1280	—	1.0	—	7.0	. . .	+
	R_5	A2	1280	—	3.0	—	8.0	. . .	+
	R_6	A	1280	—	1.0	1.0	8.0	120	+

Abbreviations: Ag, HA, I, Pm_{in}, Pche, $T_{56}HA$ – as in Table 1.

Pm_{ic} – as in Table 2
TC_{cf} – Cytopathogenic activity in chick embryo fibroblasts
. . .   – Not studied

5 recombinants out of 6 acquired the ability to kill mice on intranasal inoculation (Pm_{in} character), two of the recombinants acquired the ability to do this on intracerebral inoculation (Pm_{ic} character).

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Table 4

Recombination between the RNA of WSN Virus and the RNA of F_3 Virus

Strains	Ag	HA	I	Pche	Pm_{in}	Pm_{ic}	$T_{50}HA$	Pl_{km}
WSN	A	1280	—	8.0	4.0	4.0	10	+
F_3	A2	20	+	5.0	—	—	120	—
R_1	A	2560	—	8.0	4.0	—	120	—

Abbreviations – as in Tables 1 and 2.

In subsequent experiments, the infectious Altai strain of A2 influenza was used as the recepient. In these experiments we aimed to investigate the possibility of transmission of Pm_{ic}, I^-, TC_{ef} and Pl characters. Recombinants were obtained, which possessed the antigenic properties of the recepient, but acquired I^- and Pm_{ic} markers. Cytopatic activity was observed only on primary infection of tissue cultures. No transmission of the Pl character was observed.

Thus, by these experiments the possibility was established of regular transmission of certain genetic characters on crossing non-infectious viral RNA preparations of influenza A with different strains of influenza A2.

In later experiments, RNA was isolated from purified virus suspensions using detergents and phenol, according to the procedure of Sokol F. and Schramek S. (1964). The virus purification was carried out using fibrous DEAE-cellulose and differential centrifugation. Concentration of the virus in purified preparations varied from 4.8 to 6.0 mg/ml. The purified virus suspensions possessed high infectious and hemagglutination titers. Purification with respect to protein reached 97%. The detergent-phenol method provided for maximal extraction of RNA from virions. Isolated RNA preparations possessed similar parameters with those mentioned above. The RNA purification procedure is shown in Figure 1.

Besides the changed method for RNA isolation, a modified procedure was used for detecting infectious properties of the RNA (initial infection of cell cultures with DEAE-dextrane treatment, dissolution of the RNA in 0.15 M NaCl with Mg-salts, and addition of 500 μg/ml od DEAE-dextrane). The above mentioned alterations provided for regular isolation of RNA preparations possessing infectious activity. Results of these investigations were reported by us in 1968 (Menshikh L. K. et al.).

The transforming properties of the infectious RNA preparations were studied in recombination experiments with infectious FMI strain of influenza A1.

Figure 1

Procedure for RNA Isolation from Influenza Viruses

Purified and concentrated
virus preparation

1.   Desintegration of the virus
with sodium desoxycholate

2.   Phenol deproteinization

Phenol   Phenol plus
0.5% sodium
dodecylsulphate

3.   Ether and nitrogen treatment

4.   Precipitation of RNA
with 98% ethanol

5.   Solution of RNA in 0.15 M NaCl
in 0.02 M phosphate buffer, containing
0.001 M MgCl, and DEAE-dextrane
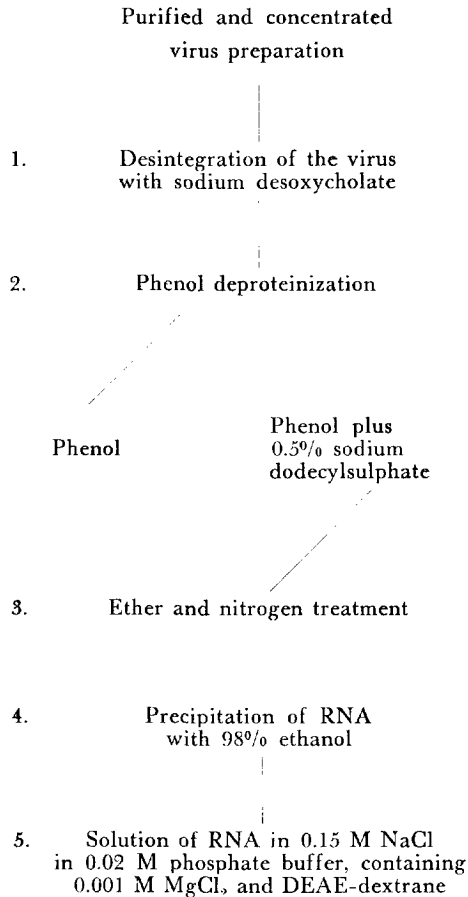
In experiments made in chick embryos, recombinants were isolated, possessing polyantigenic properties; no transmission of I$^-$ and Pm$_{ic}$ characters was observed. To investigate the possibility of transmission of Pl character, a number of experiments were made in chick embryo fibroblasts under the agar overlayer. In 4 of the 5 experiments we observed the transmission of Pl character; though in two of the experiments, when the palques were subcultured to chick embryoes, the allantoic cultures possessed no hemagglutination activity.

Thus, in our experiments the ability to be transformed has been shown for some characters of the virus, from which the RNA was isolated, with both infectious RNA and with RNA lacking this property.

III. Recombination between two viral nucleic acids

Experiments were conducted in two directions:

1. Recombination between nucleic acids isolated from influenza A and A2 viruses.

2. Recombination between nucleic acids isolated from influenza A virus and from Newcastle disease virus.

In the first series of experiments, RNA preparations were used, isolated from strains WSN (influenza) and strain F_3 (influenza A2). The experiments were made in CAM cultures and in chick embryo fibroblasts. The RNA were isolated from virus infected CAM cultures, which contained not high concentrations of infectious viruses ($10^6 - 10^7$ ID_{50}). In these particular experiments the isolated nucleic acids were not infectious. However, when these RNA preparations were introduced in CAM cultures, in 2 recombination experiments out of 5 an infectious virus was produced. One of the recombinants studied possessed a number of genetic characters specific to influenza A virus (HA, 1−, Pche, Pm_{in}), but at the same time this recombinant was not lethal to mice on intracerebral inoculation (Pm_{ic}-marker) and possesses T_{56} marker, which was characteristic of influenza A2 virus.

Thus, we have for the first time shown the possibility of genetic recombination among animal viruses on introduction into the cell of ribonucleic belonging to different varieties of influenza virus.

In subsequent experiments, recombination was carried out between RNA, isolated from influenza A virus (strain WSN) and from Newcastle disease virus. The RNA were isolated from highly purified and concentrated influenza virus preparations ($10^9 - 10^{10}$ ID_{50}) and Newcastle disease virus preparations ($10^{10} - 10^{11}$ ID_{50}). In the given experiments the nucleic acids possessed infectious activity.

There were 8 experiments of recombination between WSN RNA and NDV RNA. Polyantigenic variants were produced, which got neutralized with both antisera. The polyantigenic properties, as a rule, persisted for 1 to 3 passages.

The obtained evidence allows to suggest the possibility of recombination of nucleic acids from different virus species. Investigations in this direction are being continued.

SUMMARY

There are still many non-elucidated questions in the problem of viral recombination. To these belong, in the first place, the mechanism of recombination, the determination of species boundaries for virus crosses, the development of most effective methods of production of recombi-

nants, and other points of procedure. Investigations in genetic recombination are of great theoretical value, particularly for elucidation of viral structure and function, and primarily, of their genomes. Work on recombination may be used for development of recombinant vaccinal strains, combining in themselves characters advantageous from the point of view of immunity. According to evidence obtained both in our country and abroad such investigations are of current interest and deserve further development.

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EVALUATION OF THE EFFECTIVENESS OF LARGE-SCALE VACCINATION AGAINST INFLUENZA (RESULTS OF FIVE-YEAR OBSERVATION)

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Vaccination is at present the most promising method of influenza control. A 2-fold to 3-fold reduction in the morbidity from influenza and other acute respiratory diseases (ARD) may on the average be expected after vaccination with live influenza vaccine (Sokolov, 1954; Zhdanov et al., 1958; Smorodincev and Korovin, 1961). In most investigations the effectiveness of the vaccine has been estimated by studying the incidence of the disease among vaccinated and unvaccinated members of different sections of the community (Workers, schoolchildren, etc.). The question of the possible effect of mass influenza vaccination of the epidemic process was first raised by Slepushkin et al. (1962) in connexion with an investigation undertaken in 1959–61.

The present observation deals mainly with the effectiveness of large-scale vaccination with live divalent influenza vaccine containing vaccine strains A2 and B in industrial centres in the Smolensk region during 1965 – 3. 1969.

The towns chosen for vaccination were Smolensk and Jarcevo, both with large concentration of light and metal-working industry. Children below 14 years formed between 25% and 28% of the population.

Nearby industrial centres where no influenza vaccination was carried out were selected as controls to allow evaluation of the effectiveness of the vaccination. The control towns for Smolensk were Vitebsk and Kaluga, both of which had roughly the same industrial structure, total population and age distribution of population. The control towns for Jarcevo were Safonovo and Roslavl, which had similar populations and age distributions but a somewhat different industrial structure.

The vaccine was prepared by the Moscow Institute for Research on Virus Preparations from the virus strains: A2/Krasnodar 101/59 and B/Moscow/Lich/59 in 1964; A2/England 12/64 and B/Romania 1/63 in 1965; A2/Moscow 21/65 and B/Moscow/Lich/59 in 1966 and 1967.

The Vaccine prepared from last two strains were used for first vaccination of adult (over 12 years) population of Smolensk and for both vaccination of population of Jarcevo in 1968. For the second vaccination in Smolensk in 1968 were used the vaccine from strains A2/Moscow 21/65, A2/Leningrad 133/65, B/Dushanbe 1/66 and B/Leningrad 2/67.

This strains were more immunogenic and antigenically similar to the strains circulating in nature.

Vaccination were carried out generally in October – November twice at intervals of 25 to 30 days. Vaccination were carried out 3 times at intervals of 10 to 14 days in 1964. In view of the possibility of an outbreak of B influenza in the spring of 1966, subjects were vaccinated first with monovalent influenza B vaccine and later with divalent A2B vaccine in December 1965.

The most important task of this observation was to study practical significance of vaccination as measure of influenza control.

Therefore we did not have special control groups in Smolensk and Jarcevo from 1964 and tried to vaccinate most of people over 12 years who must be subjects of vaccination. From 1968, vaccination was carried out also among children from 3 to 12 years. For vaccination of these children we used special variant of live influenza vaccine, which was prepared by Alexandrova, Smorodintsev et all. (1965, 1967) from strains A2/Moscow 12/65/17 and B – 14/59/17.

In 1964–1967, we vaccinated 41–42% of Smolensk's population and 52–50% Jarcevo's annual. There were 49.8% of vaccinated population (41.2% children below 12 years and 51.5% of people over 12 years) in Smolensk and 46.9% of vaccinated population in Jarcevo (26.7% children below – 12 years and 52.9% population over 12 years) in 1968.

A daily record of the incidence of influenza and other ARD was Kept in Smolensk, Jarcevo and the control towns, starting from November 1964.

A special card index was also compiled for all vaccinated and unvaccinated subjects in 12–13 factories of Smolensk and Jarcevo to allow more accurate registration of influenza and other ARD cases, according to the medical certificates delivered. This survey covered about 30000 subjects.

A controlled trials was carried out in Smolensk in 1964 and in town Vjasma, Smolensk's region, in 1965–1968 annual. Shifts of workers under comparable conditions were selected at random and given divaccine, one of the monovaccines (A2 or B) or placebo.

Haemagglutination-inhibition tests were performed on paired sera from 30–50 subjects vaccinated with each of 4 and more series of vac-

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cine for estimating the immunogenity of the vaccines annual. Serums usually were sampled before vaccination and 30 days after the last vaccination. The tests were performed according to routine techniques, using standard antigens of influenza A2 and B, antigens of vaccine strains and strains circulating during the previous epidemics (A2/Gorkij 62/65, A2/Smolensk 22/67, A2/Hong Kong 1/68). The same test was used for diagnostic purposes.

— Nasopharyngeal washings from 20–30 persons vaccinated with each taken for study series were tested 3 times (on the second, third and fourth day after vaccination) for the take of the vaccine on the nasopharynx. The take rate was defined as the percentage of the persons tested in whom virus could be reisolated:

The temperature of 40–100 persons vaccinated with each serie of vaccine and with the placebo was recorded daily for 5 days in order to determine the reactogenicity, when vaccine of this composition was used for the first time.

Besides were compared the incidence of influenza and other ARD during each five — day periods before, and after vaccination for estimating influence of vaccine's reactogenicity on influenza and other ARD morbidity with temporary invalidity.

The clinical picture of influenza (not only clinical but laboratory diagnosed) were studied by doctors of infection clinic Erkina and Chamcow during 1967 and 1969 epidemics.

Statistical records on incidence influenza and other ARD and laboratory findings showed, that during this observation in selected towns there were three influenza A2 epidemics.

These findings indicated that the virus circulating during 1965 epidemic was a new variant of the influenza A2 virus (A2/England 12/64).

The same variant of A2 virus circulating during 1967 epidemic, but part of morbidity was caused by B influenza virus. '

The new variant of A2 virus — A2/Hong Kong 1/68 broke the 1969 epidemic out, which lasting from January to March 1969.

There were no influenza epidemics in 1966 and 1968, but there was some increase of influenza and other ARD morbidity from January to May 1966, and from February to April 1968 in all the control towns (Fig. 1 and 2). Thus, the period of observation was successful for study, because we could evaluate of the effectiveness of large — scale vaccination in condition when then antigenic structure of vaccines strains was the same or did not correspond on varied degree to the influenza virus strains circulating in nature.

The study of reactogenicity influenza vaccines for adults and special for children showed that the number of reactions with low fever (above 37.5^0C) depended on the serie of vaccine; it ranged from 6% to 5%. The number of reactions with fever over $37.5\ ^0$C after first vaccination ranged from 0.5% to 2.5% and generally was not exceeding the maximum permissible level — 2.0%.

Fig. 1
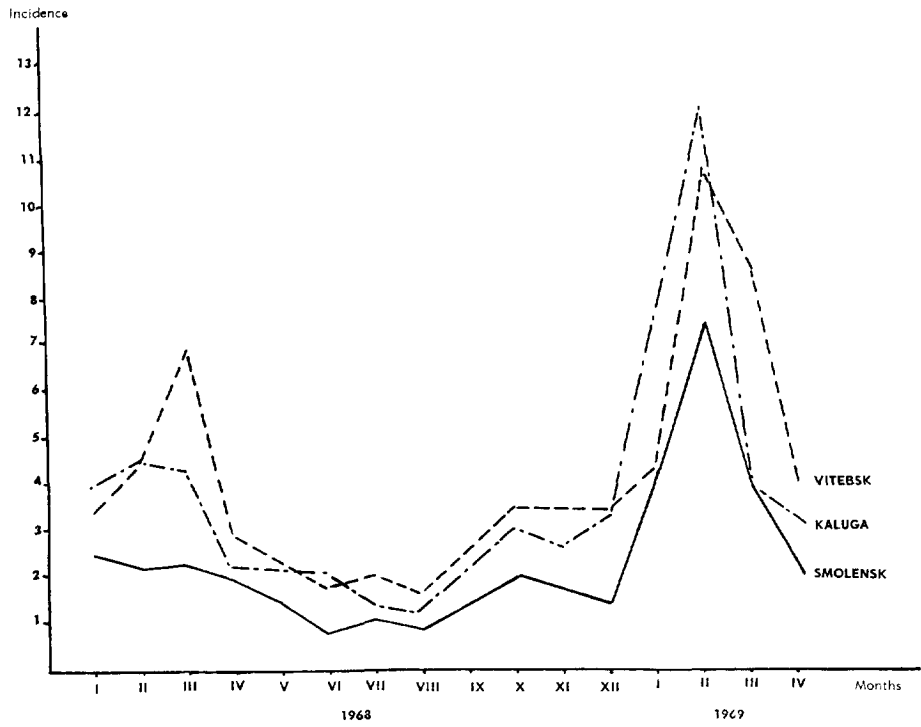
Incidence of influenza and other ARD in Smolensk, Kaluga and Vitebsk
per 100 population.

Fig. 2
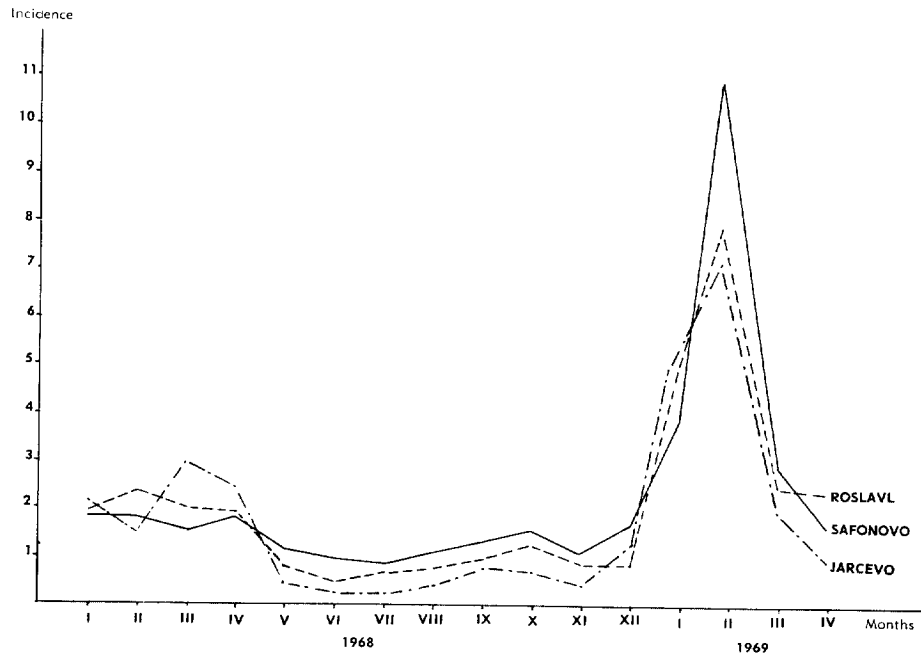
Incidence of influenza and other ARD in Jarcevo, Safonovo and Roslavl'
per 100 population.

The number of vaccinated persons confined to bed by influenza or other ARD did not differ during first five-day period after first vaccination in 1969 from the morbidity rate during five-day period before vaccination (table 1). Only during first five-day period after second vaccination, for which we used vaccine with new A2 vaccine strains, there was some little increase in morbidity rate – 0.2% which could be explain as consequence of vaccines reactogenicity. The take rates of B and A2 vaccines varied considerably with the series used and were between 40 and 90%.

Table 1

Incidence of influenza and other ARD among workers at the time of vaccination in 1968.

Five day intervals	Unvaccinated		Once vaccinated		Tvice vaccinated	
	Number of persons in group	Incidence (%)	Number of persons in group	Incidence (%)	Number of persons in group	Incidence (%)
Before first vaccination	4941	0.8	2924	0.5	19124	0.3
First after first vaccination	4941	0.5	2924	0.4	19124	0.3
Second after first vaccination	4941	0.5	2924	0.4	19124	0.3
Before second vaccination	4941	0.6	2924	0.1	19124	0.2
Fist after second vaccination	4941	0.3	2924	0.4	19124	0.4
Second after second vaccination	4941	0.1	2924	0	19124	0.2

4 – fold or greater rise in antibody titre in the blood of the vaccinated persons was observed in only 17–50% of them and in 25–65% of vaccinated persons with low initial titres before vaccination.

The two fold immunisation with vaccine from the strain A2/Moscow 21/65, which generally used in Jarcevo caused a 4 – fold or greater rise in antibody titre in the blood in 17% of vaccinated persons in the autumn 1968 (mean geometric rise of antibody 1.5 log 2).

This percentage was greater (26%), when vaccine from the strains A2/Moscow 21/65 and A2/Leningrad 133/65 was used for the second immunisation as it was in Smolensk. The mean geometric rise of antibody in the blood of tested vaccinated persons in this town was 2.0 log 2 or 4 – fold.

Thus, the take rate, and immunogenicity of the vaccine's series which were used for immunisation were generally good or satisfactory.

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The influence of vaccination on incidence of influenza and other ARD among workers of 12–13 factories in Smolensk and Jarcevo during the 1965, 1967, 1969 outbreaks and during winter of 1965–1966 was showed on the tabl. 2. There was 1.9–2.0 fold reduction in incidence of influenza and other ARD in vaccinated persons in 1966 and 1967, when antigenic structure of vaccine and circulating in nature strains was similar. The indexes of effectiveness of vaccines were lower in 1965 and 1969 epidemics, when antigenic structure of vaccine strains used in many respects did not correspond to influenza virus strains circulating in nature. Nevertheless, immunisation with live influenza vaccines had a favorable effect on morbidity rate in vaccinated persons (index of effectiveness 1.4–1.6).

Table 2

Effectiveness of vaccination with Live-Influenza vaccine (1964–1969).

Time, etiology, quantity of vaccination	Unvaccinated		Vaccinated		Index of effectivenes of vaccine	Mean error of reduction	Statistical significance of reduction p
	Number of persons in group	Incidence of influenza and other ARD (%)	Number of persons in group	Incidence of influenza and other ARD (%)			
1–3. 1965, outbreak A₂.	7989	20.0	7286	14.5	1.4	0.61	<0.001
twice three time	7989	20.0	12927	12.5	1.6	0.54	<0.001
2–4. 1966, interepidemic period, twice	6749	7.7	18656	4.0	1.9	0.31	<0.001
1–3. 1967, outbreak A₂B, twice	6716	27.4	23349	13.6	2.0	0.59	<0.001
1–3. 1969. outbreak A₂ twice	8872	24.8	21556	15.6	1.6	0.57	<0.001

The same reduction in the incidence of influenza and other ARD among vaccinated persons (1.4–1.6) was found in the limited controlled trials in 1964 and 1969 (table 3).

It must be mentioned that the higher index of effectiveness of monovaccine A2 (1.6) in comparison with divaccine A2B (1.4) may be explained as a result of interference between influenza strains A2 and B. The indexes of effectiveness of special live influenza vaccine for children were higher than indexes for adults in controlled trial which was carried out in Smolensk during the 1969 outbreak. They are: 2.2 for the monovaccine A2 and 1.7 for the divaccine A2B.

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Table 3

Results of controlled trials of Live influenza vaccines

Year outbreak, vaccine	Vaccinated	Number of persons in group	Incidence of influenza and other ARD (%)	Index of effectivenes of vaccine	t between incidence among vaccinated with vaccine A₂ or A₂B and placebo or B
1965, outbreak A₂/England 12/64, vaccine strain A₂ Krasnodar 101/59	A₂B vaccine	1196	8.7	1.6	3.3
	placebo	939	13.3	—	—
1969, outbreak A₂/Hong-Kong 1/68 vaccines for adults	A₂B vaccine	1216	13.4	1.4	3.4
	B vaccine	1284	18.5	—	—
	A₂ vaccine	1084	11.5	1,6	4.7
1969, vaccines for children	A₂B vaccine	1548	13.1	1.7	6.7
	placebo	1519	21.9	—	
	A₂ vaccine	1298	10.1	2.2	8.9

The results of live influenza vaccines effectiveness study in 1967 showed that revaccination at the next seasons increased the effectiveness of vaccine in 1964–1966, when the vaccine strains of influenza A2 (A2/Krasnodar 101/59; A2/England 12/64, A2/Moscow 24/65) were changed every year (table 4).

It should be taken into account that 2814 unvaccinated in 1966 persons were vaccinated in 1965 and 1964 or only in 1965; it would therefore be more correct to compare the morbidity rate among unvaccinated in either year (2738 persons) and varied groups of vaccinated persons is subdivided in four groups. It will be seen that 9348 persons vaccinated in all three years exhibited the highest (3.2 fold) reduction in morbidity rate 7428 persons vaccinated in last 1966 year and some of two other exhibited the 2.8 fold reduction, while 2.5 reduction was observed among persons vaccinated only in 1966.

Thus, it may be taken in account, that if the vaccination was carried out in the same collective any years repeatedly we must not only compare the incidence of morbidity among vaccinated and unvaccinated in last year persons but among persons unvaccinated during all years of observation.

The studies which were carried out during the 1969 outbreak, for the other hand showed, that there were the same incidence of influenza and other ARD among the unnvaccinated workers and workers twice vaccinated in 1966, 1967 and 1968 with vaccine from the same strain A2/Moscow 21/65 (table 5).

Table 4

Influence of vaccination in 1964 and 1965 on effectiveness of immunisation in 1966 (outbreak 1967).

| Vaccination status | Number of persons in group | % | Incidence of influenza and other ARD | | |
			Average duration of invalidity (days)	Index of effecti- veness	Statistical signifi- cance reduction in incidence between group 4,5 and group 3
Unvaccinated	2738	40.1	5.8		
Vaccinated in 1965 or 1964 and 1965	2814	16.6	5.4	2.4	
Vaccinated in 1966	6598	15.8	5.4	2.5	
Vaccinated in 1966 and 1965 or 1964	7428	14.1	5.2	2.8	0.01
Vaccinated in 1964, 1965, 1966	9348	12.7	5.4	3.2	<0.001

Table 5

Influence of vaccine composition on incidence of influenza and other ARD in 1969 outbreak.

| Composition of vaccines | Town, factory | Unvaccinated | | Twice vaccinated in 10–11. 1968 | | | |
		Number of persons in group	Inci- dence %	Number of persons in group	Inci- dence %	Index of effec- tive- ness	Statistical significance of reduction t
First vaccination A$_2$/Moscow 21/65, second vaccination A$_2$/Moscow 21/65 A$_2$/Leningrad 133/65	Smolensk	6579	26.3	17682	14.3	1.8	>10
	cotton stuff combine	1663	16.0	1545	10.7	1.5	5.2
Twice vaccine strain A$_2$/Moscow 21/65	Jarcevo	1225	23.2	3981	20.6	−1.1	1.7
	Jarcevo	3320	20.3	6382	18.3	1.1	1.9

Index of effectiveness of vaccination among the workers of the same cotton stuff combine in 1969 epidemic was 1.5, if the second vaccination in 1968 was carried out with vaccine from strain A2/Moscow 21/65 and new vaccine strain 42/Leningrad 133/65. The highest index of vaccination effectiveness (1.8) was observed among the vaccinated workers of Smolensk's factories, for the second immunization of whom the vaccine series from A2/Moscow 21/65 and A2/Leningrad 133/65 also were used.

This data led to conclusion that for effectiveness of live vaccine in conditions repited large – scale immunisation we must change the vaccine strains every two years.

The difference in effectiveness of vaccination in Smolensk and Jarcevo had the influence on morbidity rate among all population of Smolensk and Jarcevo in 1969.

The incidence of influenza and other ARD in Smolensk was about two fold lower than in control towns Vitebsk and Kaluga (Fig. I). More higher difference was in morbidity rate among adults: Smolensk 17.9%, Vitebsk 38.1%, Kaluga 41.2% (table 6).

Table 6

Incidence of influenza and ARD per 100 population from 3. 1968 to 3. 1969

Town	All population	Adults (over 14 years)	Children (bellow 14 years)
Smolensk	28.8	17.9	70.9
Vitebsk	54.3	38.1	107.3
Kaluga	48.7	41.2	76.4

The incidence of morbidity in Jarcevo during 1969 epidemic was not lower than in Safonovo and Roslavl, where vaccination was not carried out (Fig. 2).

It must be mentioned, however, that for wide – spread infection as influenza the index reduction of morbidity – 1,8 is economical expedient. The curves of last three epidemics in Smolensk 2-fold lower than before in 1962. So, in Smolensk during the week on the top of 1962 epidemic were ill 6,3% of population, in 1965 – 3,7%, in 1967 – 3,5% and in 1969 – 3% (Fig. 3). The incidence of morbidity rate among 17682 vaccinated persons was an 1.8 fold lower than among unvaccinated. From 2236 suffered by influenza or other ARD it may be think, that 2021 vaccinated were prevent from illness and have no invalidity during 12,126 days, or 53,032 days from all twice vaccinated 77,357 workers of Smolensk.

Fig. 3
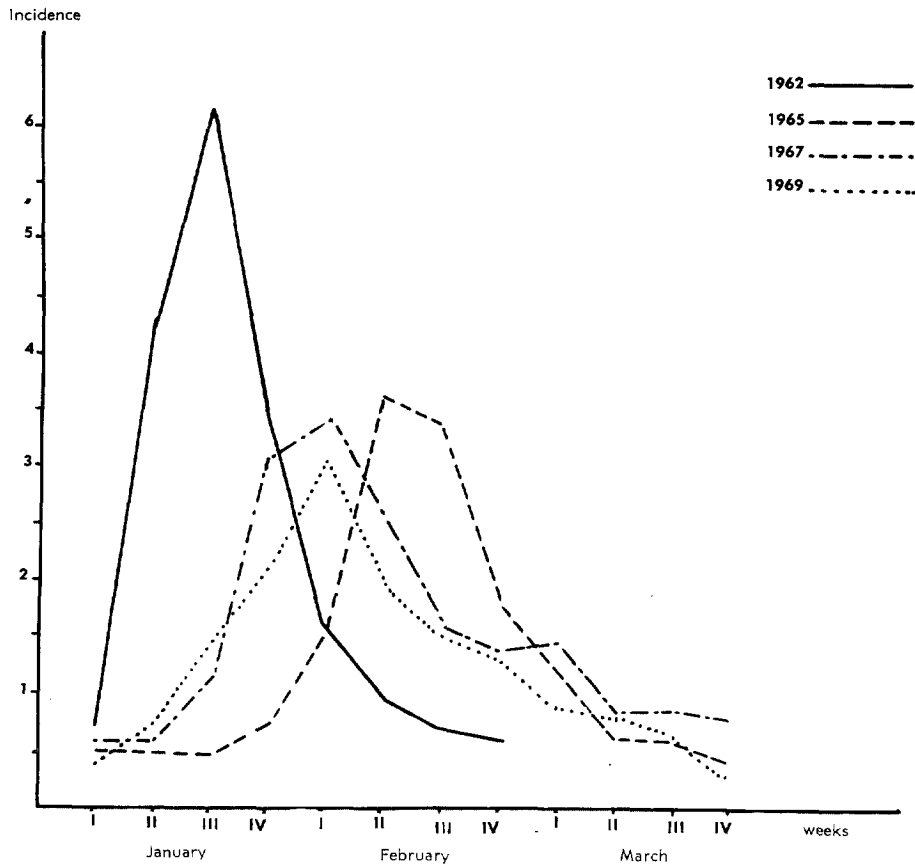
Incidence of influenza and other ARD in Smolensk during influenza outbreaks in 1962, 1965, 1967 and 1969 (per 100 population).

For evaluation of the effectiveness of large — scale immunisation during all five-year period of observation the morbidity rate in all six towns during the period of observation (1965 – March 1969): were compared with morbidity rate among all population in same by the time and epidemic situation period (1959, 1962, 1963, 1964 years and I–III 1962).

Incidence (Table 7) was somewhat lower in all towns except Kaluga at the last four and quote years.

However the decrease in incidence was higher among adults (over 14 years) in Smolensk. They were vaccinated all five year annually.

The decrease was in Smolensk on 20 percentage higher, i. e. three times as much as in Vitebsk and Kaluga.

Table 7

Incidence of influenza and other ARD per 100 population

Town	During 1965, 1966, 1967, 1968 and 1-3. 1969			During 1959, 1962, 1963, 1964 and 1-3. 1962			Difference in incidence in these two periods (%)		
Time, group of population	total	among adults (over 14 years)	among children (below 14 years)	total	among adults	among children	total	among adults	among children
Smolensk	118.4	66.8	305.7	127.7	90.6	237.7	< 7.3	<30.8	>28.7
Vitebsk	185.1	126.7	389.3	188.0	142.0	290.6	< 1.5	<10.8	>33.5
Kaluga	182.1	142.0	322.0	181.7	157.4	206.5	> 0.2	< 9.7	>55.9
Jarcevo	105.9			140.2			<24.5		
Safonovo	82.7			94.7			<12.7		
Roslavl	95.2			115.6			<17.6		

The morbidity rate among children increased in all towns and this increase is somewhat lower in Smolensk.

The difference in increase of morbidity rate on 20 percentage corresponding at 18.1% of all adults population of Smolensk or 32 000 persons. If it takes into account that all of them could be ill during about 5 days, so the large scale vaccination helped to prevent for invalidity during 160000 working days. That is three times as much as during the last influenza epidemic only.

Vaccination also had good influence on health of vaccinated persons. So, the clinical picture of influenza were more light in vaccinated persons than in unvaccinated. The average duration of fever was on 2.2 days and the average duration of invalidity on 3 days shorter among vaccinated person than unvaccinated, who were ill during 1967 epidemic.

The incidence of complication after influenza had place among vaccinated persons three time lower than among unvaccinated persons.

This difference in clinical picture ot influenza was also at the last 1969 epidemic (tabl. 8) but it was lower probably because of difference in antigenic structure of vaccines and circulating in nature strains of A2 influenza. We observed the decrease of average duration of fever on 1.8 days, average duration of invalidity on 2.4 days, and of 1.7-fold reduction in incidence of complications.

Thus, this five-year investigation has shown that large-scale vaccination with live influenza vaccine is an effective means of influenza control even under the conditions of outbreaks provoked by a new variants of the causative agent, the antigenic structure of which differs essentially from that of the vaccine strains.

Table 8

Clinical picture of influenza during 1969 outbreak

Vaccination status	Number of persons in group	Average durations of fever (days)	Average durations of invalidity (days)	Incidence of complications %	Statistical significance reduction in incidence of complications t
Vaccinated	157	4.8	8.4	32.4	
Unvaccined	222	6.6	10.8	53.6	

The influence of revaccination in the previous seasons on the effectiveness of vaccine was showed in condition of yearly change of vaccine strains Side by side of this uneffectiveness of vaccination in Jarcevo during 1969 epidemic, could be explained not only that there was a market difference in antigenic structure of vaccine and circulating in nature strains of influenza, but that here we carried out yearly twice immunisation with vaccine from the same strain A2/Moscow 21/65 during three years. It was obvious that after two years of immunisation most of vaccinated persons were so immune to this vaccine strain, that last could not live in nasopharynx of them and stimulate any new immunity.

It must be mentioned that this last conclusion showed the necessity of periodical changes of vaccine strains once from 2–3 years, which is connected not only with variation of antigenic structure of influenza virus in nature but also with impossibility to stimulate immunity by yearly large-scale vaccination of the same population with vaccine from the same vaccine strain during some years.

SUMMARY

Vaccination is at present the most important means of influenza control. This paper discusses such a trial which had place in Smolensk's region of the USSR during 1. 1964 – 3. 1969.

About 42–50% of the population of Smolensk and about 47–52% of the population of Jarcevo were vaccinated twice annually with live influenza vaccine in the autumns of 1964–1968. The incidence of influenza and other acute respiratory diseases (ARD) in these towns during 1. 1965 – 3. 1969 period and in the same by the time and epidemic situation period before observation (1959, 1962 – 1964 and 1–3. 1962) was compared with that in four nearby »control« towns. Statistical records on incidence influenza and other ARD and laboratory findings showed that during this observation in selected towns there were three A2 influenza epidemics. The new variants of A2 virus A2/England 12/64 and

A2/ Hong Kong 1/68 broke the 1965 and 1969 epidemic out accordingly. So far the antigenic structure of vaccine and circulating in nature strains was similar only in 1967 epidemic.

The five-year investigation showed that large-scale vaccination with live influenza vaccine is an effective and economical means of influenza control even under the conditions of outbreaks provoked by a new variant of the causative agent, the antigenic structure of which differs essentially from that of the vaccine strains.

Analisis of the incidence data for the towns involved of more detailed incidence data for about 30 000 workers and the annual controlled trials involving about 4000 persons indicated that the vaccination led to a reduction in incidence of about 1,5 – to 1,8 in 1965 and epidemics, when antigenic structure of vaccine strains did not correspond to strains circulating in nature; and of about 2.0–2.5 in 1966 and 1967 epidemics when antigenic structure of vaccine strains did not correspond to strains circulating in nature; and of about 2,0–2,5 in 1966 and 1967 when antigenic structure of vaccine and circulating in nature strains was the same.

The influence of revaccination in the previous seasons on the effectiveness of vaccine was showed in condition of yearly change of vaccine strains.

The observation showed the necessity of periodical changes of vaccine strains once from 2–3 years, which is connected not only with variation of antigenic structure of influenza virus in nature but also with impossibility to stimulate immunity by yearly large-scale vaccination of the same population with vaccine from the same vaccine strain during some years.

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A STUDY ON THE EPIDEMIOLOGICAL EFFICACY OF LIVE ANTI-INFLUENZA VACCINE AND SERUM

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The appearance of A_2/Hong Kong/68 variant imposed a rapid production and a comprehensive study on new, both inactivated and live influenza vaccines.

It was in 1956 that work on live vaccines started in Bulgaria. The vaccines were examined for efficacy during the A_2 Influenza epidemics in the years of 1959, 1962 and during the 1966–1967 period when the coefficient of epidemiological efficiency was found to be in the range from 1.3 to 7.9 (4, 6, 7, 9). These results with live and killed vaccines found support in the communications of other workers (3, 11, 13, 15, 16, 17), but they are considered still as unsatisfactory and work along the line of their improvement is in progress.

New data were obtained during the Influenza epidemic which started in the beginning of the month of February 1969, had a protracted course, without the characteristic high peak and a low morbidity rate (5.6%). The laboratory investigations evinced the etiological role of the new A_2 Hong Kong/68 variant. Only in rare isolated outbreaks could the circulation of the earlier A_2/64 variant be established.

The present investigations include observations on the epidemiologic efficacy of the vaccine of the earlier A2 variant, after a threefold application in the preepidemic period as a A_2B bivaccine; of the vaccines of the earlier and the new A_2 variant, after single application as monovaccine during the epidemic which had begun, and of antinfluenza serum applied during the epidemical period.

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The monovaccine of the older A_2 variant was produced from the 21st passage on chick-embryos of the A2/Sofia/472/65 strain, while the type B monovaccine was obtained from the 23rd passage of the B/Sofia/173/63 strain. Both preparations were lyophilized, the titre of the active virus being 6.0–8.0 lg 10. In the preparation of the vaccinal strain from the new A_2/Hong Kong/68 variant, we employed the A_2/AICHI/2/68 and A_2/England/344/68 standards. Studied after different passages on volunteers they manifested a high reactogenicity. This delayed the production and the application of this new vaccine. It was obtained from the 19th passage of A_2/AICHI/2/68 strain and applied immediately without lyophilization with a biological titre of 8.0. log 10.

The antinfluenza serum was bivalent (A_2B), liquid and produced in horses with a titre of the antihaemagglutinins of 1 : 1600 to 1 : 3200 to both types. The vaccinal strains A_2/Sofia/472/65 and B/Sofia/173/63 were used as antigens.

All the vaccines were applied intranasaly by means of a dose-pulverizor, in a dose of 0.5 ml containing by 0.1 ml infective dose from the respective vaccine type. The three-fold immunization in the preepidemic period started on the 11 September 1968 with an interval between the first and the second injection of 14 days, and between the second and the third injection of 40 days. The antiinfluenza serum was applied prophilactically during the entire epidemic, in establishments where the morbidity rate was high, the daily dose being 2–3 ml once weekly, intranasally.

The observation of the vaccines was carried out in 15 districts of the country on a total of 303,733 workers and employees in different establishments of which 131,408 were immunized and 172,325 Controls (internal control in the same establishments and external control in other related establishments). The observations of the serum wa carried out in 12 districts of the country on 37,226 persons, of which 17,697 were treated with the serum and 19,529 served as controls. The morbidity rate and the duration of the illness was recorded according to clinical data and the hospital cards for temporary inability for work. The epidemiological efficacy of the vaccines was calculated by comparing the morbidity rate between the immunized and serum treated patients with that of the control groups. The data obtained have been statistically worked out for the reliability of the differences.

RESULTS AND DISCUSSION

The data pertinent to the epidemiological efficacy of the vaccines and serum presented on Table 1.

It is seen from the Table, that the morbidity rate among the persons having received a threefold immunization in the preepidemic period

Table 1

Epidemiologic Efficacy of the Vaccines and Serum Studied

Groups	Preparation Kind and number of applications	Immunized			Controls			Efficacy	
		Number	Taken ill		Number	Taken ill		Coefficient	%
			Number	%		Number	%		
I	A_2 – old variant threefold.	11774	1761	14,9	9742	1866	19,1	1,3	22,0
II	A_2 – old variant Single	16558	1103	6,6	68205	6411	9,4	1,4	29,8
III	A_2 – new variant Single	103076	9612	9,3	94378	18701	19,8	2,1	53,0
IV	S e r u m Manyfold	17697	3399	19,2	19529	6388	32,7	1,7	41,3

with the vaccine of the old variant A_2, as a A_2B bivaccine amounts to 14.9%, while in the control group it is 19.1%, thus giving an efficiency coefficient of 1.3. In the case with the single immunization with the same vaccine, applied during the epidemic the efficiency coefficient is not significantly different, namely 1.4. In this case the percentage of the persons both among the immunized and the non-immunized, who had fallen ill was the lowest in comparison with the other observed groups — 6.6% and 9.4%. In the single immunization with vaccine of the new variant A_2, applied also at the beginning of the epidemic, the number of the ill among the immunized was 9.3%, while that among the Control group was 19.8%. The efficacy in this case is 53% ($K = 2.1$). The highest morbidity rate for influenza and acute respiratory diseases was observed in the fourth group where antiinfluenza horse serum was applied, it was 19.2% among the serum treated and 32.7% for the controls. The epidemiological efficiency coefficient amounts to 1.7. On the statistical elaboration of the results it was established that in all groups the differences in the percentages of the ill among the immunized and the controls were significant – $P_t > 0.999$.

The highest efficacy was established with the vaccine of the new A_2 variant, despite the fact that it was applied unrepeated at the beginning of the epidemic on a large but dispersed contingent. The efficiency coefficient of this vaccine is shown on Table 2, by regions.

It is seen from the Table that it varies from 1.1 to 3.8 the morbidity rate among the immunized being in the range from 2.4% to 23.0% and from 7.7% to 47.3% for the control groups. In two regions – the first and the fourth- the vaccine has not given any effect ($KK = 0.5$ and 0.7). Traced in separate establishments the efficiency coefficient has shown a certain tendency for a direct proportional dependence on the density of the immunization carried out at the given establishment (Table 3). This is in accord with earlier data of ours as well as with the data of other workers (7,13, 14, 16).

With the view of recording the effect of the interferon with the same vaccine, we traced the morbidity up to the tenth, and after tenth day following the immunization. The observation was carried out on 48920 immunized and on 55594 controls. Up to the tenth day the number of the ill among the immunized was 1959 or 4%, whilst after the tenth day it was 1151 or 2.4%. The number of the ill in the Control group was 6328 or 11.4% and 3199 or 5.8% respectivelly. The efficiency coefficient up to the tenth day is 2.8, and after the tenth day – 2.4. These data give reason to accept the efficacy of the vaccine up to the 10-th day a result of the induced interferon, while after the 10th day as a result of the specific immunity that has been produced (18). It is very probable that during the first few days after the immunization the antibodies produced earlier play a certain protective role (10). All this justifies the application of the vaccine even when an epidemic has begun.

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Table 2

Epidemiological Efficacy of the Vaccine of the New Variant $A_2/68$.

Regions	Immunized			Controls			Efficiency coefficient
	Number	Taken ill Number	%	Number	Taken ill Number	%	
I	2146	429	20.0	3395	355	10.0	0.5
II	21847	1447	6.7	21149	1626	7.7	1.1
III	14744	3405	23.0	15167	7173	47.3	2.1
IV	3796	690	18.2	2063	257	12.4	0.7
V	13162	720	5.5	29023	5084	17.5	3.2
VI	997	24	2.4	830	76	9.2	3.8
VII	44437	2719	6.0	16512	2895	17.5	2.9
VIII	1910	178	9.3	6239	1235	19.8	2.1
Total:	103069	9612	9.3	94878	18701	19.8	2.1

Table 3

Dependency between the Compactness of the Immunization and Epidemiologic Efficacy of the $A_2/68$ Vaccine

No.	Establishment	Immunized				Controls			Efficiency coefficient
		Number	%	Taken ill Number	%	Number	Taken ill Number	%	
1	OS	997	68.1	24	2.4	466	39	8.4	3.5
2	KK	371	63.9	17	4.6	209	19	9.1	2.0
3	VP	198	54.2	106	53.5	167	128	76.6	1.4
4	DV	982	35.5	38	3.9	1780	170	7.8	2.0
5	GV	1318	34.1	64	4.8	2546	193	7.6	1.6
6	TA	429	12.3	110	25.9	3025	295	9.7	0.3

The efficacy of the vaccine of the new variant was recorded and evaluated in terms of the duration of the illness and the complications that had arised. The average duration of the disease course was 3.7 days for the immunized and 4.2 days for the control group. The complications with the ill from the immunized group amounted to 1.7%, while for the unimmunized it was 2.8%.

The lower epidemiologic efficiency of the vaccine and the serum of the earlier variant A_2 may be explained with the incomplete antigenic conformity between the vaccinal and the epidemical strain upon establishing that the old variant A_2/64 was still partly in circulation.

The prophylactic effect of the hyperimmune antiinfluenza horse serum has been pointed both by authors of our country as well as from foreign countries (1, 2, 5, 8, 12). The efficiency coefficient, obtained after its application, as it is seen in our study, may be considered as satisfactory, varrying from 1.1 to 3.7, the morbidity rate being in the range of 3.1 to 44.2% among the serum treated and from 6.4 to 77.8% among the controls.

CONCLUSION

In testing the vaccines and sera of the old and new variants of the influenza A_2 virus, during an epidemic caused by the new A_2/Hong Kong/68 variant we were able to establish a higher efficacy of the vaccine obtained from the new variant. After a single application on a large, yet not fully covered contingents, dispersed throughout the country during an epidemic with protrated course and relatively low intensity, the vaccine has manifested good results. Its efficacy during the first days after its application may be explained with the interferon induction.

The immunization with the vaccine of the new A_2 variant has given a decrease in the morbidity rate, as well as a shorter duration of the disease and a lower frequency of complications.

The efficacy of the vaccine produced from the old A_2 variants is low and the same, regardless of the number and the time of application.

The effect of the vaccine and the serum of the earlier variant may be explained with the circulation of the old variant, as well as with the antigenic relationship between the A_2/64 and A_2/Hong Kong/68 variants.

Immunization with live anti-influenza vaccine, even when an epidemic has begun, is deemed as correct and expedient procedure.

SUMMARY

The two anti-influenza vaccines and serum, studied during an A_2/Hong Kong/68 epidemic in 1969, have given the following efficiency coefficients: A_2 vaccine produced from the A//64 variant, after a three-fold application as a bivaccine in the pre-epidemic season was 1.3; the

same vaccine after a single application as a monovaccine, after the epidemic had started, amounted to 1.4; A_2 vaccine produced from the new A_2/Hong Kong/68 variant, applied once at the beginning of the epidemic, was 2.1 and the bivalent A2B serum obtained from the earlier A_2 variant gave 1.7.

The efficacy of the vaccine from the new A_2 variant was recorded in terms of the duration of the disease and the complications which followed. The average duration of the illness with the immunized amounted to 3.7 days while with the controls it was 4.2 days. The complications in the case with the immunized ill was 1.7% and with the nonimmunized 2.8%.

The effect of the interferon with the same vaccine has been recorded up to the tenth day and after the tenth day by tracing the morbidity rate in the case with the immunized and nonimmunized patients. The data obtained give reasons to accept that the effect of the vaccine up to the tenth day is a result of the induced interferon, while after the tenth day it can be accounted only as the result of the specific immunity produced. This justifies the application of the vaccine even when an epidemic has begun.

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LIVE INFLUENZA VACCINE AND PROSPECTS OF ITS IMPROVEMENT

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At the present time active immunization with a live or killed vaccine is acknowledged to be the most prospective method of mass prophylaxis against influenza for both adults and children. Vaccination has a basic advantage over other methods of combatting influenza in that it makes it possible to increase the specific protection of the inoculated population in advance, during the quiet months of the epidemic period. All other existing methods of prophylaxis (such as chemo- and serophoplylaxis and also interferon induction), can be initiated only in the period when an epidemic has already begun. Any mass prophylactic measure during this time is complicated by the urgent deadline of the epidemic itself and by the difficult working conditions which result.

LIVE INFLUENZA VACCINE

During the past 20 years in U. S. S. R., Yugoslavia and Bulgaria, active immunization against influenza has been performed by direct administration into the upper respiratory tract of a live attenuated vaccine, a method first proposed by the author in 1937. The present allantoic vaccine includes strains of groups A_2 and B selected from antigenic variants now prevalent.

The essential advantage of a live vaccine is in its ability to produce asymptomatic immunizing infection of the respiratory tract, approximating the subclinical form of influenza often encountered during the epidemic period among naturally infected adults and children. Just as in natural subclinical infection, asymptomatic vaccine infection stimulates not only general, but also local humoral immunity, because of the intensive appearance of antibodies in secretions of the respiratory passages.

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The immunogenic activity of live vaccine, following 3-fold administration of large doses of the preparation, is characterised by the appearance of virus-neutralizing antibodies and of antihemagglutinins in the blood of 50 to 70 per cent of susceptible people, with an average of 8-fold increase in antibodies among adults and of 10 to 13-fold among children (Table 1). Although the level of antibodies in the blood may be noticeable lower in people receiving live vaccine than in those inoculated with the present killed vaccine with adjuvant, live vaccine is followed by more regular and intensive appearance of influenza antibodies in the nasal passages.

Another important method for laboratory evaluation of vaccine strains or of live vaccine is a trial of the resistance of previously vaccinated subjects to repeated administration of homogolous or heterologous strains of the same serological subtype into the frequency and severity of clinical reactions after administration of a sufficiently virulent strain or

Table 1

Relative Frequency and Intensity of Increase in Antibodies in the Blood of Adults and Children 3–6 Years after they had A_2 or B Influenza or 2-to-3-Dose Vaccination with Live Monovalent A_2 and B Vaccine or A_2 + B Divaccine

Antibodies against viruses	Composition of the group	Mean immunologic values for susceptible persons without antibodies in their blood in a 1:20 dilution before illness or vaccination			
		2 with antibody increase		Mean Factor	
		Adults	Children	Adults	Children
Strains A_2	Persons who had had influenza	82.0	84.5	18.8	20.9
	Persons who had been vaccinated with the monovaccine	61.7	91.6	6.4	18.8
	Persons who had been vaccinated with the divaccine	77.0	85.7	8.4	13.7
Strains B	Persons who had had influenza	84.0	70.7	20.6	19.3
	Persons who had been vaccinated with the monovaccine	52.4	89.1	5.4	19.6
	Persons who had been vaccinated with the divaccine	79.0	82.4	8.7	14.6

the index of reproduction of the challenge virus, determined by the frequency of virus isolation from nasopharyngeal secretions 2 to 4 days post immunization.

Most important in laboratory investigations of live influenza vaccine and its improvement are the problems of selection and periodic replacement of vaccine strains, the technology of production and methods of its use.

1. Selection of vaccine strains

Because of the inevitable natural variation of influenza virus in antigenic and biologic properties, periodically, at intervals of at least 4 to 6 years, completely new influenza vaccines must be developed and old, nonspecific vaccine strains consigned to the archives. The task of preparing a new vaccine is completely unequal in difficulty for killed and live vaccines. In the first case one virus must simply replace another, almost mechanically. In the second case it is necessary to prepare entirely new vaccine strains, harmless but highly immunogenic for man.

In essence, what the adherents of live influenza vaccine must do, every 4 to 6 years, is to develop entirely new preparations for both serological types. Our laboratory performed this task for serotype A between 1937 and 1969 a total of 8 times, and for serotype B 6 times. Added to that when development of the basic adult vaccine is completed, further attenuated strains must immediately be prepared for the children which actually doubles our work. In U. S. S. R. specialized laboratories in the All-Union Research Institute of Influenza, the Ivanovsky Institute of Virology and the Moscow Institute of Viral Preparations are occupied with completing a labor of such magnitude.

Incorporated in the live vaccine must be specific strains of types A and B which were prevalent in U. S. S. R. and other countries during the previous influenza outbreak. There is no reason to introduce into the vaccine any new variants of epidemic strains if they do not differ from the current vaccine strains by 1 : 8 or more in cross inhibition titrations with homologous sera.

The appearance of new-variants of influenza virus in several countries is a signal to begin their rapid conversion into vaccine strains. This was especially well demonstrated in 1947, when A_1 virus appeared, in 1957 for A_2 virus and in 1968 for virus A_2/Hong Kong. Under such circumstances it is especially important to obtain rapid information and the appropriate viruses themselves from the central laboratories of the World Health Organization so that the long and complicated conversion into vaccine strains can being at once.

For the production of live vaccine in U. S. S. R. no more than two strains of each type are used.

In the present vaccine type A is produced from strain A_2/Hong Kong and the older A_2 strain of 1967.

The universal method for attenuation of laboratory strains is by passage through developing chick embryos at temperatures of 32 to 34 °C. The number of passages necessary for attenuation cannot be predicted: sometimes 6 od 8 passages have sufficed to eliminate virulence, or sometimes, as we found for strains of A₂/Hong Kong virus, about 30. Consequently, there are serious differences in the periods of time required for attenuation which have varied for different strains from 3 to 6 months.

The development of harmless and immunogenic vaccine strains cannot proceed without the wide use of volunteers, because there is no laboratory method which replaces direct trial on human subjects. Any laboratory method must be preliminary, demanding obligatory verification for man, as has already been well demonstrated in the development of live vaccines against measles, mumps and rubella. Even during the velompent of live vaccines against poliomyelitis monkeys served only as an approximate test for substantiating avirulence, and were completely worthless for characterizing the immunogenic activity of live polioviruses for humans.

Studies in our laboratory have found organ cultures of human embryo trachea not to be suitable for the differentiation of freshly recovered strains of Hong Kong influenza virus known to be highly virulent for man from harmless vaccine strains. At the same time, however, direct passages through the upper respiratory tract of volunteers proved useful in a number of cases for the enhancement of the virulence of laboratory strains for man, and of associated reproductive capacity and immunogenic activity in human volunteers (Fig. 1).

Fig. 1

Immunogenic activity of influenza strains before and after its adaptation during the 4 - 6 passages to the upper respiratory tract of susseptible volunteers (no antibody in dilution 1 : 20)

Strains: PR-8   A-9   A₁-183   A₁-650   A₂-803   A₂-140   A₂-JKsha   B₁-4945   Bm 8

Legend: 2.0 - 4.2 - mean index of antibody rise; I - before; II - after adaptation.
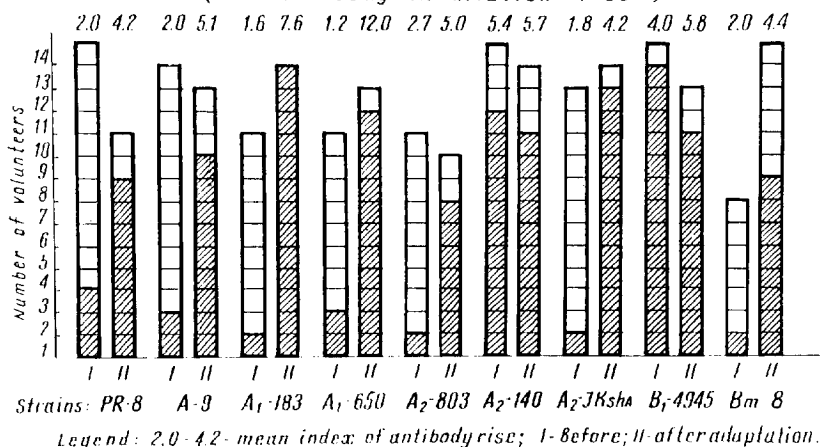
More promising methods of preliminary laboratory evaluation of virulence of vaccine strains for man may be tests of sensitivity of strains to thermolabile beta inhibitors of human serum or of changes produced in the phagocytic activities of human macrophages exposed to strains of different virulence.

At the present time our conclusive tests of the avirulence of vaccine strains is vaccination of susceptible people, among whom no more than 2% of febrile reactions are permissible, this also serves as a test of the immunogenic activity of the strains which is possible only by direct observation of antibody content in blood and in secretions of the nasal cavity in human subjects.

Current practice in U. S. S. R. provided for coded trials of candidate vaccine strains on groups of 40 seronegative adult volunteers for each strain, with clinical follow-up of temperatures and catarrhal symptoms developing during the next 7 days, investigation of the ability of the strains to multiply in and to be isolated from secretions of the nasopharynx in 2 to 4 days, and verification of antibody rise in the blood (which must be found in no less than 50 per cent of the subjects vaccinated after 3-fold inoculation with intervals of 2 weeks).

Because vaccine strains harmless for adults and adolescents (13 years old or more) often elicit clinical reactions among children, the severity of reaction increasing progressively with decreasing age, it is imperative that children be inoculated with further attenuated vaccine strains. To obtain these strains the standard strains approved for the basic vaccine for adults receive additional passages through developing chick embryos. Twenty to 30 or more passages are performed at temperatures reduced to 26 to 28 °C, until the viruses obtained have lost their reactogenic properties for children one to 6 years of age and older (Table 2, Fig. 2 and 3).

Table 2

Reactogenic and Immunogenic Activity of A_2 Vaccine Strains During the Prolonged Passages by 25–26°

Number of passages by 25–26°	Clinical reaction rate	Immunogenic activity
0	+	+
11	+	+
17	—	+
21	—	—
27	—	—

Fig. 2

Febrile reactions in children after live influenza vaccine or placebo (normal allantoic fluid)

1, 2, 3 - time in days after immunization or placebo .
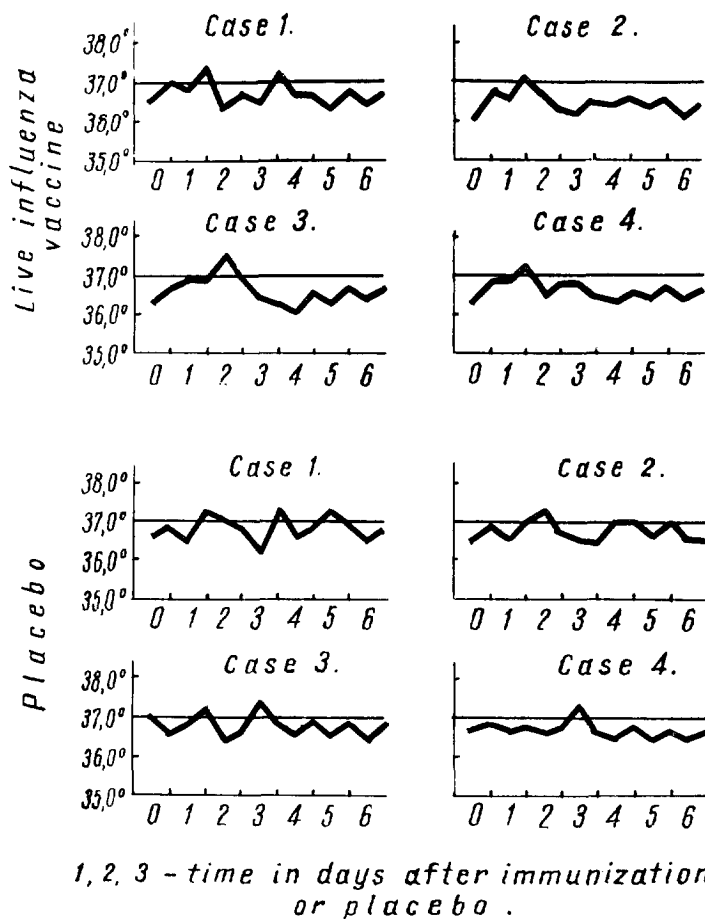
The immunogenic activity of the special variant in children vaccinated is regularly higher than that of standard vaccine in adults (Fig. 4).

Because of existing rigid restrictions of permissible reactogenicity of live influenza vaccines, associated with their respiratory route of administration, maximally attenuated vaccine strains must be used. Repro-

Fig. 3

Number of children with t°rise 1-5 days after administration of live influenza divaccine $A_2 B_2$ or placebo.
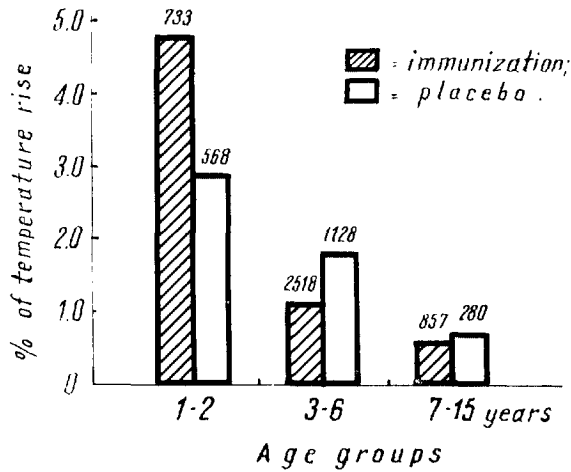
Fig. 4

Hemagglutination-inhibiting antibody response to live influenza vaccine of A_2 and B_2 in children of preschool age with low (▢), middle (▨) and high (▮) titer of antibodies prior to the immunization
(each column includes 45-100 children with paired sera)
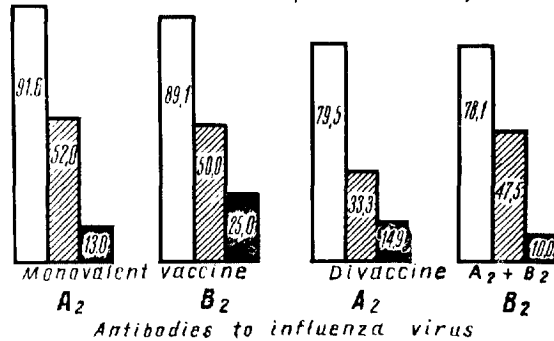
91,6 ; 52,0 - % of paired serum tested with fourfold or greated rise of H.J. titer in postvaccination serum .

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duction of these strains in the nasopharynx among highly susceptible people can take place only after administration of relatively massive doses of virus, consisting of 100,000 to 500,000 EID_{50} for both types A and B.

Because of the low effectiveness of a single intranasal immunization, 3-fold administration of the live divaccine is recommended. Under practical conditions of mass immunization roughly 70% of participants complete the full 3-fold vaccination, and 30% receive two immunizations. According to data of A. N. Slepushkin the results of 3-fold immunization with intervals of 2 weeks differ very little from those of 2-fold immunization with an interval of one month.

For production of live influenza vaccine ordinary fertile eggs may be used in place of the significantly scarcer and more expensive leukosis-free eggs. Ten years' observation in Leningrad of a group of some 40,000 children who had been inoculated subcutaneously with live mumps vaccine prepared in eggs showed the same number of cases of leukoses as in an equivalent control group not receiving injections of the egg vaccine.

EFFECTIVENESS OF LIVE INFLUENZA VACCINE BY EPIDEMIOLOGICAL OBSERVATIONS

The epidemiological effectiveness of live influenza vaccine has been studied for the last 15 years by observations of homogeneous and comparable contingents of the population in U. S. S. R. during influenza A and B outbreaks. Among these were also coded observations organized as »blind« studies.

Part of the published material is difficult to interpret because of low morbidity in control groups, a situation in which the percentage of extraneous illnesses ordinarily exceeds significantly that of influenza. Some of the experiments were initiated in periods when and epidemics was already starting, when the effects of active immunization are concealed by natural clinical and subclinical infections and confused by a second protective mechanism, the induction of interferon. Some of the experiments were conducted as total immunization of fixed collectives (factories and small cities) comparing their morbidity with that of other collectives where immunization was not carried out; these studies are open to objection because of the well-known variability of influenza in striking separate groups of people. When live vaccines of specific antigenic composition and potency are used properly, a significant degree of protection is assured. But in the face of epidemics and pandemic provoked by entirely new antigenic variants live influenza vaccines usually failed to produce protective effect.

The statistical evidence of the effectiveness of influenza vaccines is definitely limited, because of common errors in the clinical diagnosis of influenza in the course of an epidemic.

Even during the most intensive outbreaks, at least 15 to 25% of clinically diagnosed cases of influenza have diseases other than influenza by laboratory criteria. For this reason we cannot expect unquestionable statistical evidence of intensive protection by influenza vaccines on the basis of clinically diagnosed cases not confirmed by the laboratory, even when the vaccines were highly effective.

Epidemiological field trials with influenza vaccines must be based on strictly controlled double blind data and also include serological confirmation of the majority of clinically diagnosed cases. Only laboratory confirmation allows conclusive analysis of influenza vaccine efficiency from reliable data concerning the real number of specific influenza cases in vaccinated and placebo groups.

The effectiveness of immunization against influenza by use of live vaccine also depends considerably on the percentage of vaccines in the observed group of a population. There is a constant and distinct rise in effectiveness proportional to the extent of immunization as it was shown very distinctly here in Yugoslavia.

The standard live influenza vaccine used for the protection of adults from 16 years upwards, cannot be applied to children, in view of the progressive increase in the clinical reaction rate of this vaccine with decreasing age.

However, where vaccinations were performed at favourable times, with the lost of preparations specific for the epidemics which followed, with properly selected groups of internal controls and sufficiently high morbidity in the control groups, decreased morbidity was regularly observed among these vaccinated with indices of effectiveness of 1.5 to 2 or 3 as it was shown.

In the Smolensk district during the 3 to 5 year trials of a live vaccine, organized by the Ivanovsky Institute of Virology as well as in Yugoslavia and Bulgaria.

Definite effectiveness was also observed in studies of a children's variant of live influenza vaccine in 1962, 1965 and 1967 in Leningrad (Table 3, Fig. 5, 6, 7 and 8).

Effectiveness of the live vaccine has become more clearly apparent when vaccination has included the predominant part of an experimental group, because of a diminished reservoir of virus among the nonvaccinated (Fig. 7). Considering the objective difficulties in preventing influenza by vaccination which has a limited and inconstant level of effectiveness, it is advisable to consider any system of influenza prophylaxis as complex in character. The struggle with influenza should be based on the intensive use of vaccination, supplementing its effects with other effective prophylactic measures, such as chemoprophylaxis, interferon induction and seroprophylaxis. In this way the effects of active immunization and supplementary prophylaxis will yield results inaccessible to each method alone.

Table 3

Results of Strictly Controlled Trial of the Effectiveness of Live Influenza Vaccine in 1964 through 1967 in Smolensk (A. N. Slepushkin)

Year of epidemic	Causative agent	No. of immuniz- ations	Number of influeza cases and similar infections						Reliability of results		
			Unvaccinated		Vaccinated		Coeff. of effect- iveness	Index of protect- ion	Differ- ence in % of taken ill	Mean error of differ- ences	P
			Number	% of taken ill	Number	% of taken ill					
1965 (January– March)	A_2	2	7989	20.0	7286	14.5	27.5	1.4	5.5	0.61	0.001
1967 (January– February)	A_2 (Jan.) B (Feb.)	2	6780	21.1	23450	11.0	48.0	1.9	10.1	0.54	0.001

Fig. 5

*Incidence of influenza during
the epidemic of type A_2 in January-
-February 1965 in different age
groups vaccinated or non
vaccinated in the same children
institutions .*

Twofold live vaccine

in December 1964   in October-November 1963

% of influenza cases

Age group - 1-2   3-6   7-15   1-2   3-6   7-15
                   years   years
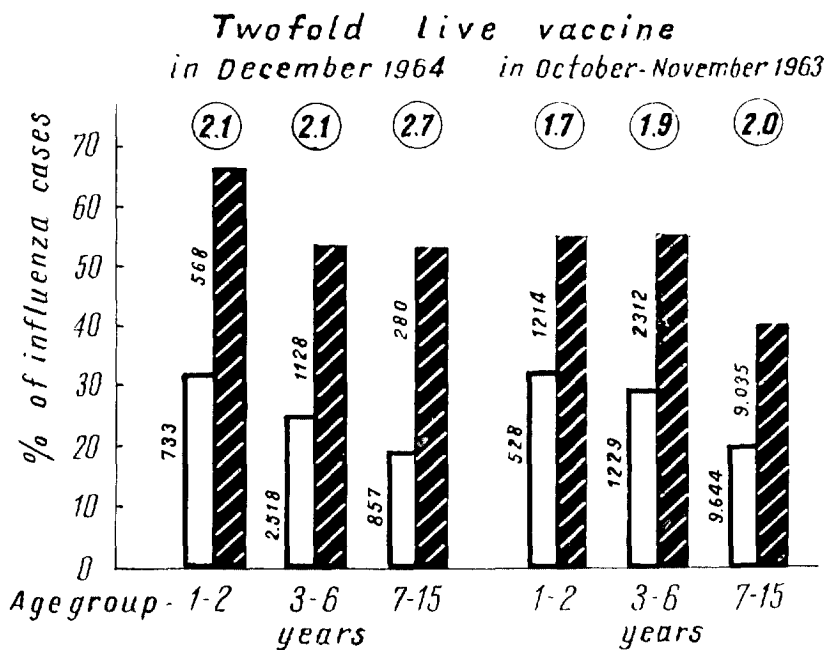
Legend: 733, 568 - total children in the group ;
(2.1) - index of effectiveness.

Fig. 6

Incidence of influenza A_2 and B in immunized and non immunized preschool children during the epidemic of A_2 and B_2 type in 1962 .

Decades - I II III I II III I II III I II III

Epidemic of A_2
(January- February 1962)

Epidemic of B_2
(March - April 1962)

Legend: —————— - immunized A_2 (682 pers.) or B (283 pers.) ;
— — — — - non immunized (932 persons).
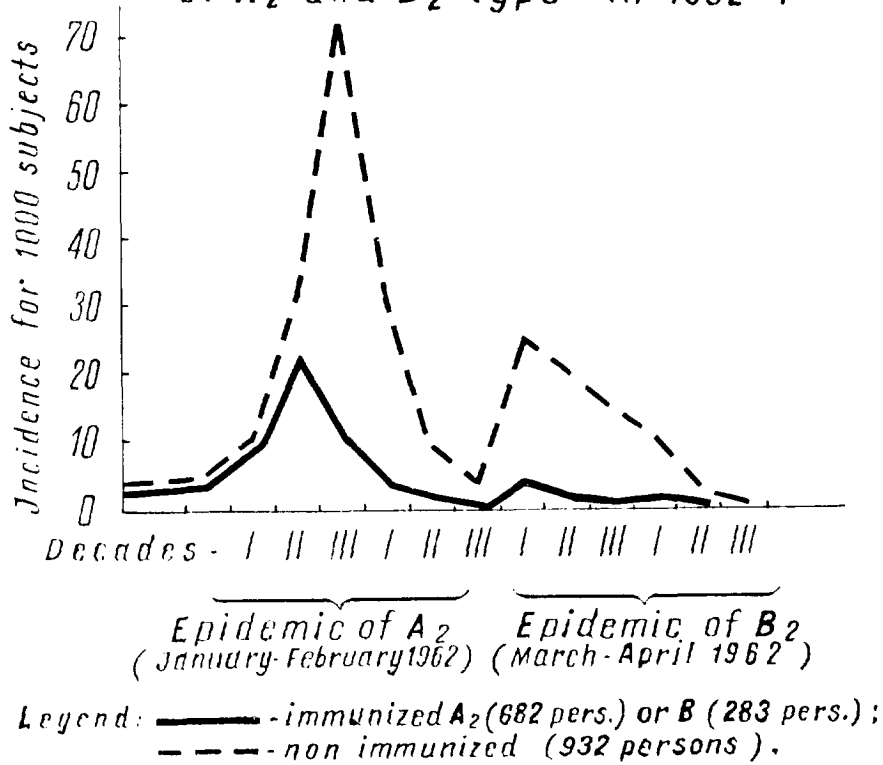
Fig. 7

Attack rate (per 1000) of influenza A_2 and B in vaccinated and non vaccinated students during epidemic in 1962.

Influenza A_2 (Jan.-Febr. 1962)

Non immunized (16052 persons)

156.8

Immunized (12601 persons)

41.3

Non immunized values: 482.5, 438.9, 269.8, 264.8, 245.4, 224.4, 214.2, 211.9, 180.0, 125.1, 115.0, 78.1, 66.6, 66.1, 47.2, 41.6, 15.1, 10.0

Groups: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Immunized values: 299.2, 130.8, 86.0, 72.5, 48.0, 35.0, 13.8, 11.3, 10.1

Groups: 20 21 22 23 24 25 26 27 28 29 30 31 32 33

Influenza B (March-Apr. 1962)

Non immunized (18180 persons)

58.7

Immunized (10473 persons)

4.7

Non immunized values: 355.4, 207.7, 145.2, 135.0, 131.6, 131.4, 104.9, 89.9, 78.7, 51.2, 42.5, 21.8, 12.0, 11.3, 10.0

Groups: 5 24 8 33 7 10 2 28 12 23 1 13 4 6 14 3 9 16 18 19 26

Immunized values: 32.0, 11.4, 10.7, 10.6

Groups: 11 29 22 20 21 25 27 30 31 32 15 17

No of groups

Attack rate per 1000 subjects

Legend: **156.8, 41.3**... mean rates per 1000 persons.

Fig. 8

Incidence of influenza during epidemic A₂ in January 1965 in nurseries and kindergartens where 60 and more % had been immunized .

Nurseries   Kindergartens
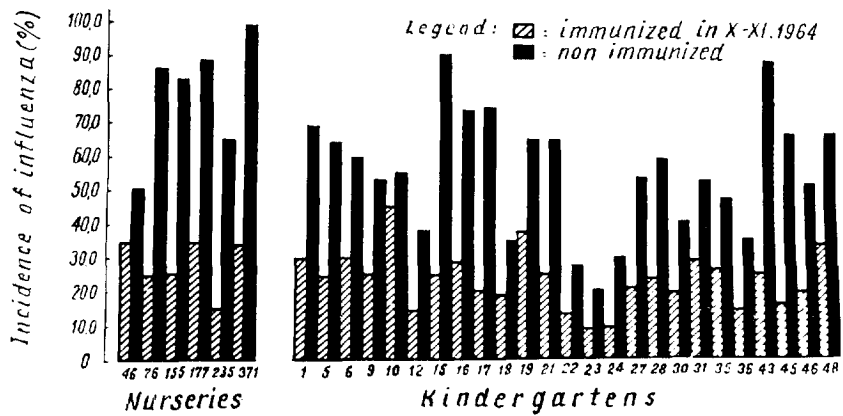
STUDY OF THE PROTECTIVE EFFECT OF LIVE INFLUENZA VACCINE (1964–68)

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Since 1962 the Institute of Immunology has been producing live influenza vaccine with the intention to reduce the number of acute respiratory diseases of the population on a wide basis, particularly in factories and enterprises.

The application of live influenza vaccine is simple and painless, mass vaccination campaigns are easily organized and the production of vaccine is comparatively quick.

Vaccination with the live influenza vaccine has been conducted in the S. R. Croatia and S. R. Slovenia since 1962.

Vaccination was voluntary and was carried out mainly in large establishments of the above mentioned republics. So far, more than 9,000,000 doses of vaccine have been administered.

The effectiveness of the vaccine was studied twice in the field – in 1964 and in 1968 – when influenza appeared in this country in an epidemic form.

MATERIAL, METHODS AND PLAN OF STUDY

Vaccine

Seed Viruses. Between 1962 and 1965 only the strain A$_2$ Krasnodar (101) 1959 was used, received from the USSR as an attenuated vaccinal strain. In order to obtain a sufficient quantity of seed virus, this strain underwent one allantoic passage in our Institute.

From 1966, RIF-free chicken eggs have been used for the production of the vaccine. Therefore the A$_2$ Krasnodar(101)1959 was passed twice

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in the allantois. Since that time, in addition to the strain A_2 Leningrad (29)1965 obtained from the State Control Institute has been used. This strain underwent there six passages in chick embryos in the presence of normal horse serum and was obtained as an inhibitor resistant variant. In our Institute the A_2 Leningrad(29)1965 underwent four embryonic calf kidney tissue culture passages and two allantoic passages and was named L-Z/12/1965. The 12th passage was used as seed virus. The vaccine represents the 13th passage.

Preparation of Vaccine. The substrate for the virus growth is the epithelium of the inner wall of the allantoic sac of 11-day chick embryos.

The multiplication of the virus A_2 is allowed to proceed for 48 hours in an incubator at a temperature of 35 ^{0}C and 80% humidity.

The dose for infection is 100 $ID_{50}/0.1$. ml.

After the aforementioned egg incubation, the allantoic fluid is harvested by means of a vacuum system and filled into bottles of 500 ml. After testing the sterility of each individual bottle, pooling takes place. Such a pool represents one lot of vaccine. Up to 1963, 15% egg yolk and 10% saccharose were added as stabilizer, and after 1963, 5% pepton (Difco) is added.

Of antibiotics, between 1963 and 1968, penicillin and streptomycin were added, 100 IJ/1 ml. of each. Since 1968, only Aueromycin 100 Y/1 ml. has been added.

After titration and testing of sterility, 1 ml of the preparation is filled into ampoules and freeze-dried.

Titration and Vaccine Control. The vaccine is titrated in eggs. Dilutions 10^4, 10^5, 10^6, 10^7 are made from vaccine samples in a broth-saline solution. Out of each dilution, 0.1 ml is inoculated intraallantoically into each of ten 11-day-old chick embryos. After 48 hours of incubation at 35–36 ^{0}C, the eggs are opened and examined for the presence of virus in the allantoic fluid. One or two drops of allantoic fluid are poured into a test tube with 0.5 saline solution. Then 0.5 ml of 0.5% chicken erythrocytes are added. After one half to one hour at $+$ 4 ^{0}C, the presence or absence of hemagglutination is read. The titre is obtained by calculation according to Reed Muench, and represents that dilution of the vaccine which has resulted in a 50% infection of chick embryos.

Vaccine sterility is tested in the usual way, and the innocuity of the vaccine is tested in mice.

The declared type of virus in the vaccine is identified by means of the hemagglutination inhibition test with sera specific for type A_2.

The above tests are carried out before freeze-drying and after freeze-drying only the titre and sterility are tested.

Multiplication of the vaccinal virus on nasal mucous membranes and antibody response in humans represent the tests for immunogenicity of the vaccine.

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Vaccination. The vaccine is diluted with sterile saline solution according to instructions and poured into an atomizer. Each nostril is sprayed with 0.2 to 0.3 ml vaccine and the total dose per person amounts to 0.4 to 0.6 ml.

The vaccine is administered at an interval of 10 to 14 days, between individual doses. Only adult healthy persons are vaccinated.

Reactions. Reactions were followed-up usually after the first dose of vaccine during the first 72 hours. Temperature was taken every day axillary. The reactions were registered by the physician who had conducted the vaccination.

Reactions were encountered in approximately 3% of vaccinees, manifesting themselves by a short lasting fever up to 37.5 °C, increased nasal secretion or other symptoms of nasopharyngitis. Some vaccinees complained of headaches and sore throat.

Information on the Epidemics of 1964 and 1968.

Epidemic of 1964. The first cases of influenza were registered in the first days of the second half of February 1964. The epidemic broke out in Croatia in the region of Rijeka and in Slovenia in the surroundings of Ljubljana. In both Republics the epidemic spread very quickly. It reached its peak in the second week of March and lasted until the second week in April. In that week the number of patients abruptly dropped, so that only sporadic cases were registered.

It is very difficult to assess the accurate number of diseased in the epidemic. The data are most or less accurate for working people. The data on the other categories – mainly family dependants – are only approximate, so that the number of registered cases of influenza at the time of the epidemic was considerably smaller than the actual number.

According to information furnished by the Institute of Public Health of SR Croatia and SR Slovenia and the Bureaus of Social Insurance of both republics, in Croatia there were 371,000 and in Slovenia about 147,000 registered cases of influenza among working people.

The highest number of cases was registered in large enterprises and factories and among school children.

Laboratory tests, the aim of which was to identify the virus type which caused the influenza epidemic in 1964, were carried out in the laboratories of the Republic Institute of Public Health of Croatia and Slovenia. Blood specimens for serological examinations were taken from apprentices at the PTT Home at Ljubljana – Bežigrad who fell ill with influenza in February 1964. None of these persons had been vaccinated against influenza. Two samples of blood were taken from each person. In that institution the disease, with the typical clinical signs of influenza, broke out suddenly, and morbidity at the time of taking the first blood specimens amounted to 60%. The serum was separated from the coagulum within 24 hours and frozen at —20 °C until further processing.

Of seven patients in Zagreb throat washings were taken for virus isolation.

Epidemic of 1968. Influenza did not have an epidemic character in the whole territory of Croatia. In certain big centres (Zagreb, Rijeka, Sisak) the frequency of cases of respiratory diseases rose in January 1968. A definite epidemic character was noted only at Osijek. The first cases were registered on 22 December 1967 and the epidemic lasted until 25 January 1968. In this month of the epidemic, a total of 12,000 cases were registered. Owing to the fact that in no epidemic can all the diseased be registered, it is considered that the actual number of cases was about 20,000, i. e. about 20% of the population of Osijek, which has about 90,000 inhabitants.

From nine patients at Osijek and 12 at Rijeka throat washings were taken for virus isolation. From 12 patients at Rijeka, two blood specimens were taken – acute and convalescent blood – for serological examinations.

Isolation of Epidemic Virus. For virus isolation the following method was used: The patient gargles with a 15 ml saline solution and to this throat washing 2 ml of broth are added. Two ml of such throat washing are centrifuged for 5 minutes at 2,000 r. p. m. The supernatant is poured into a test tube and 0.2 ml of a solution of streptomycin and penicillin each of 10,000 IJ/ml concentration are added, so than in 1 ml of washing there are 1000 IU of penicillin and 100 IU of streptomycin. 0,1 ml are inoculated intraallantoically and 0.1 ml intraamniotically into 8-day-old chick embryos. Through five successive days after inoculation, the perishing of the embryos is followed and the amniotic and allantoic fluids of the perished are examined for hemagglutination. If in the first egg passage the hemagglutination test was negative, another blind egg passage is made. If this passage shows the characteristic perishing of embryos 48 and 72 hours after inoculation, further passages are made in spite of the absence of hemagglutination. After the appearance of hemagglutination, the identification of the virus is carried out, only if the hemagglutination titre is higher than 1 : 27.

Determination of antibodies. Serological tests were made on patients in order to prove influenza, where acute and convalescent blood were taken. Antibodies in the serum were determined by the CFR, the hemagglutination inhibition test and the neutralization test.

The HI test was done by means of a microtitre according to Takatsy in cups with conically shaped bottoms of transparent plates. As antigens were used: A_2 Krasnodar/101/1959, resistant to serum inhibitor; A_2 L-Z/12/1965, resistant to serum inhibitor; A_2 A-Z/11/1968 variant resistant to serum inhibitors of guinea-pigs.

In the test a 1% red blood cell suspension obtained from 18-day-old chick embryos (Leghorn) was used as a pool of five embryos.

To 0.3 ml of blood taken from the finger, 0.3 ml of Hank's with heparin are added. After centrifuging and removal of erythrocytes, the dilution of the serum is 1 : 4.

Because of using inhibitor resistant antigens, the serum did not have to be previously freed from serum inhibitors.

The volume unit in the test was one drop = 0.025 ml.

The dilution is carried out in a saline solution buffered with phosphates. The dilutions of the serum are two-fold (1 : 2, 1 : 4, etc.) in the volume of one drop. After that,one drop of antigen containing four HA units is added to each dilution. After shaking and incubating for half an hour at room temperature, one drop of erythrocyte suspension is added. After shaking the plates are kept at +4 °C for 1/2 to 3/4 hours until the erythrocytes are sedimented.

The total reaction volume of this microtest amounts to three drops = 0.075 ml. The final dilution of the red blood cell suspension in this volume amounts to $1/3^0/_0 = 0.33^0/_0$.

As the end point of the hemagglutination inhibition the highest dilution of the serum is taken in which there still exists a total of hemagglutination inhibition. As HI titre we take the initial serum dilution of the endpoint (i. e. before adding one drop of antigen and one drop of erythrocytes to one drop of serum dilution.

The paired sera of patients were tested at the same time. An at least four-fold increase in antibodies was considered conversion.

Laboratory Investigations into Influenza Etiology. A significant rise of antibody titres for type A_2 influenza virus was obtained in 16 of 18 sera of diseased persons in the epidemic of 1964 in the CFR and in 17 of 18 sera in hemagglutination inhibition test as well as in the neutralization test. Two sera with a negative CFR, showed a significant titre increase in HI test and in the neutralization test, and the serum in which the HI and the NT were negative, showed a high rise of antibody titre for type A_2 in CFR (0–256).

In all applied methods for antibody determinations the average rise of titre was four-fold or greater.

The rise of antibody titre was demonstrated in HI test when the vaccinal A_2 strain and the A_2 strain isolated in the 1964 epidemic were used as antigens.

From seven throat washings taken from seven influenza patients in the epidemic of 1964, in one only the influenza virus was isolated and was identified by the HI test with diagnostic sera as influenza virus A_2.

In 1968, of 12 patients at Rijeka, eight had a four-fold increase in the antibody titre for type A_2 in the HI test and CFR.

In six throat washings out of 12, the influenza virus was isolated which in the HI test with diagnostic sera showed up as type A_2. Isolated strains had very low hemagglutination titres.

Of nine patients at Osijek from whom throat washings were taken, the virus was isolated in three washings. Of these three isolates, only in one a sufficiently high HA titre was obtained. The isolated virus was identified as an A_2 influenza virus.

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Survey of the Incidence of Influenza in Vaccinated Persons. The effectiveness of live influenza vaccine was studied in epidemic years. The study was carried out in establishments vaccinated against influenza. In order to make the information obtained by the survey as reliable as possible, we included in the survey only those establishments which have their own, well organized health service, where the diagnosis was made by the physician of the establishment and where the data required by the survey were available from a well kept individual card index of workers held by the health service of the establishment. The data collection was conducted by our teams consisting of epidemiologists and physicians. while some establishments sent us the filled-in survey forms by mail. The form contained questions on the total number of workers in an establishment, on the number of once, twice and three times vaccinated workers, the number of unvaccinated workers, as well as on the number of diseased in the course of the influenza epidemic in each of these groups.

The diagnosis did not present particular difficulties at the time of the epidemic and the clinical diagnosis of influenza could be made with a considerable degree of certainty. We endeavoured to have consistence in the criteria for the diagnosis of influenza in all those who made them. This we tried to ensure by drawing the attention of the health officials of establishments when taking over the vaccine to the importance of an accurate diagnosis of influenza during a possible epidemic.

Influenza includes those diagnostic terms which are incorporated in Manual of the International Statistical Classification of Diseases, Injuries and Causes of Death of 1955 (7) under code numbers 480 (influenza with pneumonia), 481 (influenza with other respiratory manifestations and unqualified influenza) 482 (influenza nervous manifestations), 470 (acute nasopharyngitis), 471 (acute sinusitis), 472 (various forms of pharyngitis), 473 (acute tonsilitis) 474 (acute laryngitis and tracheitis), 475 (acute upper respiratory infections of multiple or unspecified sites).

RESULTS

Effectiveness of the Live Influenza Vaccine in the 1964 Epidemic

Table 1 shows the results of the study carried out in 29 vaccinated establishments in Croatia and Slovenia. The total number of employees amounted to 51,625. Of these, 29,791 were vaccinated three times, 21,834 were not vaccinated, while the rest were incompletely vaccinated.

In the three times vaccinated group 5.00% fell ill whereas in the unvaccinated group 19,39% fell ill.

The morbidity ratio between the three times vaccinated and unvaccinated persons is shown in Table 2. Of 1000 vaccinated employees, 50 fell ill, and of 1000 unvaccinated employees, 194 i. e. 3.9 times more.

An analysis of data from Table 1 is shown in Table 3.

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Table 1

Incidence of Influenza in Vaccinated and Unvaccinated Persons
(1964 Epidemic)

Total number of persons	3 × vaccinated			Unvaccinated		
	No. persons	No. cases	%	No. persons	No. cases	%
51.625	29.791	1.488	5.00	21.834	4.233	19.39

Table 2

Morbidity ratio Between Unvaccinated and Vaccinated Persons

	Cases per 1000	Morbidity ratio unvacc./vacc.
3 × vaccinated	50.0	3.9
Unvaccinated	193.9	

Table 3

Analysis of Data on the Incidence of Influenza in Vaccinated and Unvaccinated Persons

No. surveyed establishments	3.9	
No. establishments with significant difference in the incidence of influenza in vaccinated and unvaccinated persons	27	$P < 0.001$ (20) $P < 0.02$ (4) $P < 0.05$ (3)

The incidence of influenza in three times vaccinated in relation to unvaccinated persons was significantly lower in 27 out of 29 followed-up establishments, and a high significance ($p < 0.001$) was obtained in 20 establishments.

Somewhat poorer results were obtained in twice vaccinated persons. Out of 29 establishments, the incidence of influenza in twice vaccinated persons was significantly lower in 20 establishments, and highly significantly lower in 14 establishments. In persons vaccinated once, the incidence was significantly lower in 12 establishments and highly significantly lower in eight of them.

Separately we tried to show the incidence of influenza in the 1964 epidemic, in relation to the total number of vaccinated persons regardless of the number of doses received. The results are shown in Table 4. A

Table 4

Influence of the Percentage of Vaccinated Persons on the Reduction of Influenza

Group	% vaccinated	No. persons in group	No. cases	% cases	Incidence reduction group V = 100
I	80	33.428	844	2.52	11.2
II	60–80	14.091	730	5.18	23.1
III	40–60	16.404	1.905	11.61	51.8
IV	40	5.559	754	13.56	60.5
V	0	17.723	3.976	22.43	100.0

total of 87,205 persons were covered. This number includes some establishments which were not vaccinated; they were included in our survey to enable us to compare the incidence of influenza in vaccinated establishments with the incidence in unvaccinated ones. According to the number of vaccinated, the establishments were divided into five groups:

    I. Establishments with 80% or more vaccinated,
   II. Establishments with 60–80% vaccinated,
  III. Establishments with 40–60% vaccinated,
  IV. Establishments with less than 40% vaccinated,
   V. Establishments which had no vaccination at all (control group).

The association between the percentage of vaccinated employees and the percentage of employees who fell ill, is highly significant ($P < 0.001$), which means that the incidence of influenza depended upon the number of vaccinated persons in the establishment.

The percentage of sick persons was highest in those establishments in which vaccination was not carried out, and lowest in those with 80% or more vaccinated employees. In this group there were 11 sick against 100 in group of unvaccinated, which means a reduction of 88.8%.

Effectiveness of Live Influenza Vaccine in the 1968 Epidemic

In the epidemic of 1968, an epidemiological survey was conducted in Croatia in two vaccinated establishments at Osijek. The total number of employees in these two establishments was 933.

The incidence of influenza in the group of three times vaccinated. incompletely vaccinated and unvaccinated is shown in Table 5.

Table 5

Incidence of Influenza in Vaccinated and Unvaccinate Persons (1968 Epidemic)

Total number of persons	3 × vaccinated				Unvaccinated			
	No. persons	%	No. cases	%	No. persons	%	No. cases	%
933	322	34.51	16	4.97	320	34.20	51	15.94

In the group of three times vaccinated, only 5% of the employees fell sick, somewhat more in the group of once or twice vaccinated, while in the group of unvaccinated employees 16% fell sick.

The ratio of the frequeny of sickness in unvaccinated employees and three times vaccinated is shown in Table 6.

Of 1,000 three times vaccinated employees, 49.7 fell ill and of 1,000 unvaccinated ones 159.4, i. e. 3.2 times more.

Table 6

Morbidity Ratio Between Unvaccinated and Vaccinated Persons

	Cases per 1000	Morbirity ratio (unvacc./vacc.)
3 × vaccinated	49.7	3.2
Unvaccinated	159.4	

DISCUSSION

The evaluation of the effect of vaccination against influenza is far more difficult than it is in those communicable diseases (Smorodintsev 1954; Okuno et al. 1966) which in contrast to influenza are far easier to determine clinically.

The diagnosis of influenza is established most frequently on the basis of the symptoms of acute infection of the upper respiratory tract and is not often confirmed by laboratory findings (Andrews et al. 1966; Lancet 1968).

Symptoms very similar to those of influenza can be caused by a great number of other viruses, so that it is hardly possible to establish a proper diagnosis without laboratory examinations.

However, it is much more easier to evaluate the effect of vaccination against influenza during an influenza epidemic (Zaksteljskaja et al.

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1969). It appears justifiable to assume that the majority of cases clinically diagnosed as influenza in the period of an influenza epidemic are virtually caused by the influenza virus. Such an epidemic caused by the influenza virus type A_2 occured in the spring of 1964 and 1968 and since the epidemic broke out a month after the accomplished vaccination it was an ideal opportunity to evaluate the influence of vaccination on the incidence of the disease. Our data set out in this report are exclusively based on the investigations performed during these epidemics (Ikić et al. 1966; Pasini et al. 1964).

In all the years since the introduction of the vaccination against influenza we have tried to carry out the vaccination in all larger factories and establishments givig preference to those with well organized health service, most frequently in the form of factory out-patient clinics. In such a way we have tried to secure a high reliability of data which could help us in evaluating the effect of vaccination. By vaccinating such selected groups of the population we intended to protect the most active part of the population from a possible epidemic and to prevent the stoppage in industrial production and dislocation of essential public services.

In our trial of the effectiveness of live vaccine against influenza we needed control groups composed of unvaccinated workers both within the vaccinated establishments and in some others not subjected to vaccination. For this reason we did not insist on vaccination of either all establishments or of all workers in an establishment. The Managing Board of individual establishments was supposed to decide whether a collective would be subjected to vaccination or not, and how many workers would be included into it.

The influenza epidemics that occurred in this country have made it feasible for us to collect a sufficient amount of information according to which it has been possible to evaluate the effect of vaccination.

The findings presented indicate a substantial reduction of the disease among the vaccinated. This refers especially to those vaccinated three times.

The analysis of the relation between the percentage of the vaccinated and the decrease of the incidence of influenza has shown that in order to obtain the greatest degree of protection i. e. to reduce morbidity from influenza more than 80% of workers of a collective should be vaccinated.

SUMMARY

The effectiveness of the live influenza vaccine was studied twice in the field – in 1964 and in 1968 – when influenza appeared in this country in an epidemic form. The vaccination was carried out in larger factories and establishments giving preference to those with well organized health service.

Control groups were composed of unvaccinated both within the vaccinated establishments and in some others not subjected to vaccination. The findings in both epidemics indicate a substantial reduction of the disease among the vaccinated. This refers especially to those vaccinated three times and to collectives where more than 80 per cent of workers have been vaccinated.

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THE ATTENUATION OF A₂ (HONG KONG) VARIANT OF INFLUENZA VIRUS

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The appearance of the new variant of influenza virus A₂ (Saenz et al. 1969; Coleman et al. 1968) offered us a possibility to try and answer the question how long it takes to attenuate the virus (Beare et al. 1968) and prepare large amounts of live influenza vaccine. Are we able to prepare large amounts of vaccine, to organize a large scale vaccination campaign and face an epidemic, in the interval from the appearance of the new variant until the epidemic wave has reached our country? Influenza epidemic in this country usually begins by the end of February and ends in the first half of April (Ikić et al. 1966).

In the middle of August in 1968 we were informed by WHO from Geneva about the isolation of a new variant of influenza type A₂, antigenically considerably different from the then prevailing A₂ strains.

Early in September 1968 we asked for the new variant of type A₂ from the Influenza Centre in London. In the middle of October we received two strains of this new antigenic variant, A-2/Aichi/2/1968, with one amniotic and three allantoic passages in the chick embryo and A-2/England/68, with one amniotic and two allantoic passages in the chick embryo. Both of them are antigenically indistinguishable from the new Hong Kong varian. To gain in time we decided on the strain with the more passages in eggs, and on 11th November 1968 we started the attenuation of the A-2/Aichi/2/68 strain.

The design of attenuation envisaged three parallel processes:

1. The allantoic passages of the virus in chick embryo at the temperature of 35 °C (Zhdanov 1967).

2. The allantoic passages of the virus at a lower temperature, i. e. 27 °C (Shvachkina et al. 1968; Alexandrova et al. 1965).

3. The allantoic passage of the mixture of a given dose of the virus and boiled guinea-pig serum, which was left to stand for some time (30 minutes) at the thermostate temperature (37 °C) or overnight at room temperature (Kolchurina 1965; Alexandrova 1962).

Soon we gave up the attenuation of the virus at the low temperature. The virus could no more be proved by the hemagglutination reaction. We feared that too short a time was left for such a way of attenuation requiring 15 to 20 passages.

As early as in the first passage we tried to mix the strain with the normal guinea-pig serum in order to obtain an inhibitor resistant variant. The results were of two kinds but always negative: either the vi-rus did not propagate in the egg and the hemagglutination of the allan-toic fluid was negative, or the virus did propagate but still remained (in the test of hemagglutination inhibition with the guinea-pig serum) sensitive to the serum inhibitor.

Such attempts to obtain the inhibitor resistant variant were made in all first four passages through which this strain passed in our labora-tory. The attempts were further continued and in 17th passage we ob-tained the inhibitor resistant variant which retained such properties even in the absence of guinea-pig serum. It was, unfortunately, in the middle of February, too late to investigate the inhibitor resistant virus in the field and to use it for mass application.

From 8th allantoic passage of virus at 35 °C seed virus was made for the preparation of the first batch of vaccine to be used for the testing of reactions, virus multiplication in vaccinees and immunogenicity of the strain. In the majority of the vaccinated groups or in particularly sensitive persons the reactions were more pronounced. This was the reason why a further attenuation of the strain was undertaken. For the mass production of the vaccine the seed in the 11th allantoic passage at 35 °C was used.

By the middle of January the preparation of vaccine was completed: 10 million doses were prepared. The vaccine was tested according to the requirements for live influenza vaccine. Post-vaccinal reactions are pre-sented in Table 1. The virus concentration was 5 log for adults and 4.2 log for children, 7–16 years old. The intensity of postavaccinal reactions was evaluated on the basis of the rise of temperature which was taken once daily over 4 days following vaccination. The highest temperatures were considered. The reactions were mild. There was almost no diffe-rence between the control group and the vaccinated group.

After the other field examinations (virus multiplication and antibody conversion in vaccinees) had been successfully completed a mass vacci-nation was begun. An inquiry was made among nearly 100,000 vacci-nees and the results confirmed the experiences presented above.

Additional field and laboratory examinations proved the immunizing value of the vaccine.

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Age	ID$_{50}$ per dose	No. vaccinated	Temper. higher than 37.0°C		Mean duration of fever
			No.	%	
7–11	10$^{4.2}$	127	6	4.7	1.4
	S. S.*	121	5	4.1	1.2
12–16	10$^{4.2}$	618	36	5.8	1.3
	S. S.*	365	20	5.5	1.3
≥ 17	10^5	2.211	159	7.2	1.1
	S. S.*	400	29	7.2	1.2

* S. S. = saline solution (control group)

These experiences show that in the interval between the identification of the new variant of influenza A₂ in Asia and the epidemic caused by strain in this country it was possible to prepare such amounts of live influenza vaccine that made the organization and carrying out of the mass vaccination possible.

Such activities are always preformed when the time is pressing and under the pressure of health service demanding the weapon against the coming epidemic. That is why the success of such an action depends in the first place on as quick as possible advising of manufacturing institutes about the appearance of a new variant and on its promptest delivery to manufacturing institutes about the appearance of a new variant and on its promptest delivery to manufacturing institutes for attenuation.

It would be useful if WHO had a list of institutes engaged in large scale preparation of vaccine and served as a mediator in the exchange of their results in the course of virus attenuation.

Influenza is a global problem. From the epidemiological point of view the world represents a single epidemiological unit and therefore only by prompt exchange of information among the interested institutions can we face new epidemics with more success.

SUMMARY

In the interval between October 1968 when we obtained the A₂/Hong Kong/68 and January 1969 this strain was attenuated, an experimental lot of vaccine was prepared and tested in volunteers and then 10 million doses of vaccine were made. It was shown that it was possible to prepare enough influenza vaccine for carrying out the programme of mass vaccination within the interval from the appearance of the new variant of epidemic strain in Asia until the epidemic reached Yugoslavia.

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EXPERIENCES WITH LIVE INFLUENZA VACCINE IN 1969

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In the season 1968/69 in this country about 2.5 million doses of live freeze-dried influenza vaccine prepared from the strains A_2L-Z/12/1965 and A_2/A-Z/11/1968 – Hong Kong variant – were distributed. From the total quantity of the distributed vaccine only in the territories of SR Croatia and Slovenia, with a total of about six million inhabitants, more than 1.5 million doses of influenza vaccine were used. This is the first time in the seven years of influenza vaccination that such a large quantity of the vaccine was used in these two republics. This is also the reason that in some areas in which vaccination was intensive the number of vaccinated inhabitants is comparatively high for our circumstances.

In the season 1968/69 the mass vaccination of school children was performed for the first time. For this purpose about 100,000 doses of vaccine were used. For vaccination of children the concentration of virus amounted to 4.2 log ID_{50} per dose and for adults to 5.0 log.

The expected Hong Kong influenza epidemic did not appear in the extent as we had expected. Only some territories in Dalmatia were attacked.

MATERIAL AND METHODS

Vaccine

Seed Virus. From the beginning of 1969 only the A_2-AICHI/2/1968 strain – Hong Kong variant – received from the World Influenza Centre of London from Dr Pereira as the fourth passage in chick-embryo – 1 amniotic and three allantoic has been used. In our Institute this strain

underwent another seven allantoic passages in chick embryos and was named A-Z/11/1968. The 11th passage of the virus represents the seed virus and the vaccine the 12th passage.

Preparation of Vaccine. The substrate for the virus growth is the epithelium of the inner wall of the allantoic sac of 11-day old chick embryos.

The multiplication of the virus is allowed to proceed for 48 hours in an incubator at a temperature of 35 °C and 80% humidity.

The virus dose for infection is 100 $ID_{50}/0.1$ ml.

After the aforementioned egg incubation, the allantoic fluid is harvested by means of a vaccum system and filled into bottles of 500 ml. After testing the sterility of each individual bottle, pooling takes place. Such a pool represents a series of vaccine and 5% pepton (Difco) is added as stabilizer. Of antibiotics, only Aureomycin 100 Y/1 ml has been added.

After titration and testing of sterility, 1 ml of the preparation is filled into ampoules and freeze-dried.

Titration and Vaccine Control. The vaccine is titrated in eggs. Dilutions 10^4, 10^5, 10^6, 10^7 are made from vaccine samples in a broth saline solution. Out of each dilution, 0.1 ml is inoculated intraallantoically into each of ten 11-day old chick embryos. After 48 hours of incubation the eggs are opened and examined for the presence of virus in the allantoic fluid. One or two drops of allantoic fluid are poured into a test tube with 0.5 saline solution. Then 0.5 ml of 0.5% chicken erythrocytes are added. After one half to one hour at $+4$ °C, the presence or absence of hemagglutination is read. The titre – obtained by calculation according to Reed Muench, is that dilution of the vaccine which has resulted in a 50% infection of chick embryos.

Vaccine sterility is tested in the usual way.

Innocuity of the vaccine is tested on mice.

The declared types of virus in the vaccine are identified by means of the hemagglutination inhibition test with specific sera.

The above tests are carried out before freeze-drying and after freeze-drying only the titre and sterility are tested.

Multiplication of the vaccinal virus on nasal mucous membranes and antibody response in humans represent the test for immunogenicity of the vaccine.

Vaccination. The vaccine is diluted with sterile saline solution according to instructions and poured into an atomizer. Each nostril is sprayed with 0.2 to 0.3 ml vaccine and the total dose per person amounts to 0.4 to 0.6 ml.

The vaccine is administered at an interval of 14 days. Only adult healthy persons are vaccinated.

Serology. From vaccinated persons, the blood was taken directly before each vaccination, which was performed in intervals of 14 days, and 14 days after the last vaccination.

For the titration of antibodies in the serum the Takatsy microhemagglutination test was used.

Information on the epidemic 1969. In 1969, influenza in an epidemiological form appeared only at Split and to a lesser degree in Zadar and Šibenik. In the first half of April frequent cases of febrile conditions were beginning to appear, accompanied by respiratory symptoms. In the second half of April, and in May of 1969, the disease took on an epidemic character and the number of patients rose to 600 to 700 a day. Official registration started on April 17 which is considered to be the date of the beginning of the epidemic. The epidemic reached its peak about May 10, when more than 1000 cases were registered daily. The epidemic lasted until 27 May and in that period the number of diseased reached a total of 19,193 The number of diseased very probably exceeds the number of registered patients.

Although the number of 19,193 patients is quite high, it represents only 12.7% of the total population of the municipality of Split, which numbers 150,000.

In the majority of enterprises and factories the daily absence from work did not exceed 15% of all the employed, although in some enterprises even 30% of the workers were taken ill.

The average duration of the disease in patients without complications was 3.7 to 4.0 days.

Complications were registered in 21.05% of the diseased (4041). The most frequent complication was pneumonia and bronchopneumonia. The average duration of the disease in patients with complications was between 7 and 21 days, depending on the kind of complication.

Survey on the Incidence of Influenza in Vaccinated Persons.

The study was carried out in enterprises vaccinated against influenza. In order to make the information obtained by the survey as reliable as possible, we included in the survey only those enterprises which have their own, well organized, health service where the diagnosis was made by the physician of the enterprise and where the data required by the survey were available from a well kept individual card index of workers held by the health service of the establishment. The data collection was conducted by our teams consisting of epidemiologists and physicians.

The diagnosis did not present particular difficulties at the time of the epidemic and the clinical diagnosis of influenza could be made with a considerable degree of certainty. We endeavoured to have consistence in the criteria for the diagnosis of influenza in all those who made them by drawing the attention of the health officials of establishments to the importance of an accurate diagnosis of influenza during a possible epidemic.

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Table 1 presents the results of vaccination with the vaccine prepared from the strains A_2 Krasnodar (101) 1959 and A_2 L–Z (12) 1965. The concentration of the vaccinal virus A_2 L–Z (12) 1965 was 6.7 log per dose.

Table 1

Effectiveness of Live Influenza Vaccine prepared from Strains A_2 Krasnodar/101/1959 and A_2 L–Z/12/1965 and Live Influenza Vaccine prepared from Strain A_2 A–Z/11/1968 in the 1969 Epidemic

Vaccine	Total No. persons	Vaccinated					Unvaccinated			
		No. persons	%	No. cases	%		No. persons	%	No. cases	%
A_2 Krasnodar/ 101/1959 A_2 L–Z/12/1965	6.340	4.230	66.7	227	5.4		2.110	33.3	506	24.0
A_2 A–Z/11/1968	3.400	1.650	48.5	9	0.5		1.750	51.5	146	8.3

From 6,340 workers a total of 4,230 was vaccinated. Average vaccination rate in the group of 6,340 employees amounted to 66.7%. Those non-vaccinated constituted the inner control group. The percentage of the affected in this group was 24%. Since the percentage of the affected in the vaccinated group amounted to 5.4, the vaccine conferred a considerable protection against the Hong Kong influenza.

The protection has to be attributed in the first place to the strain A_2 L–Z (12) 1965 since in the testing with sera A_2 (Krasnodar) 101/1959, A_2 L–Z (12) 1965 and A/ A–Z (11) 1968 its evident antigenic similarity to the strain A_2 A–Z (11) 1968 was determined.

With the vaccine prepared from the strain A_2 A–Z (11) 1968 – Hong Kong variant – a total of 1,650 out of 3,400 employees were vaccinated

The protection conferred by the vaccine is statistically highly significant.

Table 2 presents the results of the analysis of data on the morbidity of vaccinated and non-vaccinated persons in five establishments during the epidemic in Dalmatian towns.

The protection of the vaccinated with the strains A_2 L–Z (12) 1965 and A_2 A–Z (11) 1968 is highly significant in all five establishments $(P < 0.001)$.

Table 2

*Analysis of Data on the Incidence of Influenza in Vaccinated
(A₂ Krasnodar/101/1959 and A₂ L–Z/12/1965; A₂ A–Z/11/1968)
and Unvaccinated Persons*

No. surveyed establishments	5
No. establishments with a significant difference in the incidence of influenza between vaccinated and un-vaccinated persons	5 (P < 0.001)

It seems that the vaccine prepared from strain A_2 A–Z (11) 1968 – Hong Kong variant – is highly immunogenic. The high percentage of seroconversion in vaccinated persons speaks in favour of this statement.

Table 3 summarizes the results of the study of sero-conversion in 20 vaccinated adults. The four-fold increase of antibodies is shown after the application of the first, second and the third dose of the vaccine prepared from the strain A_2 A–Z (11) 1968. The concentration of virus amounted to 5 log per dose.

Table 3

*Fourfold Conversion Rate after the 1st, 2nd and 3rd Dose of Vaccine
and mean Antibody Titres*

Blood specimen	No. persons	Fourfold conversion		Mean titre*
		%	%	
PV	20			5.1
1D	20	(PV:1D) 35		10.6
2D	20	(PV:2D) 88	(1D:2D) 83	26.9
3D	20	(PV:3D) 82	(2D:3D) 00	35.5

* Geometric mean
PV = prevaccinal
1D = after 1st dose
2D = after 2nd dose
3D = after 3rd dose

As sero-negative persons are considered those with the titer lower than 1 : 8.

After the first dose fourfold seroconversion occured in 35% of the vaccinated and after the second dose the percentage increased to 88%. The application of the third dose did not cause further increase of percentage.

The group of sero-negative persons who remained negative after the application of the first dose of vaccine (we have mentioned before that the conversion after the first dose amounted to 35%) reacted to the application of the second dose so that the seroconversion rate increased from 35% to 83%. This fact shows that the first dose of the vaccine, although it does not bring about an increase in titre in a number of vaccinees, sensitizes the organism and the whole group in the new contact with the vaccine reacted with a much higher percentage of seroconversion. On the contrary, those who remained negative after the application of the second dose did not react to the application of the third dose either.

The difference between the mean antibody titre before the vaccination and the mean antibody titre 14 days after the application of the first dose is statistically highly significant.

The same results is obtained when we compare the geometric mean titre 14 days after the first dose with the geometric mean titre 14 days after the second dose. The difference is highly significant.

On the contrary, the difference between the geometric mean titre 14 days after the second dose and the geometric mean titre 14 days after the third dose is not statistically significant.

Table 4 presents the seroconversion after the first and second dose of vaccine in the group of 49 adults vaccinated with the same vaccine with the same virus concentration (5 log. per dose).

Table 4

Fourfold Conversion Rate after the 1st and 2nd Dose of Vaccine and mean Antibody Titres

Blood specimen	No. persons	Fourfold conversion		Mean titre*
		%	%	
PV	49			7.5
1D	49	(PV:1D) 44		16.0
2D	37	(PV:2D) 91	(1D:2D) 88	34.4

* Geometric mean

The results comply with the results presented in the table 1. After the application of the first dose the percentage of vaccinees with fourfold conversion amounted to 44% and after the second dose 91%.

The group of sero-negative persons who did not react to the first dose of the vaccine, reacted very well to the second dose, so that the percentage of persons with a four-fold increase of titre amounted to 88%

The difference in the geometric mean titre before vaccination and 14 days after the first dose of vaccine is highly significant.

The difference in the geometric mean titre 14 days after the application of the first dose and the geometric mean titre 14 days after the second dose is also highly significant.

Table 5 presents the seroconversion after two doses of vaccine in 115 persons vaccinated with the same vaccine with the same concentration of the virus (5 log. per dose).

80% of sero-negative persons became positive after the second dose. The difference between the geometric mean antibody titre before vaccination and the geometric mean titre 14 days after vaccination is highly significant.

Table 5

Fourfold Conversion Rate after the 2nd dose of Vaccine and mean Antibody Titres

Blood specimen	No. persons	Fourfold conversion %	Mean titre*
PV	115	(PV:2D) 80	7.1
2D	115		23.8

* Geometric mean

Table 6 shows the seroconversion after the third dose of vaccine of 72 adults vaccinated with the same vaccine (A_2 A–Z (11) 1968, of 5 log. per dose).

It can be seen from the table that 81 per cent of seronegative persons became positive. The difference between the geometric mean titre before vaccination and the geometric mean titre a fortnight after the third vaccination is highly significant.

Table 6

Fourfold Conversion Rate after the 3rd Dose of Vaccine and mean Antibody Titres

Blood specimen	No. persons	Fourfold conversion %	Mean titre*
PV	72	(PV:3D) 81	6.2
3D	72		44.5

* Geometric mean

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Table 7 presents the sero-conversion in 60 school children after the first dose of the same vaccine (A_2 A–Z (11) 1968, 4.2 log. per dose).

In school children the rate of four-fold seroconversion after the first dose is considerably higher. It amounts to 78% and approaches the percentage of sero-conversion which in adults is attained with two doses.

The difference between the geometric mean titre before vaccination and the geometric mean titre 14 days after the first dose is highly significant.

Table 7

Fourfold Conversion Rate after the 1st Dose of Vaccine and mean Antibody Titres

Blood specimen	No. persons	Fourfold conversion %	Mean titre*
PV	60	(PV:1D) 78	8.2
1D	60		21.0

* Geometric mean

DISCUSSION

In the course of the seven years' large scale application of live influenza vaccine we had the opportunity to study the effectiveness of the vaccine three times during A_2 influenza epidemics. (Ikić et al. 1966; Pasini et al. 1964). The protection was highly significant with all the three strains used – Krasnodar (101) 1959, L–Z (12) 1965 and A–Z (11) 1968 (Hong Kong variant). In the immunogenic respect the Krasnodar strain was the poorest and the A–Z (11) 68 the best. Therefore, when applying the Krasnodar strain we insisted on three vaccinations in order to obtain high protection; in the case of the A–Z (11) 68 (Hong Kong variant) we considered two doses to be sufficient. This is also confirmed by the results of positive seroconversion produced by the Hong Kong variant. There was no increase of positive seroconversion after the third dose.

The protection conferred by the live vaccine is high although the concentration of antibodies in serum is lower than in the case of killed vaccine. The influenza virus infection induces the formation of antibodies in the mucous membranes of the respiratory tract. We believe the live vaccines to act in the same way, so the amount of the circulating antibodies is of minor importance for the protection of the organism when live vaccine is used.

Presumably the antibodies on the surface of the mucous membrane are those that inhibit infection.

It is also probable that endogenous supplies of interferon in the tissue of the respiratory tract play a role in the defence mechanism as well as other unspecific factors. (Solovev et al. 1968).

The postvaccinal reactions caused by the live vaccine depend on the pathogenicity of a strain, on the virus concentration, on the immune status of the vaccinated groups and on the age of vaccinees. It has been observed that persons vaccinated for the first time experience stronger reactions.

The serial passage of influenza virus isolated from the patients results in a comparatively quick loss of both pathogenic and immunogenic properties of the virus for man. The virus loses both properties at different times and to a different degree. The virus first loses pathogenic properties for man although it retains the capability of multiplying in the human nasopharynx and of provoking a mild local, and sometimes even general, reaction followed by immunological reaction. By further passages the virus loses also its immunogenic properties. (Beare et al. 1968; Shvachkina et al. 1968; Zhdanov 1967).

The attenuated strain is obtained by dissociation of the pathogenic and immunogenic properties of the influenza virus during serial passages. We lack approved methods for measuring in the laboratory the changes in the pathogenic and immunogenic properties of the virus and for stabilizing the immunogenic properties at a satisfactory level. This, of course, can be achieved by testing the strain in man at different passage levels and by preparing large amounts of vaccine in the passage shown to be optimal, without further passaging the virus. This is not a good solution. It is clear that research must concentrate upon genetic markers for the determination of pathogenic and immunogenic properties of the virus and on the evaluation of the suggested markers (Kolchurina et al. 1965).

It is possible to choose strains which multiply in the nasopharynx and cause febrile reactions in no more than $2\%-3\%$ vaccinees, while hemagglutination inhibiting antibodies are produced in a high percentage of vaccinees. The question is how long this road is and how often we shall experience disappointment before we succeed at last, by careful combination of clinical and laboratory research, in well defining attenuated strains with low pathogenic properties and satisfactory immunogenic qualities.

Every age group is exposed to influenza but the morbidity rate is the highest in the 5–14 year age group.

In the last season we also set to vaccination of children seven years old or more. Perhaps the persistent immunization of children up to their being adults is that which can protect us against new antigenic variants of influenza. The vaccination of children with live vaccine is perhaps a very useful task with which we are faced. (Alexandrova et al. 1965).

In conclusion, I want to stress that the defence mechanism of the body against acute respiratory diseases is such that both specific and unspecific

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factors in the peripheral barrier, at the respiratory infection entry, play a decisive role in the defence of the body. This offers a chance to the live vaccine.

The simple manner of application, the imitation of the natural path of infection, the exploitation of local defence mechanisms, as well as the possibility of producing large quantities of live vaccine – these are the reasons why we should focus our further efforts on the problems of the live upper respiratory virus vaccine development.

SUMMARY

In persons vaccinated with live influenza vaccine prepared from the strains A_2 (Krasnodar) 101/59 and A_2 (L–Z) 12/65 a substantial protection was observed in the epidemic caused by the strain A_2 (Hong Kong) 68. The protection is ascribed to the vaccinal strain A_2 (L–Z) 12/65 which shows some antigenic relationship with the Hong Kong strain.

The vaccine made from the strain A_2 (A–Z) 68 – the Hong Kong variant – was shows to be highly immunogenic since it produced seroconversion in a high percentage of the vaccinated. After two doses seroconversion was observed in 80 per cent of the vaccinated. The third dose did not bring about any increasement of seroconversion. As there was no influenza epidemic the epidemiological effectiveness of this vaccine could not be studied.

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SPECIFIC AND NONSPECIFIC PROPHYLAXIS AND CHEMOPROPHYLAXIS OF INFLUENZA

STUDY ON ANTIBODY LEVELS AFTER COMBINATION OF INFLUENZA VACCINATION AND ADMINISTRATION OF 1-ADAMANTANE AMINE HYDROCHLORIDE IN MAN

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1-Adamantane amine has been shown to be highly effective in providing protection against clinical influenza A_2 (Wendel et al., 1966, Quilligan et al., 1966, Finklea et al., 1967, Floor et al., 1967).

Administration of 1-adamantane amine for 2 weeks immediately following a vaccination against influenza may present a valuable supplement to the influenza prophylaxis by vaccination, when an influenza A epidemic is present, as an influenzal illness can be prevented in the period during which vaccination fails to give protection.

The aim of the double blind study reported here was to find out whether administration of 1-adamantane amine interfered with the serological antibody response to vaccination with an inactivated influenza vaccine.

MATERIALS AND METHOD

Subjects: Young healthy volunteers, students of a medical technical school in Zagreb (Yugoslavia), participated in this study.

These students were of both sexes, ranged from 14 to 23 years in age and had not been vaccinated against influenza in the previous season.

Investigational setting: The study was carried out with placebo controls under double blind conditions.

A total number of 250 subjects were assigned at random to two groups of equal size, treated with either 1-adamantane amine tablets or placebo tablets.

200 Subjects, 100 of the 1-adamantane amine group and 100 of the placebo group, being randomly selected from the total group of 250, were vaccinated with inactivated influenza vaccine.

The non-vaccinated group consisting of 50 subjects served as a control for possible spontaneous antibody rises, which would indicate natural influenza infection.

The investigation was planned to be carried out at a time when there was no evidence of an influenza A or B epidemic.

The tablets were dispensed at school and were swallowed with some water under supervision of a staff member of the school, daily for 6 days per week. On Saturday the students received their medication for Sunday with the instruction to take the tablets at home.

Three days after the vaccination the students were seen for possible local reactions.

At three, six and fourteen days after the start of the trial the subjects were questioned on occurrence of disease or adverse reactions.

Medications: 1-Adamantane amine was administered in 200 mg tablets once daily for 14 days. The placebo tablets, containing lactose, were of identical appearance.

The administration of the tablets to the subjects who were vaccinated, started on the day of the vaccination.

One ml of an inactivated influenza vaccine (Influvac) was given by subcutaneous injection. The vaccine was composed as follows:

A_2 – 68 (Netherlands) 65 : 150 C. C. A.,

A_2 – 1 (England) 66 : 150 C. C. A.,

B – 33 (Johannesburg) 58 : 100 C. C. A.,

B – 78 (Netherlands) 66 : 100 C. C. A.

Serological tests: From every subject two specimens of blood were taken, the first on the day of the vaccination, respectively the day of the start of treatment, the second 21 days later.

The centrifuged sera were stored at a temperature of —20⁰C and dispatched to the Netherlands within 24 hours in isolated boxes filled with ice cubes.

The paired serum samples were tested by complement fixation (C. F.) test against influenza A antigen (S-antigen) and influenza B antigen (S-antigen) and by haemagglutination inhibition (H. I.) against influenza A_2–1 (England) 66 and B–77 (Netherlands) 66. The B-strain antigenically identical to the B–78 (Netherlands) 66 strain, which was present in the vaccine.

RESULTS

The trial was started in December, 1967. The vaccination of the 200 subjects took 3 consecutive days to be completed. The medication was started on the day of the vaccination. Also the first blood sample was taken on the same day. From the 50 subjects, who were not vaccinated, the first blood specimen was taken and treatment was started concomitantly on one of these 3 days. The second blood samples of all 250 subjects were taken 3 weeks later, again on 3 consecutive days.

The 1-adamantane amine group and the placebo group turned out to be comparable with respect to age, sex and H. I. titre of the first serum sample, which was an indication that the intended randomization was adequate (table I).

The medication of 1-adamantane amine and placebo was taken according to the prescription for at least 12 days by 94% of the students

Table 1

Distribution of sex, age and H. I. titre ranges of first serum sample in both treatment groups

sex	1-adamante amine		Placebo	
	male	female	male	female
number of subjects	17 (3)c	108 (22)	29 (4)	96 (21)
mean age (years)	17.2 (17.7)	16.9 (16.7)	17.1 (17.2)	16.8 (16.5)
range	15–19 (16–19)	14–20 (14–19)	14–19 (16–19)	14–23 (15–19)
total number of available paired serum samples	13 (2)	102 (22)	25 (3)	93 (19)
H. I. titre[a] I[b] influenza A_2				
< 9	0 (0)	0 (0)	0 (0)	0 (0)
$9 — 100$	3 (0)	13 (4)	3 (0)	17 (5)
> 100	10 (2)	89 (18)	22 (3)	76 (14)
H. I. titre I influenza B				
< 9	1 (0)	19 (5)	4 (1)	25 (7)
$9 — 100$	4 (1)	40 (8)	4 (1)	29 (5)
> 100	8 (1)	43 (9)	17 (1)	39 (7)

a : titres expressed as the reciprocals of the serum dilutions;
b : I means first serum sample;
c : the numbers within brackets apply to the non-vaccinated subjects. The main number applies to all subjects (vaccinated plus non-vaccinated).

in both treatment groups. The remaining 6% of the subjects took their tablets for a period of at least 8 days. The data of these subjects were included in the analysis of the results.

Antibody response to vaccinations: Paired blood samples of 233 subjects (115 1-adamantane amine and 118 placebo) were available for serological testing. The results of the serological analyses by H. I. tests are shown in table II.

The geometric mean titres of H. I. antibodies against influenza A_2 and B in the first serum samples showed no statistically significant[1]

Table 2

Geometric mean titres of H. I. antibodies against influenza A_2 and B and corresponding titre rises in both treatment groups, together with significance of the corresponding differences

	vaccination	serum sample[a]	1-adamantane amine	placebo	P[b]
geometric	influvac	I	185 (165–207)[d]	197 (172–226)	0.50
mean H. I. titre[c],		II	758 (617–929)	837 (704–994)	0.50
Influenza A_2		rise (II/I)	4.1 (3.3 – 5.1)	4.2 (3.6 – 5.1)	0.80
	none	I	189 (142–251)	167 (122–229)	0.55
		II	200 (147–273)	187 (136–258)	0.75
		rise (II/I)	1.1 (0.9 – 1.3)	1.1 (1.0 – 1.3)	0.60
geometric	influvac	I	62 (47 – 83)	60 (45– 79)	0.80
mean H. I. titre,		II	204 (166–251)	279 (227–344)	*0.04*
Influenza B		rise (II/I)	3.3 (2.6 – 4.2)	4.6 (3.7 – 5.9)	*0.04*
	none	I	61 (33 –111)	43 (23– 84)	0.40
		II	64 (35 –115)	52 (27–100)	0.60
		rise (II/I)	1.0 (0.9 – 1.2)	1.2 (1.0 – 1.4)	0.16

a : I means first serum sample.
  II means second serum sample.
b : P values according to Student's t-test (two sided) applied to individual data transformed to logs.
c : titres expressed as the reciprocals of the serum dilutions.
d : within brackets: 95% confidence limits.

[1] = According to the Student's t-test (two sided).

differences in both treatment groups. This applied both to the sera of the vaccinated and those of the non-vaccinated subjects.

The serological response to vaccination consisted of a 4.1 and 4.2 fold increase in geometric mean titre of H. I. antibodies against influenza A_2 in the subjects treated with 1-adamantane amine and placebo respectively. This difference was far from statistically significant[1] (P = 0.80). The geometric mean titer of H. I. antibodies against influenza B showed a 3.3 fold rise in the 1-adamantane amine group and a 4.6 fold rise in the placebo group after vaccination. This difference turned out to be statistically significant[1] (P < 0.04).

In the subjects, who were not vaccinated the geometric mean H. I. titre values of the second serum samples did not show significant increases in both treatment groups. From the individual data it appeared that in none of these subjects a 4 fold or greater rise in H. I. antibody titre against influenza A_2 or B occurred.

The 233 paired sera were additionally tested by complement fixation against influenza A and B. A 4 fold or greater rise did not occur in any of the sera.

These findings show that apparently no spontaneous influenza A or B infection occurred during the trial period.

Adverse reactions and concomitant diseases: Although it is difficult to distinguish clearly complaints resulting from a concomitant disease and adverse reactions, an attempt has been made to classify the reported complaints arbitrarily into three main categories:

1) upper respiratory and febrile illness
2) central nervous system (C. N. S.)-symptoms
3) gastro intestinal (G. I.)-symptoms.

The number of subjects complaining of adverse reactions or symptoms and signs of clinical illness, together with the nature and frequency of the complaints, are shown in table III.

Common cold and malaise were reported less frequently, whereas headache and disturbances of sleep were reported more frequently in the 1-adamanatane amine group than in the placebo group. The difference attained statistical significance[2] with regard to common cold – symptoms (P = 0.05). The total symptom-score in the category of complaints relating to non-influenzal upper respiratory and febrile illness was significantly[2] lower in the 1-adamantane amine treated group (P = 0.005).

The incidence of C. N. S.-symptoms was slightly higher in the drug-treated subjects. This difference did not attain statistical significance.[2]

No effect of 1-adamantane amine treatment was found on the local reactions after vaccination. The incidence of local reactions was 50% in both treatment groups. The detailed results are shown in table IV.

[2] == According to the chi-square test.

Table 3

Adverse reactions and symptoms and signs of clinical illness: number of subjects complaining, nature and frequency of each complaint and of categories of complaints, in both treatment groups

Complaints	1-adamantane amine	placebo	Pa
common cold	12	22	*0.05*
cough with or without fever	2	4	
laryngitis, pharyngitis	3	3	
sinusitis	0	1	
conjuctivitis	1	0	
fever	5	7	
feeling feverish	1	2	
malaise	0	4	0.07
UPPER RESPIRATORY AND FEBRILE ILLNESS	24	43	*0.005*
headache	18	14	
dizziness	4	4	
sleepiness	30	24	
insomnia	3	0	
reatlessness	1	1	
C. N. S. – COMPLAINTS	56	43	0.06
abdominal complaints	4	4	
diarrhoae	0	1	
G. I. – COMPLAINTS	4	5	
hunger	0	1	
thirst	1	3	
dry nose	1	0	
toothache	3	0	
furuncle	0	1	
MISCELLANEOUS	5	5	
total symptom score	89	96	
total number of subjects complaining	64	69	

a : P values according to the chi-square test.

Table 4

Incidence of local reactions after vaccination in both treatment groups

	1-adamantane amine	placebo
number of vaccinated subjects	100	100
number of subjects with local reactions	50	50
nature of local reaction:		
local swelling	14	15
pain	45	44
erythema	17	17
symptom score:	76	76

DISCUSSION

Challenge experiments with live attenuated influenza virus in man have shown that a decreased antibody response occurs in the group treated with 1-adamantane amine (Jackson et al., 1963, Togo et al., 1968).

It has been suggested that this lower antibody response to infection caused by the offending virus in the treated groups resulted from a reduction in virus multiplication by 1-adamantane amine.

Evidence against the hypothesis, that 1-adamantane amine reduces the antibody response by a direct effect on the antibody synthesizing system is provided by laboratory experiments in animals which showed that 1-adamantane amine had no effect on the antibody response to various kinds of antigen (Muldoon et al., 1967, Maciag et al., 1968).

The results in human subjects of the study reported here indicate that the H. I. antibody response to killed influenza vaccine with regard to influenza A_2 was not affected by the administration of 1-adamantane amine, which is in agreement with the evidence obtained so far.

On the contrary, the finding that the H. I. antibody response to influenza B was significantly reduced in the drug treated group does not fit in with the hypothesis generally accepted until now.

As the combination of 1-adamantane amine and vaccination is only to be advised during an influenza A epidemic, this decreased anti influenza B response in treated subjects is of no practical importance with regard to prophylaxis against clinical influenza B illness by vaccination.

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CONCLUSIONS

1. Spontaneous influenza A or B infection did not occur during the trial period.

2. The 1-adamantane amine group and the placebo group turned out to be comparable through adequate randomization.

3. 1-Adamantane amine treatment did not influence the H. I. antibody response to vaccination with regard to influenza A_2.

The antibody response to influenza B was, however, significantly decreased in the treated group.

4. The results indicate a favourable influence of 1-adamantane amine treatment on complaints relating to non-influenzal upper respiratory and febrile illness.

SUMMARY

The influence of 1-adamantane amine treatment on the serological H. I. antibody response to vaccination with an inactivated influenza vaccine, containing influenza A_2 and B antigen, was studied in a double blind placebo controlled trial in healthy volunteers.

1-Adamantane amine was given in 200 mg tablets once daily for 14 days.

The nature and frequency of local reactions to vaccination, complaints related to illness and adverse reactions in both treatment groups are reported.

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PREVENTION OF INFLUENZA A BY AMANTADINE (OBSERVATIONS ON VOLUNTEERS AND FIELD TRIALS INFLUENZA A₂ HONG KONG EPIDEMIC)

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INTRODUCTION

Influenza represents one of the few infectious diseases known to cause short-term, explosive mass outbreaks. Influenza A epidemics occur regularly at intervals of 2 to 3 years and effect up to 50% of the population of all age groups. Hence, more general and specific measures are required to control influenza which is a problem of special scientific and practical importance.

In recent years a new promising drug, amantadine hydrochloride, was developed by E. I. du Pont de Nemours & Co. and used for the oral chemoprophylaxis of influenza.[1—12]

Investigations carried out in 1967 and 1968 by Smorodintsev et al.,[13] showed amantadine hydrochloride to be an effective prophylactic drug when administered to volunteers in daily doses of 100 or 200 mg begun 24 hours before challenge with aerosols of live influenza A2 virus vaccines amade up separately of the strains A2/21/1965, A2/133/1967, and A2/Hong Kong/1/1968. The medications, either placebo or amantadine HCl at a daily dose of 100 or 200 mg, were continued for 11 days.

In the placebo group 80% or more of the susceptible subjects responded to the live influenza vaccine challenge with general constitutional and catarrhal clinical symptoms of various intensity. Amantadine administered at a dose of 100 mg per day was found to prevent clinical illness in 55% of the volunteers. Furthermore, the severity and duration of the symptoms in the subjects who became ill were diminished in the drug-dosed when compared to those given placebo.

A sharp 2-fold decrease in the frequency and intensity of the immune response to the live virus inoculum was also observed in the drug group when compared to the placebo group.

The marked protective effects of amantadine observed in clinical trials on volunteers provided the bases for further studies of the epidemiological effectiveness of the drug during an extensive outbreak of influenza A2/Hong Kong in January–February, 1969 in Leningrad.

MATERIALS AND METHODS

The experiments were carried out by the staffs of the Epidemiological and Clinical Departments of the All-Union Research Institute of Influenza with the participation of specially organized epidemiological teams.

Eight groups of students belonging to the similar professional engeneering high schools aged between 18 and 25 years were selected to participate in the study.

The total study population consisted of 10053 subjects. Individual populations ranged in size from 1200 to 2000 people. Their working and living conditions were essentially the same. They were regularly observed by qualified medical personnel, and those who developed influenza during the study in January–February, 1969 were hospitalized.

The majority of students were living together in the dormitories of each school campus in condition of temporary quarantine during the outbreak semiisolated from the close contact with the Leningrad city population. They were divided randomly into two comparable study groups: one under the code »A«, other under the code »B« which received either amantadine or placebo in one part of study groups and placebo or amantadine in other part of groups. Placebo represented the first control group.

The second control group (the internal control group) comprised 1011 students from the 7 schools but living in their families in the city and received no medication (amantadine or placebo). They were in close contact both with the subjects of study groups and also with the city population. Total number of this group corresponded 10% of the whole study group.

The third control group (external control group, 775 persons) represented students in one similar school (N 8) living in the campus and identical in all living conditions with the study groups but no given any medication (amantadin or placebo).

Each population was divided randomly into two comparable groups of equal size – the experimental (amantadine-dosed) study group and the 3 various control groups (Figure 2). The subjects in the control groups received placebo (placebo group) or no medication (the internal control

group), or comprised a separate group of subjects excluded from any medication and who participated in the study as external controls identical in all living conditions with the other study groups.

Figure 1
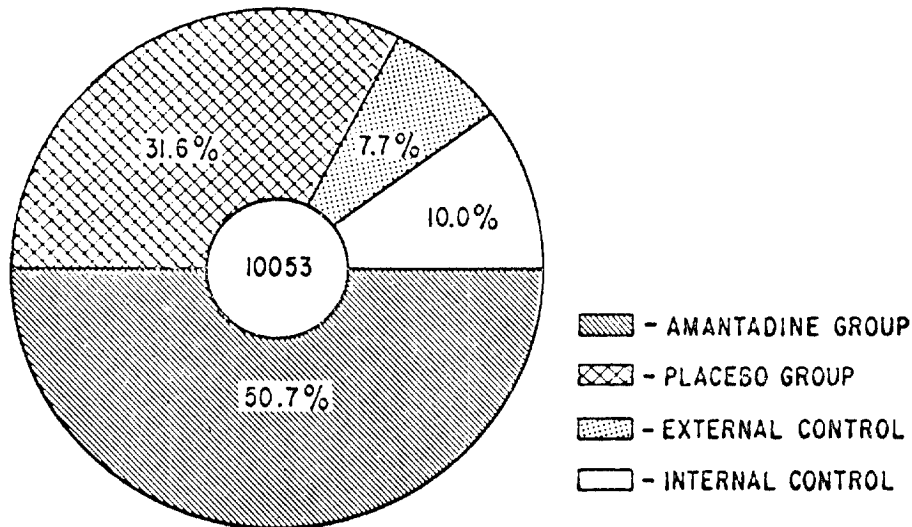
The distribution of the study populations involved in a clinical of the epidemiological effectiveness of amantadine HCl during an outbreak of influenza A2/Hong Kong in Leningrad during January–February, 1969.

Of the 10053 observed unvaccinated individuals, 50.7% received amantadine, and 31.6% placebo medications; 10% of the total population was involved as the internal control and 7.7% as the external control groups. The internal control group differed from the amantadine and placebo groups, being less isolated from the municipal population while the external control group was similar to amantadine or placebo groups in this respect.

The influenza A2/Hong Kong[*] epidemic occurred in Leningrad at the end of the first week of January, 1969 and reached its peak by the end of that month. The populations in the different institutions were involved in the localized outbreaks at different times. A morbidity rate twice normal was an indication for the start of study medication. In three groups, the initiation of dosing coincided with the initial stages of the development of the outbreaks, and in three groups prophylaxis was begun just before the peak of morbidity was reached.

[*] Influenza A-Type viruses were isolated from the populations and were determined to be antigenically similar to influenza A2/Hong Kong/1968 strains supplied by Dr. H. Pereira, WHO Influenza Surveillance Laboratory, London, England. Isolates from the present study outbreak have been identified as influenza A2/Leningrad/69.

Amantadine hydrochloride, supplied by E. I. du Pont de Nemours & Co., was given in capsules or as a syrup; corresponding placebos were of identical appearance.

The amantadine WCl (100 mg) or placebo capsules were administered orally once daily after the evening meal. Amantadine was also successfully administered as a syrup in fruit juices in the same dose of 100 mg once daily. In the majority of the groups dosing continued for 30 days; in populations 3 and 4 amantadine administration lasted for 12 days.

Medications in the various study populations were randomly coded and administered by a double-blind design. Coding was different for each population. The drug code was broken only after all statistical analyses of the morbidity findings were completed.

Medication was distributed by the trained staffs of the epidemiological teams. Using special charts, the same teams kept accurate records of the drug distributions. Medical personnel in charge of the populations observed and the staff from the Influenza Institute continuously controlled the correct distributions of medication.

After termination of the preventive program, 1825 nonill subjects participating in the study were randomly interviewed by the staff, who collected and analysed the answers recorded in special coded charts to ensure double-blind evaluations of side effects.

Since all study groups were under complete medical supervision, it was possible to detect and to hospitalize patients with clinically diagnosed influenza. A case history was recorded for each ill subject. The results of dosing were evaluated taking into consideration the duration and regularity of dosing, any side effects, the general clinical picture of the disease, and serological findings.

The etiology of the illness was confirmed as influenza A2 by using both the complement fixation and hemagglutination inhibition serological tests performed on paired heated sera. The acute specimens were collected during the first days of illness followed by the convalescent specimens 3 to 4 weeks later.

RESULTS

During the course of the preventive program with amantadine some of the subjects missed regular medication due to their temporary absence from the groups. Considering it significant that amantadine is cleared from the organism in approximately 3–5 days, the analyses distinguished between those on continuous or irregular medication schedules. Those who took amantadine without interruption during the entire period of study comprised the majority of participants (84.9%); 10.4% were given medication irregularly or were absent from the study for 1–7 days. Amantadine or placebo dosing was not given for 8 or more days in 4.7% of the subjects; hence, they were not included in the analyses of the results.

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The analyse of the epidemiological effectiveness of amantadine is shown in Table 1.

The findings presented in Table 1 suggest that the amantadine effectiveness in the group which regularly received the drug was almost two times as high as in the placebo group (1.95) and even more (2.15) with the serological correction. Among the subjects irregularly receiving amantadine effectiveness was lacking (IE = 1.1).

The results of the protective effect of amantadine are much more pronounced when comparisons are made of the morbidity rates among the subjects of the amantadine and of the internal control groups with an index of effectiveness of 5.32 and a protective coefficient of 81.2%. Comparisons with the external group give an IE of 3.08 and protection of 67.6%; with the municipal control (an additional external control) an IE of 5.34 and protection of 81.3%.

The morbidity rates in the total study populations, i. e., for the amantadine and placebo groups, were initially similar with the external con-

Table 1

Epidemiological effectiveness of amantadine HCl among subjects regularly receiving the drug during influenza A_2 outbreaks in Leningrad in January–February, 1969

Groups	No. of subjects observed	Clinical findings			
		No. of cases	Morbidity per 100	Effectiveness	
				Index of effectiveness	Index of protective effect (%)
Experimental	3885	156	4.01		
Control (placebo)	2498	195	7.81	[1]1.95 [2]2.15	48.7 53.49
Control (external)	775	214	27.62	[3]3.08	67.6
Control (internal)	1011	216	21.36	5.32	81.2
Municipal Population			[4]21.4	5.34	81.3

1) Level of effectiveness based on clinical diagnosis only.
2) Level of effectiveness corrected by serological results for **confirmation of influenza illness.**
3) These figures are derived from comparative data on morbidity among subjects of external control group No. 8 (8.8 per 100).
4) Index of morbidity for municipal population was calculated during the period of observation of the experimental group.

trol group in the beginning of the outbreak (Figure 2). Later the dosed groups showed only an insignificant rise in morbidity after initiation of treatment while a higher incidence of illness was observed among the unmedicated subjects in the external control group. The difference (more than two-fold) in the intensity of the development of the outbreaks in these groups was statistically significant.

Figure 2
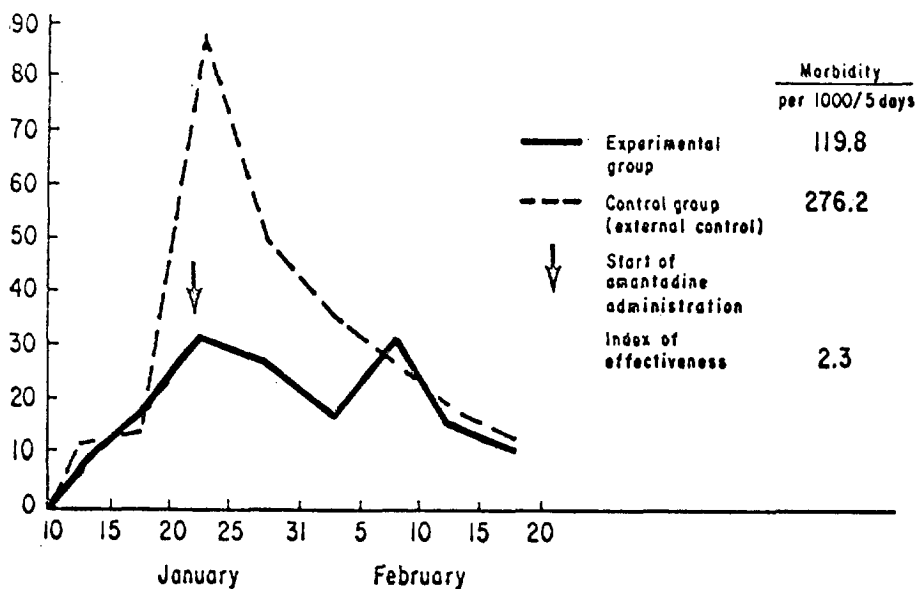
Morbidity of influenza among subjects of the experimental (dosed) and control groups during the epidemic of influenza A2/Hong Kong in Leningrad, January–February, 1969.

It is noteworthy that the levels and durations of influenza morbidity were different in study populations in the groups receiving amantadine, placebo, or no medication (internal control). Figure 3 presents the morbidity curves in one of the populations observed prior to the initiation of medication in the experimental group (from 10th to 20th January) and also after the start of the amantadine or placebo program (from 21st January). After the drug was started, the patterns of the development of the outbreak changed in these dosed-groups. Morbidity among the subjects of the placebo group continued to rise, while it dropped in the amantadine group and remained at the lower level during the entire period of the outbreak.

The duration and intensity of the outbreak in the internal control group not given medication were quite different from those observed in
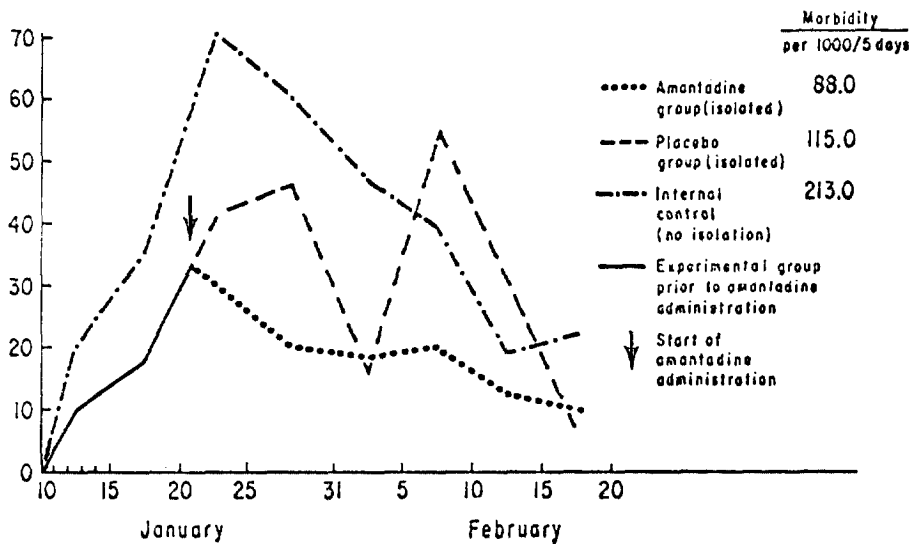
Figure 3

Morbidity of influenza A2 among subjects of three subgroups of Population Group No. 6 in January–February, 1969.

the previous groups given amantadine or placebo. The rise in morbidity occurred at an earlier time and reached a higher peak than the level of morbidity seen in the placebo group. It seems likely that persons from this control group living in more intensive contact with the manicipal population were responsible for the introduction of the influenza A2 virus into their institutions. The total morbidity rate in the internal control group was two times as high as in the placebo group and exceeded that of the group protected by amantadine almost 3 times.

Table 2 presents dates on the morbidity rates of the amantadine and placebo groups in all 7 schools studied. The index of effectiveness calculated according the clinical findings ranged from 1.15 to 2.53 and according the serological correction from 1.57 to 3.44. That means the regular reduction of influenza cases in all 7 schools studied.

Of special interest is the influence of prophylactic amantadine treatment on the course of influenza in those patients who developed disease in spite of dosing.

The clinical pictures of the influenza which developed in those subjects receiving amantadine or placebo were studied by examinations of the clinical symptoms and of the results of serological assay of 400 influenza cases selected at random. Among them 200 patients received

Table 2

The Effectiveness of Amantadine in 7 Groups Studied

Groups	Medication	No. of subjects observed	Morbidity per 100 before amantadine or placebo were given	Subjects Regularly Receiving the Drug			Index of Effectiveness	
				No. of subjects observed	Morbidity		according the clinical findings	according the serological findings
					total	per 100		
N. 2	amantadine placebo	680 271	16.74 12.53	621 238	13 10	2.09 4.20	2.0	2.73
N. 3	amantadine placebo	590 455	12.19 24.32	504 366	21 28	4.16 7.65	1.83	2.50
N. 4	amantadine placebo	502 471	5.28 5.56	377 407	32 46	8.48 11.30	1.33	1.81
N. 5	amantadine placebo	651 510	3.24 2.65	629 498	7 18	1.10 1.27	1.15	1.57
N. 6	amantadine placebo	705 391	2.60 2.46	635 348	57 40	8.97 11.49	1.28	1.74
N. 7	amantadine placebo	296 263	2.62 2.91	247 234	14 35	6.17 15.38	2.53	3.44
N. I received before influenza live vaccine	amantadine placebo	1135 443	10.53 10.44	872 407	12 18	1.39 3.50	2.51	3.43
Total	amantadine placebo	4559 2804	8.66 9.66	3885 2498	156 195	4.0 7.8	1.95	2.15

amantadine and 200 placebo (Table 3). The severity of the symptoms in the amantadine and placebo groups differed so that 56% of the subjects from the amantadine group and 38% of those from the placebo group developed mild symptoms (P 0.001). Severe symptoms were observed in 9% of the subjects in the amantadine group and in 19% of the subjects from the placebo group (P 0.01).

Table 3

Severity of the Symptoms in Those Subjects WHO Became Ill While on Amantadine HCl or Placebo

Symptoms	Amantadine		Placebo		Statistical significance	
	No. of cases	%	No. of cases	%	t	P
Mild	112	56.0	76	38.0	3.83	<0.001
Moderate	70	35.0	86	43.0	1.6	>0.05
Severe	18	9.0	38	19.0	2.9	<0.01
Total	200	100.0	200	100.0		

The findings obtained suggest that milder forms of influenza developed in the amantadine group compared to those given placebo. Such symptoms as chilliness, pain in the eyes, nausea, malaise, and some catarrhal symptoms (rhinitis, wet-cough, pharyngitis) were significantly less frequent in the amantadine group (Table 4). More severe forms (+ + + constitutional symptoms rating) of influenza disease were found

Table 4

Severity Ratings of the Constitutional and Catarrhal Symptoms in Those Subjects Who Became Ill While on Amantadine HCl (Experimental Group) or Placebo (Control Group)

Group	No. of cases	Constiutional symptoms				Catarrhal symptoms			
		0	+	+ +	+ + +	0	+	+ +	+ + +
experimental	200	30	33	28	9	19	58	23	—
control	200	7	36	38	19	8	49	35	8
t		6.2	0.6	21.	2.8	3.3	1.8	2.6	
p		<0.001	>0.05	<0.05	<0.01	<0.001	>0.05	<0.01	

in 9% of the subjects given amantadine and in 19% of those from the placebo group, a significant difference (P 0.0.). While catarrhal symptoms were lacking in 19% of the amantadine group, this was evident in only 8% of the placebo group (P 0.001).

Reductions of the general signs and symptoms of illness, as well as of the catarrhal symptoms, associated with prophylactic amantadine administration can be related to the initial severity of the disease (Table 5). Thus, the mean durations of fever were 2.6 days in the amantadine group and 3.2 days in the placebo group. Mean durations of the influenzal syndrome were 2.5 and 3.4 days and of the catarrhal syndrome 4.2 and 5.7 days respectively for the drug- and placebo-dosed groups.

Random serological examination of the patients with clinically diagnosed influenza revealed that a high proportion of erroneous clinical diagnoses were associated with respiratory infections of non-influenza etiology. Among the 291 patients studied, 12 had adenovirus, 9 – parainfluenza, 12 – respiratory-syncitial, 3 – influenza type B, 1 – mycoplasma, and 10 – mixed infections. Thus, 47 (16.1%) of the 291 ill subjects examined were not involved with an influenza A2 infection but had acute respiratory diseases of different etiologies which would not be expected to respond to amantadine. In this context, it must be borne in mind that the preventive effectiveness of amantadine, and its clinical effectiveness in the ill subjects, were evaluated in populations and patients which included considerable numbers of instances of respiratory illnesses in which amantadine could not obviously prevent or relieve symptoms.

Table 5

Durations of the Febrile Reaction, Influenza Syndrome And Catarrhal Symptoms in the Amantadine or Placebo Groups Related to the Severity of the Disease

Types	Groups	Mean duration (days)			
		Mild disease	Moderate disease	Severe disease	Mean
Febrile	Amantadine	2.0	3.4	3.7	2.6*
reaction	Placebo	2.7	3.6	3.4	3.2*
Influenza	Amantadine	1.9	3.1	4.1	2.5*
syndrome	Placebo	2.7	3.6	4.6	3.4*
Catarrhal	Amantadine	4.2	3.8	5.4	4.2*
symptoms	Placebo	5.2	5.5	7.2	5.7*

* = P < 0.05

Serological findings suggested involvement of influenza A virus in 42.1% of the subjects from the amantadine and in 58.4% from the placebo groups (Table 6). However, as has been reported previously[8, 13] there was a significant difference in the intensity of the immune responses between the groups dosed with amantadine or placebo.

Table 6

Serological Findings Among Subjects of the Experimental (Amantadine)
and Control (Placebo) Groups

Groups	No. of patients examined	With 4-fold or more rise in antibodies		Mean geometric titers of serum antibodies		Mean geometric value of multiplicity of rise in antibodies
		No. of cases	%	Acute	Convalescent	
Experimental	107	45*	42.1	2.1 lg 2 = = 1:4.0	4.71 lg 2 = = 1:26	1.52
Control	137	80*	58.4	3.7 lg 2 = = 1:13.9	5.9 lg 2 = = 1:60	2.06

* = P < 0.01

Since some authors have reported amantadine to produce side effects,[12, 14] particular attention was given to this area in the course of these studies. 1825 subjects from the amantadine or placebo groups who did not become ill were asked to fill out anonymous patient check lists. The results thus obtained are presented in Table 7. The total number of subjects in the amantadine group who registered complaints in excess of the placebo group was 1.9%. Most reliable were the complaints of various sleep disturbances and of dyspepsia described by 1.14% and an excess of 1.7% of the subjects in the amantadine group respectively. Other side effects were statistically nonsignificant.

DISCUSSION

Epidemiological and clinical observations of the effectiveness of amantadine HCl were carried out in January–February, 1969 in Leningrad during intensive outbreaks of influenza due to influenza A2 viruses related to the A2/Hong Kong/68 variants. Of the 164 virus strains isolated from patients in this period, about half of whom belonged to the patients included in this study, all were related to the influenza A2/Hong Kong/68 viruses according to their antigenic and biological properties.

The same etiology was confirmed by serological examinations of paired heated sera in hemagglutination inhibition tests which showed a distinct prevalence of antibodies that neutralized A2/Hong Kong/1968–1969 strains. This was in contrast with a lower level of antibody to influenza A2/21/65.

Table 7

Side Effects of Amantadine Under Conditions of Prophylactic Administration with Daily Doses of 100 mg

Groups	No. of subjects questioned	No. of complaining subjects		Complaints of:					
				Dyspepsia		Sleep deterioration-malaise		Others	
		Total	%	Total	%	Total	%	Total	%	Total	%
1	2	3	4	5	6	7	8	9	10	11	12
Experimental	1313	94	7.1	43	3.28	15	1.14	27	2.06	9	0.68
Control	512	26	5.2	8	1.56	—	—	14	2.74	4	0.78

Differences in figures indicated in columns 6 and 8 are statistically significant (with confidence level of 5% and 1% respectivelly).

CONCLUSIONS

1) During an epidemic of influenza due to influenza viruses related to influenza A2/Hong Kong/1968 variants the prophylactic effectiveness of amantadine HCl was studied under conditions of double-blind, inert placebo-controlled tests involving adults in six similar institutions. In all, 10,053 persons participated in the study.

2) According to the summarized clinical findings the index of amantadine effectiveness in the total group was 1.7. When only those subjects are considered who received amantadine regularly, the index of effectiveness based on clinical diagnosis was increased to 1.95. When the clinical diagnoses are corrected by serological confirmatory tests, the index of effectiveness rises to 2.15. A comparison of the drug-treated group with the nonmedicated internal control group showed an index of effectiveness of 5.32.

3) Preventive treatment with amantadine resulted in the reduction of influenza A2 morbidity not only in the drug group, but also in the side-by-side placebo-dosed controls.

4) Clinical examination of patients continuously receiving amantadine or placebo beginning prior to the influenza attack showed that regular administration of amantadine at a daily dose of 100 mg reduced not only

the incidence of influenza infection, but also the severity and duration of symptoms in those subjects who became ill.

5) Administration of amantadine to healthy young subjects at a daily dose of 100 mg after a meal was associated with a 1.14% increase in the number of complaints of various sleep disturbances with no interference with working capacity. This quite insignificant difference in the amantadine group cannot be considered a contraindication to the wide general application of the drug.

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TO THE PROBLEM OF DEVELOPMENT ON PARAINFLUENZA VACCINES

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The great role of parainfluenza viruses in ethiology of acute respiratory diseases raises the problem of prophylactic measures, and particularly, that of developing parainfluenza vaccines. The problem can be solved on the basis of knowledge of basic regularities of creating immunity in separate organisms, in population as a whole, by developing laboratory models for experimental studies.

Appearance of circulating antibodies serves one of the basic indications of immunological response. Applying this criterion to parainfluenza infections one may state that each of the 4 Types of parainfluenza viruses is sufficiently immunogenic, since all virologically proved cases of infection described in literature were accompanied by vigorous increases in antibody levels during the convalescence period (Chanock et al., 1956, 1958, 1959; Petersen, 1959; Bukrinskaya and Pactoris, 1960; Zlatkovskaya et al., 1961; Zakstelskaya et al., 1963).

How long are the antibodies maintained? According to Rendfort et al (1963), antibodies to the 3rd Type of parainfluenza virus persist for 16 months. We had under observation for about a year 14 children recovering after infections caused by Type 3 virus, and 8 children-reconvalescents after infections caused by Type 1 virus.

Complement-fixing antibodies to Type 3 virus appeared to be more stable and were maintained without change in the course of 3 months in 11 children out of 14, later they gradually decreased but did not disappear completely, and could be detected in titers of 1 : 20 – 1 : 40 even after 8 to 10 months. With parainfluenza virus of Type one, on the contrary, the antibodies acquired as a result of infection decreased in titer already in the second month, and by 4 to 6 months disappeared completely or dropped to the level of 1 : 10.

In addition, Dr. B. Arnaudova together with workers of our laboratory (1969), on material collected in Bulgaria, made special observations on persistence of antibodies to parainfluenza viruses which pass to newborns from their mothers. By regular observations on 22 infants, with monthly blood tests, it was shown that antibodies to Type 1 parainfluenza virus were lost most quickly and that antibodies to Type 3 virus were maintained longer than others (Fig. 1). This data corresponds to

Figure 1
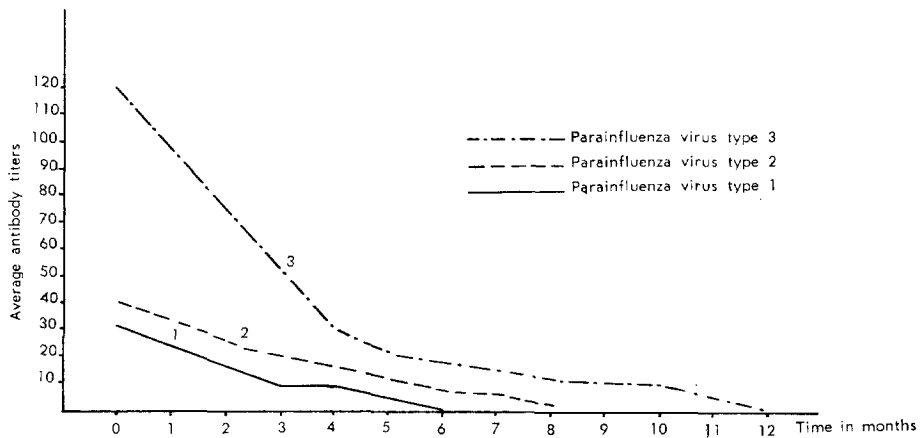
The persistence of passively acquired antibodies to parainfluenza viruses in infants' blood

experimental observations of Cook and Chanock (1963) who showed that antibodies developed in hamstars after infection with Type 3 parainfluenza virus persisted without considerable change during 3 months, while those developed to Type 1 virus dropped almost to zero already in a month.

Next, we (Efimova et al. 1963) studied age distribution of antibodies (antihemagglutinins) to parainfluenza viruses in blood sera of subjects from 0 to 90 years of age. It was established that only in infants under 1 year and in persons aged 26 to 30 years could there be found sera free of antibodies to all three types of parainfluenza viruses (Table 1).

In children up to three years of age antibodies were found only to one of the types, mostly to Type 3, or to two types of parainfluenza viruses; while at the ages 4–6, 8–10, and over 26 years the majority of sera contained all the three types of antibodies.

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Table 1

Comparative frequency of detection of parainfluenza virus antihemagglutinins
in people of different age groups

Age in years	Number of sera tested	Sera free from anti-bodies	Sera possessing antibodies		
			To one of the virus types	To two of the virus types	To three virus types
0— 1	10	3	6	1	—
1— 2	6	—	5	1	—
2— 3	5	—	2	3	—
3— 4	7	—	2	4	1
4— 5	8	—	3	1	4
5— 6	5	—	—	2	3
6— 7	8	—	2	6	—
7— 8	7	—	1	5	1
8— 9	4	—	—	2	2
9—10	7	—	—	4	3
10—11	5	—	—	5	—
22—25	7	—	2	4	1
26—30	13	2	2	5	4
31—35	18	—	4	8	6
41—45	11	1	2	5	3
36—40	22	1	4	6	11
46—50	13	—	2	5	6

When serogramms characterizing distribution of average antibody levels with age were determined (Fig. 2), it was found that the curves of Type 2 and 3 of parainfluenza viruses were of usual character, peculiar to agents epidemically circulating in population and invading human organism at an early child age. The serogramms of parainfluenza Type 1 virus were different from others and resembled those peculiar to influenza viruses. The reason for that is not quite clear and seems to be connected either with poor immunogenicity of Type 1 parainfluenza virus (Jensen et al., 1962) or with the fact that with this Type of the virus, just like with influenza viruses proper there are years of high and low circulation among population. However it may be, the investigation of age distribution of antibody levels to parainfluenza viruses has revealed that antibodies gained after natural infection are sufficiently stable, and being supported by encounters with circulating viruses, can be maintained until an old age.

What is the role of antibodies in immunity? Observations made in our work, which fully agree with findings of other authors (Dick, Mogabgab,

Figure 2
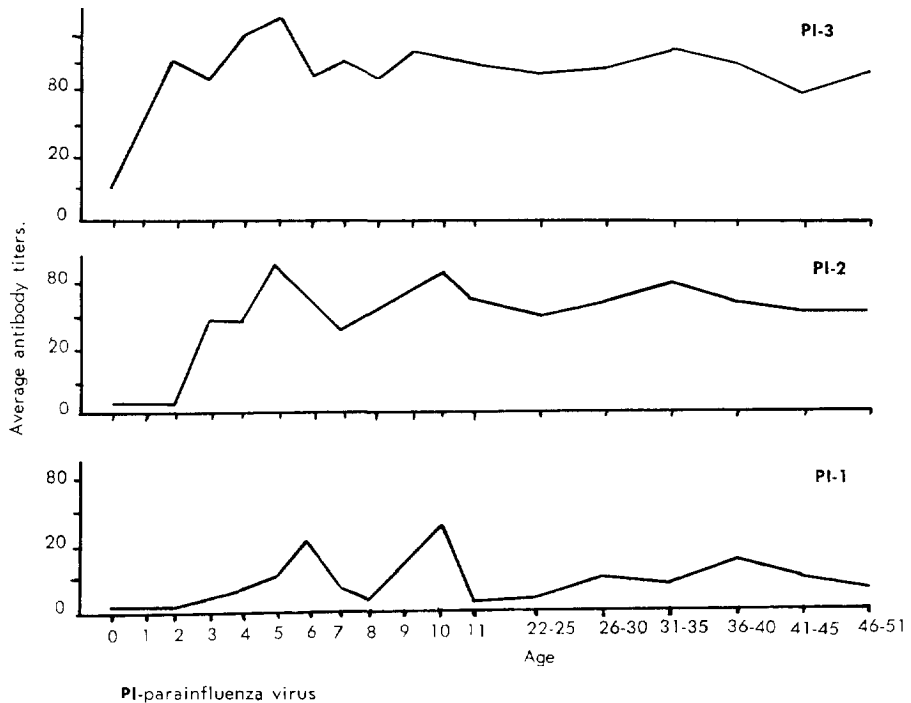
The age distribution of average antibody titers to different virus types

1961; Evans et al., 1963; Chanock et al., 1965), showed that in children of young age development of parainfluenza infections, of Types 1, 2 and 3, took place as a rule at the background of absence of homologous antibodies in their blood.

Children of older age and adults fall ill with the disease in spite of the presence of antibodies in their acute period sera in titers 1 : 20 to 1 : 40, and subsequently develop increases to the level of 1 : 60 to 1 : 320 (Table 2). Since patients' sera are not tested before the disease one cannot state whether the initial antibodies were present before the disease or were developed as a result of rapid mobilization of immune mechanisms on reinfection of adult persons. Dick and Bogabgab (1961), comparing antibody levels in students who fell ill and did not fall ill with the disease, found that, as a rule, sick persons either had no antibodies or had them in low titer.

These findings allow to suppose that antibodies in parainfluenza infections are indication of immunological response and constitute one of

Table 2

The correlation between age and antibody level in sera of parainfluenza patients

Age in years	Virus Type	Number of patients	No anti-bodies in sera	Possessed homologous antibodies in the titer of:				
				10	20	40	80	160
0— 3	PI–1	13	13	—	—	—	—	—
	PI–2	13	13	—	—	—	—	—
	PI–3	19	13	3	2	1	—	—
3—10	PI–1	14	12	1	1	—	—	—
	PI–2	19	2	4	4	4	3	2
	PI–3	26	2	4	10	4	5	1
Over 10 years	PI–1	15	8	1	3	3	n. s.	n. s.
	PI–2	22	1	3	4	8	5	1
	PI–3	23	—	3	6	6	5	3

Note: n. s. – denotes not studied.

basic factors of acquired immunity, though not determine it completely. There is sufficient ground, therefore, to believe that artificial immunity to parainfluenza infections can be created by immunization.

The following problems have to be solved for development of parainfluenza vaccines:

a) A convenient and cheap source of virus-containing materals is to be found;

b) Methods of purification and concentration of antigens should be developed;

c) A laboratory model should be chosen for testing immunogenic properties of preparations;

d) The possibility should be tried of combining parainfluenza antigens with preparations already in use in child immunizations, as well as with antigens of other respiratory viral infections.

Our investigations of sensitivity of several tissue cultures to parainfluenza viruses showed that primary cultures of kidney tissue of new-borne guinea-pigs could be used as the virus source. Viruses of the 2nd and 3d Types yielded in these conditions 1,000 to 10,000 Tissue-Infecting Doses (TID) per ml, while parainfluenza Type 1 virus reached the concentration of 10,000,000 TID/ml.

Sedimentation in supercentrifugals is most frequently used to concentrate antigens of parainfluenza viruses, but the method is not practicil with large volumes. A study of purification methods (Zakstelskaya L. Y. et al., 1963) showed that highly concentrated parainfluenza virus antigens could be obtained using adsorbtion on formalized guinea-pig

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erythrocytes followed by elution with hypertonic NaCl solution. The antigens have been successfully used as standardized diagnostics and as the starting material for immunizing preparations, which were found highly specific and possessed strong immunizing potency. In experiments with rabbits, rats, and mice (Table 3) it was shown that the antigens stimulated rapid production of antibodies and could be used both for primary vaccination and for the booster-dose.

Table 3

Immunizing activity of native and purified antigens of parainfluenza viruses

Virus	Animal	Preparation	HA-inhibition titers	
			Before immunization	After immunization
PI–1	Rabbits	Native	1:10	1:80
		Purified	1:10	1:320
PI–2	Rabbits	Native	1:10	1:640
		Purified	1:10	1:2560
PI–3	Rabbits	Native	1:10	1:320
		Purified	1:10	1:640
PI–3	Rats	Native	1:10	1:2560
		Purified	1:10	1:2560
PI–3	Mice	Native	1:10	1:640
		Purified	1:10	1:1280

For testing immunogenic properties of vaccinal preparations it is necessary ot have a laboratory model with an animal which would be susceptible to the particular virus and sufficiently responsive to its antigen. Our investigations have shown that albino mice and white rats provide such a model. They both can be infected with parainfluenza viruses and andergo symptomless infection accompanied by antibody response.

Intensive antibody formation takes place in mice and rats given the antigen by intranasal, subcutaneous or intraperitoneal route. Clear-cut response is obtained with single administration of the antigen, which allows to use mice for estimation of immunogenicity of killed parainfluenza virus vaccines by end-point titrations. These findings allow, in our opinion, to recommend albino mice as a model for studying immunogenic properties of parainfluenza viruses.

An essential point for development of parainfluenza vaccines is the possibility of adsorbtion of the purified antigens on a deponating ma-

terial – the alum (Al_2O_3). Experiments made in our laboratory in this respect showed that purified and concentrated parainfluenza antigens were fully adsorbed on the alum.

Further, we tested the possibility of associating the parainfluenza antigens with those of other respiratory viruses and of agents of bacterial child infections. The experiments showed that the purified and concentrated parainfluenza antigens continued to be adsorbable totally on the alum which had already been used for adsorbtion of influenza virus antigen or adenovirus antigen, or both. Besides, it appeared possible to adsorb the parainfluenza antigens of the alum loaded with the diphtheria-tetanus toxoid. The parainfluenza virus antigens, adsorbed on the alum, iritiated antibody response when used for immunization of animals.

Experimental findings reported in this communication may be of some contribution to solving the problem of prophylaxis of acute respiratory diseases.

SUMMARY

Some problems concerning parainfluenza vaccines were discussed on the base of experimental studies of parainfluenza viruses, data of distribution by age of antibody to parainfluenza viruses in people and analysis of literature.

The following steps in the development of parainfluenza vaccines have been found to be most important:

a) A convenient and cheap source of virus-containing material is to be found;

b) Methods of purification and concentration of antigens should be developed;

c) A laboratory model should be chosen for testing immunogenic properties of preparations;

d) The possibility should be tried of combining parainfluenza antigens with preparations already in use in child immunizations, as well as with antigens of other respiratory viral infections.

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FREE THEMES

THE CHOICE OF RUBELLA VACCINES

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Rubella is not a serious disease for children but it can cause tragedies when a woman in early pregnancy comes into contact with the virus.

One vaccine, Cendehill grown on rabbit kidney, has been licenced in Switzerland and Belgium whereas a different strain HPV 77 has been approved for use in America. It is pertinent, therefore, for us to review the availability of vaccines and consider what may be the most expedient choice.

There have already been two major conferences, one in London (November 1968) and one in Washington (1969) at which the detailed findings were presented. My intention today is to crystalize a few thoughts many of which may be personal.

There are 4 factors that must be taken into acount when choosing a vaccine. These are:

    I freedom from reactivity – both short term and long term

    II acceptability

    III antibody response and persistence of immunity

    IV the age group requiring immunization.

I *Freedom from reactivity*

The freedom of reactivity can be measured only by inoculation into humans. It is an unusual event when animal tests can tell us how reactive a product may be in man and it is much more likely that they are capable only of rejecting the most toxic materials. Such was the case with measles in which Enders Edmonston B, Beckenham 31 and Schwarz

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strains behave identically in animals whereas their reactivity in children is quite different, largely due to the pyrexia caused by the viraemia. Since pyrexia in young children may lead to convulsions such a complication caused by a vaccine cannot be predicted in laboratory animals. Nevertheless, animal tests are most useful especially where there is a measurable parameter showing a difference between a wild and an attenuated strain, we are most fortunate if the degree of attenuation can be determined in this way.

Thus the HPV 77 strain was considered satisfactory for use in children when it no longer produced an exanthema and virus excretion in monkeys. Similarly the Cendehill strain after attenuation no longer infected monkeys or rabbits.

The short term reactions to rubella vaccine are arthralgia and arthritis. These are sequelae of the natural disease but it was hoped that they would disappear with attenuation of the strain and the three virus strains HPV 77, Cendehill and RA 27/3 have different degrees of reactivity in this respect. The majority of work with HPV 77 was done in children, in which there are no such complications, but in adult females the incidence of reactions has been as high as 30% some of which have been most uncomfortable requiring hydrocortisone therapy. The other two strains although not entirely free from complications the incidence and severity are much reduced bringing them into the realms of acceptability. Of course in children they are practically asymptomatic.

We can not leave the section on reactivity without considering the long term effects of the vaccine. The modern techniques of virology such as immunofluorescent staining and electron microscopy are now detecting virus like particles in cell substrates that have hitherto been undetectable. Thus monkey kidney cell cultures are shown to be contaminated by such viruses on frequent occasions. Monkey kidney, as a cell substrate, therefore, is now being looked upon with some degree of suspicion and with it the virus harvests from such tissue can not be regarded as being free from such viruses.

For this reason the use of HPV 77 virus must be reviewed with caution. This virus has had 77 opportunities of hybridising with a monkey passenger virus and although it may not have happened, and indeed such a hybrid may be harmless, nevertheless there is a possibility of such an event. Growth of the virus on a tissue different from monkey kidney may help to suppress a monkey passenger virus but such a virus harvest can never be regarded as being clean. Indeed in the recent Hong Kong influenza episode American manufacturers were forbidden to use HK/1/68 strain for vaccine production because the initial isolation was made on monkey kidney cell cultures. Here we have a situation of a virus population passaged once on monkey kidney cells which would subsequently be inactivated and given to adults being banned, whereas another virus passaged 77 times in monkey kidney cell cultures is being given live to children. I am still searching for the logical explanation of this.

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In this respect the Cendehill strain was also originally isolated on monkey kidney cell cultures but since that time it has been attenuated by 51 passages on rabbit tissue. The only strain that has never been in a foreign tissue is the RA 27/3 strain which has been attenuated in human tissues by growing at a low temperature.

II *Acceptability*

Acceptability of any vaccine is a balance between the possibility of having in untoward reaction and the severity of the disease. Two centuries ago variolation, that is passing the infected puss from a case of smallpox into a healthy subject, was acceptable. In those days the chances of dying by variolation was much less than falling victim to the disease. With the eradication of smallpox, however, such a procedure is totally unacceptable and indeed the use of vaccines available today with their very low rate of reactions are being regarded as presenting an unnecessary hazard.

Rubella is not a danger to children and therefore the use of any vaccine giving complications is not acceptable. Fortunately none of the 3 strains cause reactions in children. As we have said the danger of the disease is to the adult female but only when she is in the first trimester of pregnancy. The problems of vaccinating adult females therefore is in ensuring that the vaccine virus does not cross the placenta and cause similar damage as a wild virus to the foetus. Making sure that all vaccinees are not pregnant in this early phase of motherhood is not easy and several procedures have been resorted to in order to avoid this. Clearly it is much more satisfactory to know that the vaccine virus is safe even in the pregnant female. Such data are available only on a limited number of subjects and more data are required.

Also under the heading of acceptability we must consider the method of administration. The majority of studies have considered only the parenteral route. One strain, however, Ra 27/3 has been successful when given as a spray intranasally. This may become a more acceptable method of administration and these trials are being followed with great interest.

III *Antibody response and persistence of immunity*

In the many trials that have been undertaken it seems a general finding that about 95% of seronegative subjects, children or adults, give a good antibody response. There is nothing to choose between the vaccines although claims have been made for marginal superiority of HPV 77.

The crux of the matter is the persistence of antibody and immunity. The vaccines are made from live attenuated strains which simulate a mild natural infection by multiplying in the nasopharyngeal area, giving a viraemia and stimulate antibody production. It is hoped therefore,

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that lifelong immunity will be conferred upon the vaccinee but this can not be assumed. Using measles as a parallel it is known that imunity lasts for several years but this is not sufficient to be regarded as long lasting. This question with rubella as with measles must remain constantly under review since none of the vaccines has been used for a sufficiently long time to provide useful data.

IV *The age group requiring immunization*

There is a strong argument in favour of eliminating the disease at source, that removing the virus from the child population so much responsible for the spread of the disease. However, this is a unique disease and there are more facets to be considered. Since it is such a benign illness in children it will be the first time that it must be suggested that half the child population, the boys, should expose themselves to the risk, small though it may be of vaccination simply for the sake of being public spirited. The vaccine can be of no material benefit to them and yet the disease will not be eliminated by vaccinating only the girls. A further important concern about immunization of children is the persistence of immunity. If a child is given vaccine in the second year of life on the assumption that life long immunity will follow then it would be tragic for her to contract the disease at a time when she was having her first child. The proponents of immunization in childhood feel that in such cases a booster dose should be given but there seems little point in protecting a child against a benign disease through a period of life when protection is not necessary. Indeed if left unprotected she may have the natural disease that would certainly give life long immunity.

In England we have not defined a policy of immunization so far but it is probable that immunization will take place in three phases.

a) The group at greatest risk are the 15% of pregnant women about to deliver their baby and who are known to be seronegative. Such women will be offered vaccine in the immediate postpartem period since they will be at greatest risk when their babies grow up and bring the disease into the homes.

b) The girls at school between the ages of 11 and 13 when pregnancy is a rare event would be offered vaccine. This could well be fitted in with their visits for BCG vaccination and such a scheme is currently on trial.

c) It is understood that immunization of the groups in (a) and (b) will not remove the disease from the community but only in the event that the groups (a) and (b) were successfully immunized without untoward reactions would children be considered.

There will be much discussion in the U. K. before rubella immunization takes a place in the routing immunization schedules for children.

The most important immediate aim is to make sure that all women have rubella antibodies before they become pregnant.

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ATTENUATION OF RUBELLA VIRUS IN HUMAN DIPLOID CELLS

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Recently, rubella vaccines were licensed in several countries. Although the considerable publicity which attended these events gave the impression that the rubella problem had been solved, there are still a number of questions concerning vaccination which must be considered. This should not be surprising as it is a well-known fact that licensing of a vaccine does not signify that all problems concerning its use are solved.

The most pressing uncertainty about rubella vaccine at the moment is the definition of the adequacy of vaccine-induced immunity. The HPV-77 strain, recently licensed in the United States, is given by the subcutaneous route. After subcutaneous vaccination, viremia occurs occassionally, and in the majority of cases, virus excretion is detected in the nasopharynx. In several studies of volunteers who had been previously vaccinated with HPV-77 and then challenged with a wild rubella virus strain, it was disturbing to note that a significant proportion of subjects developed virus carriage in the nasopharynx and serologic evidence of reinfection. Moreover, even more disturbing was the observation by Grayston and his group in Taiwan that school children previously vaccinated with HPV-77 were re-infected during the epidemic of natural rubella, despite the fact that they themselves were largely protected against clinical manifestations of rubella. Thus, HPV-77 induced immunity seems to be incomplete at least with regard to what might be called mucosal immunity.

Recently, the concept of mucosal immunity has become much better understood. Most viral pathogens have as their primary sites of multiplication either the intestines or the respiratory tract. Although dissemination of the agent may occur from the sites, the fate of the infecting

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organism is decided at the mucosal surfaces. Resistance or susceptibility to the virus is thus a function of local immunity. In recent years, it has heen shown that local immunity depends on the secretion of specific antibodies contained in the IGA fraction of gammaglobulin. The function of local nasal antibodies in rubella has not yet been defined. It now seems urgent to fill this lacuna in our knowledge. Our own work with the RA 27/3 strain of rubella vaccine has bearing on this problem, for this particular strain has the ability to immunize by the intranasal route.

The results achieved with the RA 27/3 strain given subcutaneously were as satisfactory as those reported for other strains. Large-scale studies using RA 27/3 subcutaneously are going forward in Britain, France, and elsewhere. Recently, we have been endeavoring to define the immunogenicity, reliability, and feasability of the intranasal method of administration.

A number of titrations of virus given by the intranasal route have been performed, both in Philadelphia and elsewhere. The first Table shows a titration performed in school girls in a school in Philadelphia. The subcutaneously vaccinated girls all developed antibodies as did the girls given 1,000 tissue culture doses of virus and those given 500 tissue culture doses of virus. On the other hand only 50% of the girls given 100 tissue culture doses of virus developed antibodies.

Table 1

Titration of intranasal vaccine in seronegative schoolgirls-trial J

Route of Vaccination	Dose in PFU	No. Seroconv. No. Vacc.	HAI Titers Post-Vacc.* Median	Range
Subcutaneous	500	7/7	40	20–80
Intranasal	1,000	6/6	40	40–160
Intranasal	500	5/5	40	20–80
Intranasal	100	4/8	(120)**	40–320**

* Four weeks
** Only those subjects who responded with antibody

Another similar titration was performed in an institution for retarded children as shown in the next table 2. One ml of the virus administered was equivalent to 1,000 tissue culture doses. All the children given 1,000 or 500 tissue culture doses developed rubella antibodies, whereas as many of those given 100 tissue culture doses failed to develop antibodies. In this particular study an attenmpt was made to atomize virus into the nose by aerosol. Inconstant results were obtained probably because much of the dose was lost in the process of atomization in the De Vilbiss atomizer.

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Table 2

Hamburg Trial 11/5/68 Vaccine J2500

Child No.	Route	Dose	HAI Antibodies	
			Pre	Post
PA 231	IN–drops	1.0 ml	< 10	80
PA 236	IN–drops	1.0 ml	< 10	80
PA 237	IN–drops	1.0 ml	< 10	80
PA 177	IN–drops	1.0 ml	< 10	80
PA 146	IN–drops	1.0 ml	< 10	160
PA 238	IN–drops	0.5 ml*	< 10	80
PA 239	IN–drops	0.5 ml*	< 10	80
PA 240	IN–drops	0.5 ml*	< 10	80
PA 227	IN–drops	0.1 ml*	< 10	< 10
PA 228	IN–drops	0.1 ml*	< 10	< 10
PA 243	IN–drops	0.1 ml*	< 10	80
PA 246	IN–drops	0.1 ml*	< 10	10
PA 169	IN–drops	0.1 ml*	< 10	80
PA 164	IN–drops	0.1 ml*	< 10	40
PA 170	IN–atomizer*	1.0 ml	< 10	80
PA 88	IN–atomizer*	1.0 ml	< 10	80
PA 83	IN–atomizer*	1.0 ml	< 10	< 10
PA 85	IN–atomizer*	1.0 ml	< 10	< 10
PA 214	throat swab	0.5 ml	< 10	40
PA 89	throat swab	0.5 ml	< 10	< 10

* Diluted to 1 ml
— de Vilbiss

Another titration was performed in an institution for retarded children. Here the results, shown on the table 3, were slightly different. High titered virus containing 10,000 tissue culture doses was used. Nine out of ten of those who received this dose developed antibodies. On the other hand, only two out of six given 1,000 tissue culture doses intranasally developed antibodies. One out of five given 100 tissue culture doses became positive.

Table 3

Titration of intranasal RA27/3 in retarded children

Dose CTD_{50}	Lot No.		
	D 0545	D 0446	J 2500
10,000	6/6	3/4	—
1,000	0/3	—	2/3
100	1/5	—	—

The table 4 summarizes the results of vaccination with RA 27/3 done independently by Dr. A. Nicolas at the Institut Merieux in France. 114 of 115 individuals given 10,000 tissue culture doses subcutaneously seroconverted. Intranasal results also showed a high degree of effectiveness. 45 of 45 given 10,000 tissue culture doses intranasally and 14 of 14 given 1,000 tissue culture doses intranasally developed antibodies, whereas only 4 of 8 given 100 tissue culture doses also converted. There were 52 seronegative contact controls, none of whom developed antibodies.

Table 4

*Results of vaccination with RA27/3 in France**

Route of Administration	Dose TCD_{50}	Seroconversion
Subcutaneous	10,000	114/115
Intranasal	10,000	45/45
	1,000	14/14
	100	4/8
Controls	—	0/52

* Data courtesy of Dr. A. Nicolas, Merieux Institute.

The table 5 shows a small trial done in nurses in Philadelphia. The nurses were given either 10,000 or 1,000 tissue culture doses of virus. All seven developed antibodies, regardless of the amount of virus given.

Table 5

Titration of intranasal RA27/3 vaccine in adult women

Dose	Seroconversion
10,000 TCD_{50}	4/4 (40, 40, 160, 160)
1,000 TCD_{50}	3/3 (40, 80, 160)

In summary of the results of the intranasal titration we can conclude that 10,000 tissue culture doses given intranasally are almost always effective in eliciting antibody response. In many studies 1,000 tissue culture doses have also been effective, whereas in others the results have been inconstant. 100 tissue cultures doses is certainly too small an amount of virus to produce regular immunization. Therefore, it would appear that the necessary dose for regular intranasal vaccination lies between 1,000 and 10,000 tissue culture doses.

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The table 6 shows a comparative study of virus excretion in subjects vaccinated subcutaneously or intranasally. There may be an increase of virus recovery from the intranasal vaccinees. However, in both groups virus was recovered only by blind passage and therefore, only small amounts of virus were present in the nasopharynx.

Table 6

Nasopharyngeal virus excretion after intranasal and subcutaneous inoculation of RA27/3 vaccine, 27th passage* (trials E and H)

Type of Vaccination	No. Vaccines Excreting Virus Days post-inoculation:								
	0	3	7	9	11	13	14	17	21
Subcutaneous (N = 11)	0	0	0	1	5	4	2	1	0
Intranasol (N = 10)**	0	0	2	3	5	5	3	0	0

* In WI-38 cell culture.
** Excludes 3 children who did not develop antibodies.

The table 7 shows the development of antibodies in a small group of subjects vaccinated either subcutaneously or intranasally with RA

Table 7
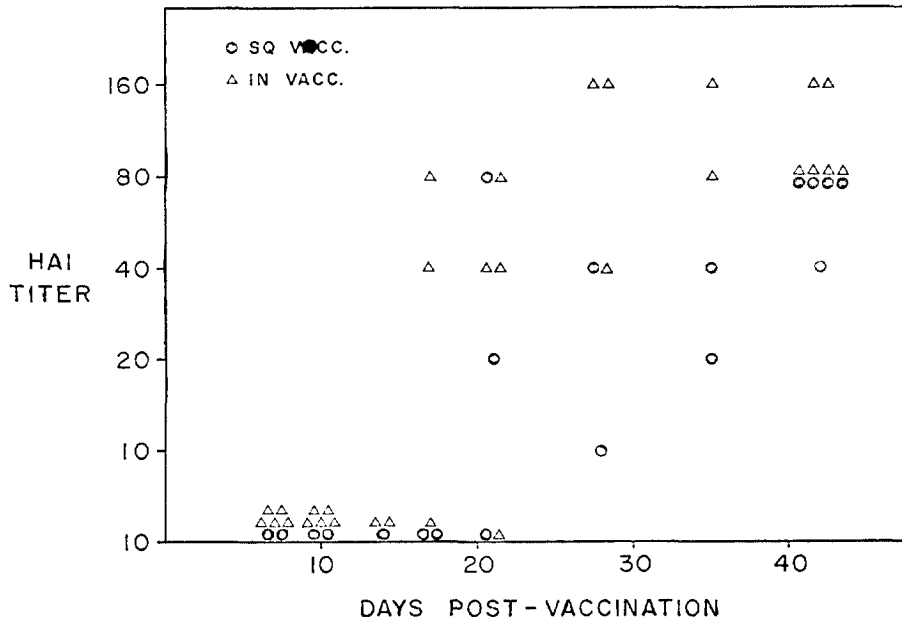
27/3. Neither group developed antibodies before the 17th day after vaccination. It appears from these data that the evolution of antibodies is much the same in the two groups. If anything, there may be a slightly earlier response in the intranasal group.

In order to show that the intranasal infectivity of RA 27/3 was a property peculiar to the strain rather than to the type of administration, we inoculated seven children, shown on the table 8, who were seronegative with Cendehill strain given intranasally in a dosage of 5,000 tissue culture doses. Only one of the seven seroconverted. The six remaining children ere given RA 27/3 intranasally and all developed antibodies.

Table 8

Sequential intranasal administration of Cendehill and EA27/3 attenuated rubella viruses-trial K

Time When Specimen Obtained	Individual HAI Titers of Vaccinees	
Before Administration of Cendehill Strain*	<10, <10, <10, <10, <10, <10, <10.	<10, <10, <10,
Six Weeks After Cendehill, Before RA27/3**	80, <10, <10, <10. <10, <10, <10.	<10, <10. <10,
Six Weeks After RA27/3	40, 40, 160, 80, 40, 80, 80	<10, <10, <10.

* 51st Passage, 5,000 PFU
** 27th Passage, 1,000 PFU

An attempt was made also to see if RA 27/3 could be given by mouth. In the first attempt, shown on the table 9, three groups of children were

Table 9

Subcutaneous, intranasal, or oral administration* of RA27/3 rubella vaccine, 27th passage

Route	HAI Antibodies Post-Vaccination	No. Seroconverted No. Inoculated
Subcutaneous	320. 80, 160, 80, 40, 80	6/6
Intranasal	80. 80, 160, 20. 20, 80	6/6
Oral	<10. <10, <10 <10. <10. <10,	0/6

* Retarded Children 6–12 yrs old

given RA 27/3 either subcutaneously, intranasally in the form of nose drops, or orally in the form of liquid. None of the children given vaccine orally developed antibodies whereas all of the other children did so. Nevertheless, tests are now going on to see if virus delivered directly into the intestines beyond the point of gastric acidity might be immunogenic.

As mentioned at the beginning of this paper, the evidence that reinfection occurs when HPV-77 vaccinees are exposed to wild rubella virus is disturbing.

The table 10 shows the results of a challenge study in which subjects previously vaccinated with RA 27/3 were given the Brown strain of wild

Table 10

Challenge of vaccinated subjects with intranasal unattenuated virus

Subject No.	Previous Vaccine	HAI Antibodies		Virus Excretion
		Pre-Vacc.	Post-Vacc.	
234	None	<10	160	+ +
226	None	<10	320	+ +
242	None	<10	160	+ +
244	None	<10	160	+ +
23	Cendehill*	160	160	0
54	Cendehill	160	80	0
41	Cendehill	<10	160	+
46	Cendehill	10	320	+
96	RA27/3**	40	40	0
80	RA27/3	80	80	0
42	RA27/3	40	160	+
52	RA27/3	10	10	0
91	RA27/3	80	80	0
113	RA27/3	20	10	0
94	RA27/3	20	10	0

* 19 months previously
** 14 months previously

rubella virus prepared by the National Institutes of Health specifically for this type of test of immunity. The subjects previously vaccinated with RA 27/3 failed to develop serologic or virologic evidence of reinfection, except in one case who had a four-fold booster response and one positive swab. The results with a small group of vaccinees given Cendehill virus (also shown in Table 19) are similar to what has been reported after vaccination with HPV-77: two of four subjects were reinfected and had substantial serologic booster responses.

More recently we have taken a group of nurses table 11 vaccinated seven to nine months previously, either subcutaneously or intranasally, and have given them another administration of 10,000 tissue culture doses of rubella vaccine. Four seronegative controls were included, all of whom developed antibodies. As can be seen in the last slide, only one vaccinated individual developed an antibody response, just four-fold. Studies of virus excretion and nasal antibodies are in progress.

In summary, then, RA 27/3 vaccine can feasibly be given by the intranasal route. This route deserves exploration because of the possible importance of nasal antibody in immunity to rubella.

Table 11

Revaccination by the intranasal route of nurses vaccinated 7 to 9 months previously

Route of Previous Vaccination	HAI Antibody Titers		
	6 Weeks Post Initial Vaccination	At Second Vaccination	4 Weeks Post Second Vaccination
None HUP 269	—	<10	20
151	—	<10	40
355	—	<10	160
261	—	<10	80
S. Q. HUP 607	80	80	80
219	80	160	40
310	80	80	80
655	20	40	20
675	80	160	80
707	80	80	80
716	80	160	40
683	320	160	80
I. N. HUP 700	320	160	80
670	80	80	40
708	160	80	40
336	80	80	80
242	80	160	80
234	80	160	80
214	320	80	320

THE ROLE OF MYCOPLASMA PNEUMONIAE IN RESPIRATORY DISEASES

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A number of studies have been undertaken in an attempt to establish the etiology of respiratory diseases. A few years ago it was discovered that a certain percent of pneumoniae (primary atypical pneumonia) was caused by M. pneumoniae.

I should like to emphasize, that the name of the genus Mycoplasma was introduced for the first time forty years ago by Julian Nowak, who did his experimental work in the Department of Microbiology in Kraków, where I am now working. Julian Nowak elaborated cellular morphology and the mode of replication of Mycoplasma mycoides in 1929 and his classical work is still quoted in contemporary references (15).

But to return to M. pneumoniae, it was not until 1962 that Chanock and his coworkers (4) succeeded in growing the Eaton agent on cell-free media. This agent was believed for a dozen years to be a virus (8), and was identified as a member of the family Mycoplasmataceae.

In previous studies, the diagnosis of Mycoplasma pneumoniae infections was based mainly on the presence in patients' sera of antibodies detected by the fluorescent antibody technique. More recently, the diagnosis of M. pneumoniae pneumonia has been based, first of all, on the isolation of the organism and on the rise in specific serum antibody. In about 50 percent of Mycoplasma pneumoniae infections, a rise of serum cold hemagglutinins is noted, and in about 30 percent, a rise of serum Streptococcus MG agglutinins occurs.

Many studies from different countries have shown that the incidence of M. pneumoniae in respiratory infections differs. Serological examination by WHO of 528 children from 10 countries with severe respiratory infections revealed that the most frequently occuring infection resulted

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from RS virus (19%), followed by that from parainfluenza viruses (16%), adenoviruses (6%), influenza (6%), Mycoplasma pneumoniae (5%), psittacosis (0,4%) and C. burneti (0,2%). Some workers indicate a much greater role of Mycoplasma pneumoniae in respiratory infections. During the examination of recruits suffering from pneumonia, Chanock and his group discovered M. pneumoniae antibodies – still known then as Eaton's agent – in 68% (5). Dowdle, employing indirect hemagglutination and metabolic inhibition tests, found serologic evidence of past infection in 70–75 percent (7). Evans, who examined 120 University of Wisconsin students with pneumonia, showed that M. pneumoniae was the etiological agent in 52% (10). The highest percent of respiratory tract infections caused by M. pneumoniae was found among populations living in close contact, such as military personel, college students, prisoners and so on, where the conditions for spreading airborne infections are especially good. This infection also spread within families (1, 2, 11) and the individuals most susceptible to infection are between 5 and 20 years of age. Mycoplasma pneumoniae was rarely isolated from children under 5 years of age (1, 9, 13). Unlike virus pneumonia no seasonal occurrence of M. pneumoniae infections have been observed.

The most typical symptoms of pneumonia caused by Mycoplasma pneumoniae are the following: slow and insidious onset, slight or absent signs on percussion and auscultation and less marked upper respiratory symptoms than in virus infections (12, 18). According to Sobeslavsky M. pneumoniae has an affinity for respiratory tract epithelium, which provides an unusual opportunity for peroxide, secreted by the organism, to attack the tissue cell membrane. This property is unique among the mycoplasmas that infect man and may play a role in virulence (17).

The fact that Mycoplasma pneumoniae infections cause considerable morbidity has stimulated search for an effective prophylaxis. For this reason attemps were made to immunize some groups of people with M. pneumoniae vaccine. C. B. Smith at al. found that 10 of 19 volunteers who were vaccinated developed growth-inhibiting antibody. After experimental infection only one of these individuals became ill, whereas, respiratory tract disease developed in seven of nine men, who failed to respond to the vaccine (16). Metzgar and co-workers prepared vaccines, which were highly antigenic in animals and in man. Especially alum vaccine gave positive results and only two doses of this vaccine were necessary to induce neutralizing antibodies (14).

Respiratory infections caused by M. pneumoniae are treated with antibiotics. In my own research carried out partly at the Wistar Institute in Philadelphia and in the Department of Medical Microbiology in Kraków, the susceptibility of mycoplasma strains to 15 antibiotics was examined. All strains, with the exception of the Mycoplasma pneumoniae group, were resistant to vancomycin, mycostatin, streptomycin, oleandomycin, erythromycin, while most strains were sensitive to the antibiotics of the tetracycline group (namely chlortetracycline, exytetracyc-

line, and tetracycline) and to chloramphenicol, tylosin, leucomycin, kanamycin, neomycin, novobiocyn and lincomycin. Mycoplasma pneumoniae strains differed from the other Mycoplasma species by their susceptibility to erythromycin, oleandomycin and also their slight sensitivity to streptomycin. Tetracycline and erythromycin are most effective, but the former is preferred because it can be used effectively against pneumonias caused by C. burneti and psittacosis. Since the diagnosis of Mycoplasma pneumoniae has been rather retrospective up to now, a wider scope for the action of tetracycline should be considered.

In the Department of Microbiology in Kraków research has been started on the etiology of acute respiratory tract infections in children hospitalized in the Institute of Pediatrics. In the first phase of the study, paired sera taken from cases with respiratory infections were examined. Complement-fixation tests were made for the following agents: respiratory syncytial virus, parainfluenza virus types 1, 2, and 3, adenoviruses, and Mycoplasma pneumoniae. Isolation of M. pneumoniae was also carried out. Sputum or oropharyngeal smears were inoculated in Trypticase Soy Broth (Difco) containing 0.5% bovine albumine, and then transfered to liquid and solid media such as PPLO-agar and PPLO-broth. Samples from broth cultures were tested for presence of mycoplasma colonies on solid media. Plates were incubated at 37°C for forty days and examined several times for the presence of mycoplasma colonies. Isolated strains are identified by the growth inhibition test, according to the method of Clyde (6). At present, complete analysis of the results is impossible, because of the limited number of tests carried out. However preliminary results indicated that Mycoplasma pneumoniae does not exceed the average estimated by WHO for the children of ten tropical countries, i. e. about 5%.

In conclusion, I should like to mention the problem which, although not directly connected with the subject of our symposium, interests most virologists, who used cell cultures in their research. Cell cultures derived from various virological laboratories in Poland, were examined in our Department. Out of 26 cell cultures examined 19 were contaminated with mycoplasma organisms. The isolated strains belonged to human species (Mycoplasma orale, Mycoplasma hominis) as well to the GDL group. The susceptibility of these strains to antibiotics in vitro revealed the presence of mycoplasma strains resistant to kanamycin and neomycin, antibiotics which were at that time used in cell media. However the addition of tylosin at a concentration of 30 mcg/ml to culture medium seemed to control mycoplasma contamination in HeLa cell line and HEp–2 cells.

In biological research carried out in cell cultures the control for mycoplasma contamination must be accomplished, otherwise undetected infections might yield completely faulty results.

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NATURAL RESISTANCE TO INFECTION

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This afternoon, I shall discuss natural resistance to infection, using mice as the model system. By natural resistance to infection, I mean genetic resistance.

In the late nineteen twenties, an American investigator from the Rockefeller Institute, Leslie Webster, wished to create a strain of mice which would be resistant to a specific group of viruses. Starting with non-selectively-bred mice, he inbred and crossbred them until, some fourteen years later, he obtained a strain of mice resistant to *Salmonella enteriditis* and to arbo B viruses, which he called bacteria-resistant-virus-resistant (BRVR) strain (1). An inbred strain, bacteria-susceptible-virus susceptible (BSVS), was also developed.

In 1925, investigators at the Rockefeller Institute in Princeton, New Jersey, wished to breed a strain for their own laboratory. They placed advertisements in local newspapers offering one dollar for each white pet mouse brought forward. The advertisement was answered by an elderly lady who sold her five pet mice for five dollars. From these five mice, a breeding colony was started. The resulting strain was found to be resistant to arbo B virus infection. Thus, a second resistant strain, Princeton-Rockefeller Institute (PRI), was developed (2). The PRI strain is out-bred; BRVR, inbred.

To study the genetic factors which influence natural resistance, we wished to create a strain of mice with a virus-resistance gene which could be grafted onto another genotype. To this end, a male of the virus-resistant strain of PRI mice (outbred) was crossed with a virus-suscep-tible C3H/He (inbred) female (3). The C3H female, though infected with mammary carcinoma virus, was utilized, nevertheless, to determine, as a corollary to these studies, whether genetic resistance to arbo B

virus has any relation to the genetic resistance to milk tumor virus. The resulting F-1 generation was tested for resistance by challenge with West Nile virus inoculated parenterally and with yellow fever virus inoculated intracerebrally and was found to be 100% resistant. Following the classic inbreeding pattern, the F-1 generation was backcrossed to the C3H/He mother. The progeny, called backcross 1 (BC$_1$), were 50% resistant. This procedure was continued until the BC$_9$ generation was reached. It, too, yielded 50% resistant mice, which was in agreement with our calculated figures, indicating the presence of one dominant gene, or, several closely related genes, which control resistance and susceptibility. Generation BC$_9$ was crossed with BC$_9$, resulting in generation G$_0$ which was then tested for resistance (4).

Males and females of the G$_0$ resistant mice were then bred with the C3H mice, and the resulting litter checked for resistance to arbo B. The G$_0$ mice which have produced 100% resistant litters were bred between themselves, giving rise to an inbred resistant line called C3H/RV. These mice are congenic with the parental C3H/He mice, differing only in the gene of virus resistance. Resistance on the level of the whole organism is reflected by resistance on the cellular level.

Figure 1 shows fibroblasts from C3H/He and C3H/RV mice exposed to West Nile virus produced in mouse brain or in monkey kidney tissue culture. Resistant fibroblasts inoculated with virus produced in mouse brain produced less virus than did susceptible fibroblasts, whereas virus production in resistant and susceptible fibroblasts inoculated with virus produced in monkey kidney tissue showed little difference. The virus-mouse brain inoculum contained a substance absent from the virus-monkey kidney inoculum, a virus inhibitor to which mouse tissue culture is susceptible – interferon (5). Thus, the difference between resistant and susceptible mice was investigated from the point of view of production of larger amounts of interferon or of greater susceptibility to interferon.

Results showed that equivalent amounts of interferon were ultimately produced by both resistant and susceptible mice, but that interferon production started later in the resistant mice, and the curve of interferon production paralleled that of virus production (Fig. 2). These results indicated that production of interferon cannot be responsible for resistance to arbo B virus. On the contrary, if interferon plays any role at all, the resistant mice would have been more susceptible because interferon production is less and occurs later than in susceptible mice (5).

To determine the effect of preformed interferon on infection in C3H/RV and C3H cells, interferon produced in mice inoculated with Newcastle virus was added to infected cultures of C3H/RV and C3H fibroblasts, and the virus yields of the two systems were compared (Fig. 3). Interferon added to the resistant fibroblasts reduced the virus yield considerably, but it had little effect on the susceptible fibroblasts. To prove that interferon is the substance involved in this phenomenon, cells were pretreated with actinomycin: in these cells the effect of interferon was eliminated (Fig. 4) (5).

Fig. 1

EFFECT OF DILUTION OF INPUT VIRUS ON GROWTH
OF WNV (BRAIN) AND WNV (MK2) IN C3H
AND C3HRV EMBRYO FIBROBLAST CULTURES
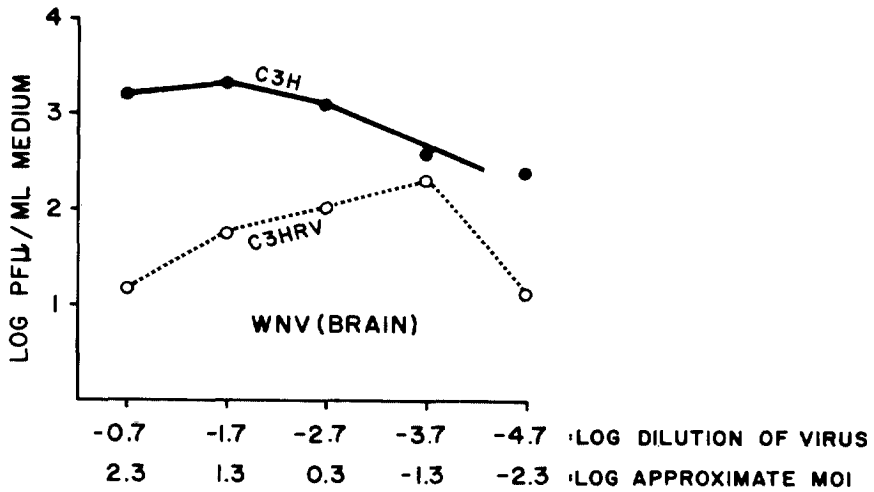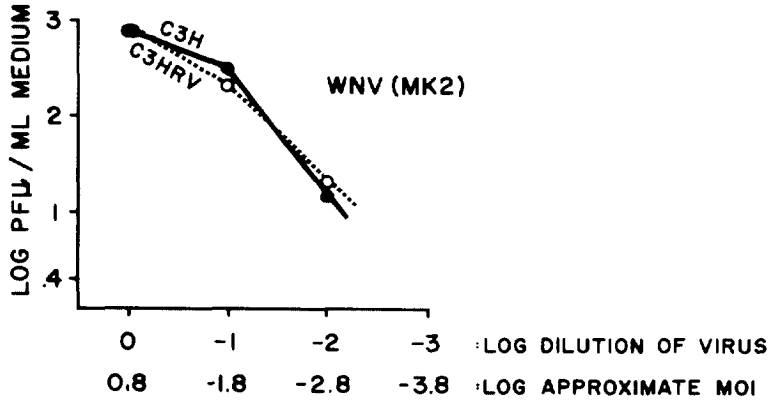
Fig. 2

GROWTH OF WEST NILE VIRUS (MK2) AND INTERFERON PRODUCTION IN BRAINS OF C3H AND C3HRV MICE AFTER INTRACEREBRAL INJECTION
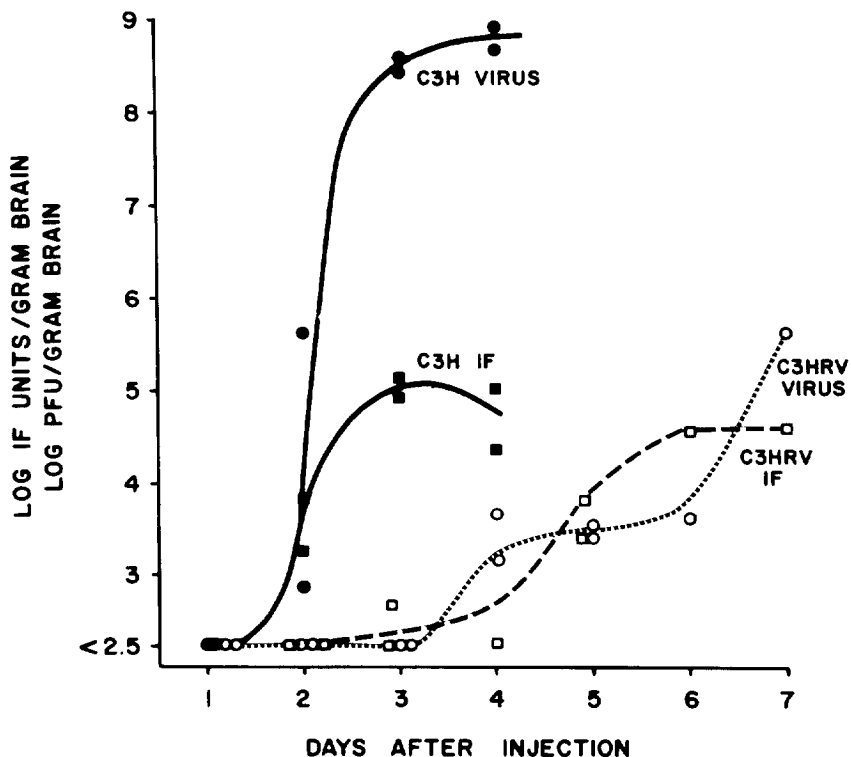
To summarize briefly, mice resistant to arbo B virus produce less interferon than do susceptible mice. However, resistant fibroblasts are much more susceptible to the action of preformed interferon than are susceptible fibroblasts.

This effect was confirmed *in vivo*. The dosage of West Nile virus was adjusted for killing approximately 70–80% of both C3H/RV and C3H mice, and interferon produced in mouse serum against Newcastle disease virus was injected simultaneously with the West Nile virus. The number of resistant mice protected by pre-exposure to interferon was greatly enhanced as compared to that of pretreated susceptible mice, though, as expected, interferon protected some of the susceptible mice (Fig. 5).

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Fig. 3

EFFECT OF MOUSE SERUM INTERFERON ON WNV GROWTH IN C3H AND C3HRV EMBRYO FIBROBLAST CULTURES

Fig. 4

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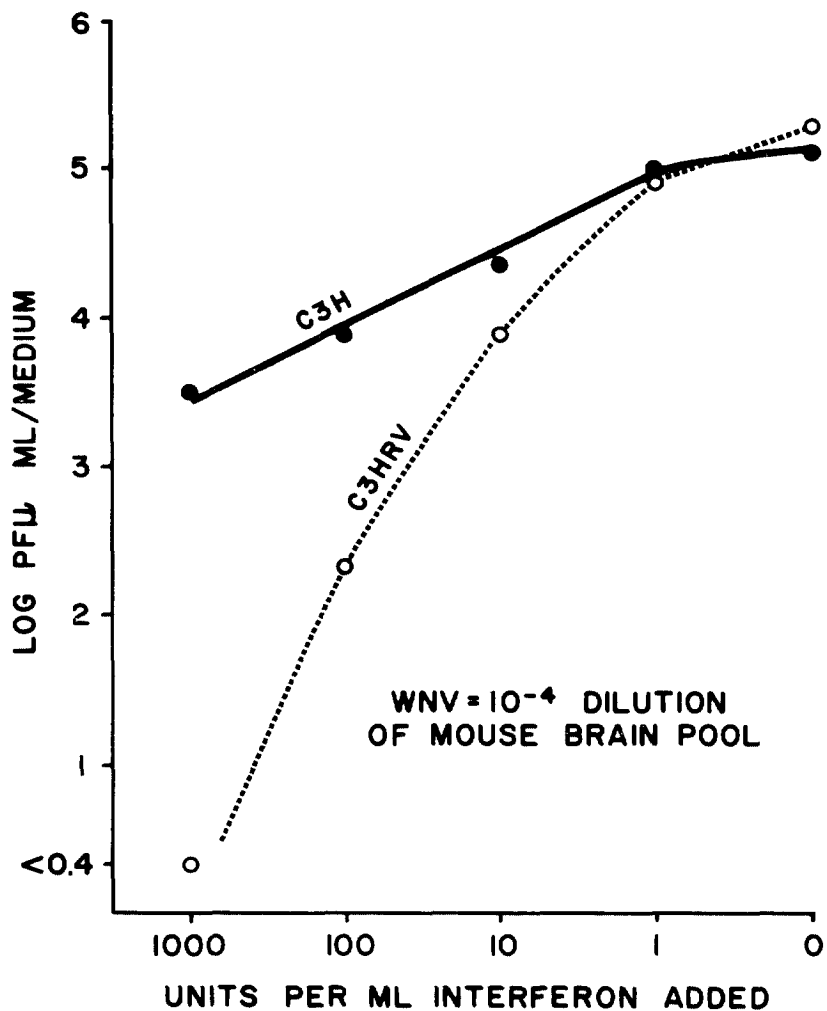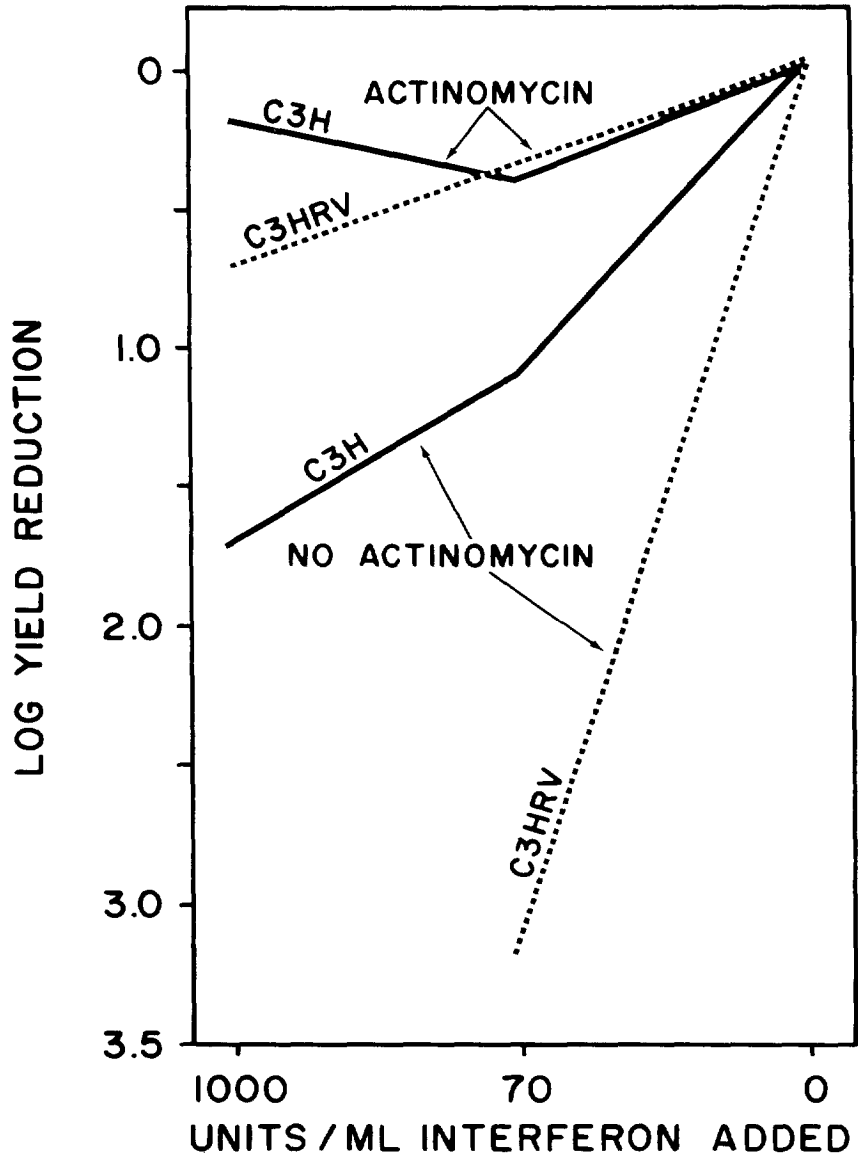
Fig. 5
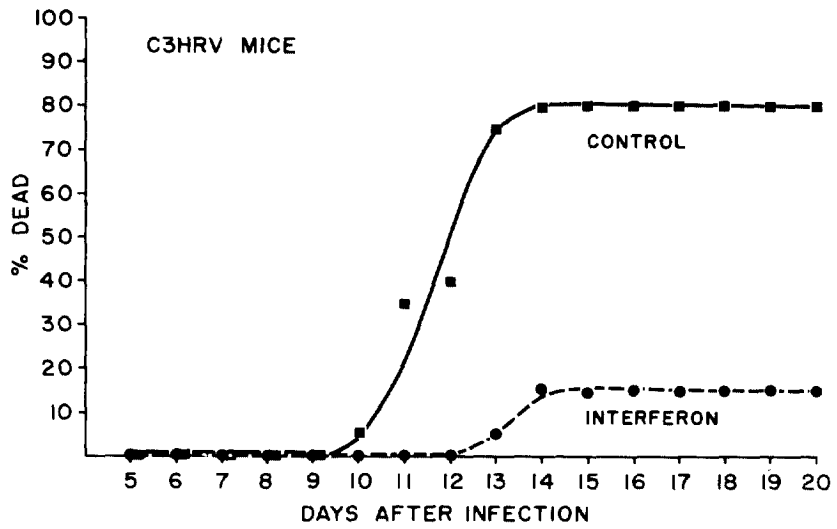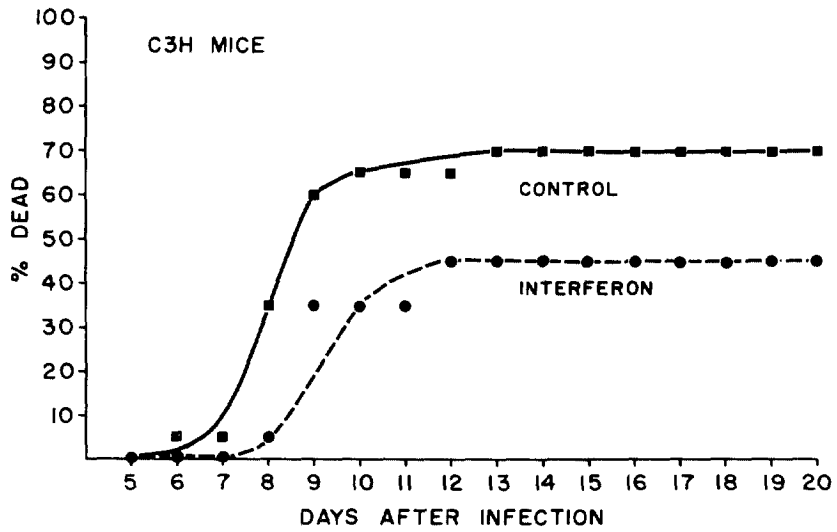
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DOCUMENT

RECOMMENDATIONS FOR FUTURE WORK ON LIVE INFLUENZA VACCINE

One of the most promising method for the large-scale prevention of influenza is active immunization with a live attenuated vaccine produced in developing chick embryos and introduced directly into the respiratory tract in a well-dispersed state.

The value of a live vaccine is not only in its greatly reduced cost and simplicity of use, but also in its ability to elicit an intense local immunity in the respiratory tract which prevents the subsequent multiplication of virus in the susceptible tissues. This is due to the massive production of antibodies of the immunoglobulin Atype at the site of primary multiplication of the vaccine virus, just as in natural infection.

The most convenient and accurate procedure for the evaluation of the immunogenic activity of vaccination is the titration of antibody rise either by hemagglutination inhibition or, especially, by neutralization using sera and nasal washings obtained before, and three or four weeks after the vaccination.

For production of a successful live influenza vaccine, it is essential: (1) To renew the vaccine strains of the A and B types, because of the gradual appearance of new antigenic variant, which assume dominant position; (2) To select the most highly immunogenic vaccine strains from the antigenic variants that have been prevalent in recent years. (At the present time these variants are represented by the subtype A_2 Hong Kong strain from 1969 outbreak and subtype B from 1966 outbreak.) (3) These strains must possess a combination of the following features: non-reactogenicity, regular and intensive multiplication in the upper respiratory tract susceptible persons, and stimulation of distinct antibody rise in 50–70 per cent of this group after a single immunization.

The efficiency of a live influenza vaccine depends on the quantity of active virus in the lyphylized preparation.

In order to elicit more intensive immunological changes, at least 100,000 infectious embryonic units of the ettenuated viruses must be introduced into the respiratory tracts of susceptible subjects.

For this purpose, the live vaccine should have a minimum of 0.5 ml of the vaccine, administered in a 1 : 5 dilution, containing 100,000 infectious units.

The close correlation that exists between the immunogenic and epidemiologic effectiveness of live influenza vaccines suggests that the primary goal of an active immunization programme should be to reduce to a minimum that susceptible part of the population which has no antibodies in serum dilutions of 1 : 5 − 1 : 20.

This task is difficult to accomplish with a single immunization. Two or three repeated administrations of a combined polyvalent vaccine preparation (divaccine containing one or two vaccine strains for each of the serotypes A_2 and B), with intervals of ten to fourteen days, sharply reduces this number to 15–25 per cent after the second or third immunization.

The effectiveness of the live influenza vaccine is increased substantially if it is introduced into the upper respiratory tract, not by instillation, but by very accurate spraying in a finely divided state through the nasal passages, since this favours the contact of the virus with the susceptible tissue and its multiplication. To meet this requirement, the manufactures should issue the vaccine together with portable spraying kits. Recently it was demonstrated that peroral administration of the vaccine is also promising method of influenza prevention in the USSR, not yet sufficiently studied in epidemiological field trials. The use of strains especially adapted to this peroral route of immunization would be helpful in reducing the immunizing dose to the levels recommended for routine intranasal spray. Only strains safe for intranasal application can be used for peroral immunization. The epidemiological effectiveness of peroral immunization is not yet clear.

During the past fifteen years many field trials of the efficiency of live influenza vaccine have been carried out in the USSR (Moscow, Leningrad, Sverdlovsk, Dnepropetrovsk) as also in Yugoslavia and Bulgaria. These trials have provided definitive data concerning the degree of protection that vaccination was able to afford under various conditions of application. From the results published following field trials with influenza A, A-prime and Asian viruses, the average protection found was close to 50%, ranging from 40 to 80%. The results were markedly better against influenza B yielding an average protection of 70–85%. These findings give evidence that when live vaccines of specific antigenic composition and potency are used properly, a significant degree of protection is assured. But in the face of epidemics and pandemics provoked by entirely new antigenic variants live influenza vaccines failed to produce protective effect.

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The statistical evidence of the effectiveness of influenza vaccines is definitely limited, because of common errors in the clinical diagnosis of influenza in the course of an epidemic.

Even during the most intensive outbreaks, at least 15 to 25% of clinically diagnosed cases of influenza have diseases other than influenza by laboratory criteria. For this reason we cannot expect unquestionable statistical evidence of intensive protection by influenza vaccines on the basis of clinically diagnosed cases not confirmed by the laboratory, even when the vaccines were highly effective.

Therefore epidemiological field trials with influenza vaccines must include serological confirmation of all clinically diagnosed cases. Only laboratory confirmation allows conclusive analysis of influenza vaccine efficiency from reliable data concerning the real number of specific influenza cases in vaccinated and placebo groups.

The effectiveness of immunization against influenza by use of live vaccine also depends considerably on the percentage of vaccinees in the observed group of a population. There is a constant and distinct rise in effectiveness proportional to the extent of immunization as it was shown in Yugoslavia.

The standard live influenza vaccine used for the protection of adults from 16 years upwards, cannot be applied to children, in view of the progressive increase in the clinical reaction rate of this vaccine with decreasing age. It is important to use therefore for immunization of children from 1 to 12 years old a further-attenuated live vaccine. These are prepared from vaccine strains by additional passages through developing chick embryos at sharply decreased temperature ranging from 30^0 to 26^0C.

Unfortunately when a major antigenic shift occurs and a new pandemic threatens it will be impossible to prepare quickly a sufficient amount of vaccine from the new strain, a procedure that takes some months after it is first isolated. For this and other reasons measures of protection other than vaccination may be very important. These measures may include enhancing the natural host resistance with interferon or effective chemoprophylaxis. Under these more complicated circumstances the susceptible groups can be protected only after an epidemic has started rather than in advance and under less urgent conditions as is possible by vaccination.

1. The vaccine should contain influenza viruses of A and B type, antigenic composition corresponding to the predominating agents of disease at the time.

2. These viruses should be attenuated to such a degree that after administration into the respiratory tract of 5.0–6.0 log 10 of active particles, only mild clinical reactions are provoked and these in no more than 2% of immunized subjects, with no significant spread of virus to healthy contacts. Both inhibitor-resistant and inhibitor sensitive strains of sub-type A_2 can be used.

3. The vaccine should contain 6.0 or more log 10 EID_{50} per ml., so that at least 100,000 EID_{50} can be administered conveniently be each immunization. (The acceptance of such vaccination by our population has been very good).

4. After two or three consecutive administrations of live influenza vaccine with intervals of 10–14 days, there should be a four-fold of greater rise in hemagglutination inhibiting (H. I.) antibodies in 50% or more of susceptible vaccinated subjects. (For the H. I. test with inhibitor-resistant influenza A_2 virus group-0 human red cells must be used instead of chicken red cells). Immunological data are even more convincing if a more sensitive neutralization test is used.

5. Vaccine re-inoculated two to three weeks after the second admistration should not be followed by recovery of virus from the upper respiratory tract of vaccinees, in contrast with 30–50% recovery of viruses for 2–4 days following the first immunization.

6. Vaccine should not be administered by simple instillation of drops into the nasal cavity but by spray, or even better, by inhalation of aerosol. For the aerosol method highly attenuated strains should be used as well as suitable administration equipment, not yet available for mass immunization.

7. The prevention of influenza which can be achieved by live vaccine is still limited by the small extent to which it has been applied. Much more intensive use is requred to cover a substantial part of the population of different ages. The highest reduction of influenza morbidity in published field trials, was observed under conditions of most intensive vaccination when live influenza vaccine was applied to 80% or more subjects in every group.

8. The periodic changes of influenza virus antigenic properties require the regular incorporation into vaccine production of the new variants: that means the development every 4–6 years of entirely new live vaccines – difficulty entirely unknown in any other disease.

9. The epidemiological role of children as the most susceptible part of the population, stresses the importance of their protection against influenza by the intensive use of a special safer variant of live vaccine.

10. The establishment of responsible specialized scientific laboratories or institutes for prevention of influenza and other respiratory virus diseases would be very important, as is close international co-operation in the development of improved live influenza vaccine and other preventive methods.

These recommendations were prepared by A. A. Smorodintsev and accepted by the Symposium.

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SPECIFIC AND NONSPECIFIC PROPHYLAXIS AND CHEMOPROPHYLAXIS OF INFLUENZA

FREE THEMES

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