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QUESTIONS OF IMMUNITY AND THE MANUFACTURE OF BACTERIAL PREPARATIONS

[VOPROSY IMMUNITETA I PROIZVODSTVA BAKTERIYNYKH PREPARATOV]

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THE INTRODUCTION OF DYSENTERY ANTIGENS INTO ANIMALS BY DIFFERENT METHODS AND THEIR EFFECTS ON THE CELL ACTIVITY

by

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Dysentery immunization is not very effective and it is very short lasting. This can be, apparently, explained by the characteristics of both the pathogenesis as well as the immunogenesis of disease. The comparative data which we obtained earlier on the reaction of the animal organism to the introduction of typhus abdominalis and dysentery vaccines support this conclusion.

It was shown that after a single subcutaneous injection of dysentery vaccine the dysentery microbe antigen is more localized in the rabbit organism than typhus abdominalis (primarily in the upper section of the large intestine, cecum, Peyer's patch, and to a lesser extent in the lungs, upper section of the small intestine and other organs).

The typhus abdominalis antigen is much less localized in the animal organs, but it is not found in the walls of the large intestines.

Both dysentery and the typhus abdominalis are consistently found in the kidneys, which could apparently be explained by the excretory function of the kidneys.

The reaction of the organism to the introduced antigen, determined by the concentration of antibodies in the tissues of organs as well as in the blood serum after the innoculations with dysentery vaccine, is also manifested in a much weaker form.

The organ tissue extracts and the blood serum of animals which were inoculated with dysentery antigen, as was previously shown by Khazanov, have weak, preventative

properties, while after the injection of paratyphoid antigens into the animals both the blood serum as well as the tissue extract of a number of organs have intensly developed preventative properties with respect to homologous microbes.

The morphological changes (proliferation of the reticuloendothelium, increase in a number of plasma cells and the concentration of ribonucleic acid in cells of the reticuloendothalial system) in the tissues of the majority of organs after the introduction of the dysentery antigen were much less pronounced than the same indices after the introduction of typhus abdominalis vaccine.*

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* F.I.Ginzburg-Kalinina, Ye.F.Kakarina and V.N.Kartasheva. "Some Aspects of the Mechanism of Innoculated Immunity" Problems of Immunity -- Transactions of the Moscow Scientific Research Institute of Vaccines and Serums im. Mechnikov. Moscow, 1960, vol. XV, pp. 7 to 21.

The above discussion indicates that the animal organism in general is little involved in the production of the reaction to the introduced dysentery antigen. This can, apparently explain the low intensity and short duration of antidysenterial immunity.

Subsequently, using the same indices, i.e., determination of the concentration of dysentery antigen and the corresponding antibodies in the organ tissues, and the blood serum of animals, we attempted to find a way to increase the reactivity of the animal organism to the introduced dysentery antigen.

On the other hand, taking into account the tropism of the dysentery antigen to the intestine walls, we

investigated what immunization method, along with the overall reaction of organisms, produces the greatest localized reaction in the intestine walls to the introduced dysentery vaccine.

In our experiments we used corpuscular vaccines from Flexner type S culture, destroyed by heating, and the complete antigen, obtained by the tryptic digestion method from the culture of the same strain. We tested subcutaneous introduction of antigens, as the most widespread method of innoculation, and the peroral method, which was used in order to bring the antigen into closer proximity to the walls of the intestinal tract. The experiments were conducted with chinchilla rabbits (weighing 1800 to 2200 g). In the case of peroral immunization the vaccine was introduced into the rabbit on an empty stomach with a pipette, after the preliminary injection of bile (4 ml) 20 minutes prior to the injection of the vaccine. Food was given 2 hours after immunization.

Prior to the experiments with rabbits the immunization dose of the complete antigen and corpuscular vaccine for subcutaneous introduction was tested titrimetrically and was selected to be sufficiently large not to cause suppression of the protective ability of the organism, which for the corpuscular vaccine was equal to 7 billion microbes and for the complete antigen -- 2.5 mg. In the case of peroral introduction the dosage of the antigens was increased: corpuscular vaccine -- 20 billion microbes and for the complete antigen -- up to 15 mg.

First of all we investigated the effect of the preparation method (corpuscular vaccine or the complete antigen) and injection method (subcutaneous or <u>per os</u>) on the distribution of the dysentery antigen in the organism of animal.

For this purpose after a single injection of dysentery

antigen the animals were killed by total exsanguination (1, 5, 10 and 20 days after the introduction of the antigen) and from their organs salt extracts were prepared according to the method described in our earlier reports. The concentrations of antigen and antibodies in the obtained extract were determined by the compliment fixation reaction at low temperature. The antigen was determined in the reaction with hyperimmune Flexner's serum, while antibodies were determined in the reaction with antigen (Flexner's corpuscular antigen).

Figure 1 shows the average data obtained in the concurrent investigations, with equal numbers of animals (total 32 rabbits).

After the injection of the rabbits with corpuscular vaccine or complete antigen of dysentery microbes, regardless of the method of introduction (Subcutaneously or per os) the antigen is distributed in the organism almost identically and is found primarily in cecum, the Peyer's patches (primarily at the base of the cecum) and the kidneys. Antigen is found in smaller amounts in the walls of the large intestine and the upper section of the small intestine, the lungs, mesentery lymphatic nodes, and in very small amounts in the spleen and occasionally in the liver. After the perioral injection of the vaccine, the antigen is found in the stomach walls.

In comparing the amount of dysentery antigen in the organisms after subcutaneous injection of the corpuscular vaccine and after the injection of the complete antigen, it was found that subcutaneous injection frequently produces large amounts of antigen in the Payer's patches, the large and small intestine walls and the spleen. Peroral introduction of the complete antigen on the other hand produces antigen in certain organs, particularly the kidneys and lungs, in amounts three times greater than peroral introduction of the corpuscular vaccine. This can be explained

on one hand by more rapid absorption and assimilation of the complete antigen in comparison with the corpuscular vaccine, which must, first of all undergo enzyme reaction in the organism, and on the other hand a more rapid excretion from the organism. This in turn results in its larger concentration in the kidneys and lungs.

The experiments on the determination of the concentration of antibodies in extracts of animal organs, the results of which are represented in Figure 2, show that dysentery antigen is a weak stimulant and produces a low antibody titer in the blood serum and the organ tissues.

It should be noted that on the first through the fifth days after the immunization in the lymphoid elements of the intestinal tract and the mesentery lymphatic nodes the antibody titer was frequently higher than the antibody titer in the blood serum. The concentration of antibodies in the spleen (the organ which according to the data of a number of investigators is participating in the production of antibodies for a number of causal organisms of infectious disease) was lower than in the Peyer's patches of the intestines.

In comparing the concentration of antibodies in organ tissues after the injection of the complete antigen and the corpuscular vaccine a number of organs (Peyer's patches of the intestine, cecum and the small intestine, mesentery lymphatic nodes, spleen) showed a certain advantage of the introduction of the complete antigen both subcutaneously as well as per os. Only in the kidneys and in the blood serum was the antibody titer higher after the introduction of the vaccine.

Thus, it was found that a single injection both of the Flexner's bacteria corpuscular vaccine as well as the whole antigen either subcutaneously or perorally produces identical distribution of the antigen in the animal organism

and causes weaker antigen stimulation, manifested as the low antibody titer in the organ extracts and blood serum.

In the attempt to increase the ability of the organism to react to the injection of dysentery antigen, it was decided to increase and prolong the antigen stimulation by increasing the number of injections and the antigen dose (provided it was introduced in the sorbed state). For this purpose for primary immunization the complete antigen was injected subcutaneously in rabbits in twice the amount used in the previous experiments -- 3 ml after sorption on hydrated aluminum oxide. The interval between the first and the second antigen injection and the method of application during the second injection were different. One group (24 rabbits) was immunized subcutaneously for the second time 10 days after the first injection using the same sorbed Flexner's bacteria antigen in 1.5 mg. dose.

Figure 3 shows the data on the concentration of compliment fixing antibodies and agglutinins in the blood serum of these rabbits. The figure shows the average data for each period of investigation: 7 days after the initial injection and 7, 14, 20, 30 and 40 days after the second injection of the antigen.

Seven days after the initial subcutaneous injection of sorbed Flexner's bacteria, complete antigen, all rabbits displayed increased antibody titer in the blood serum. It should be noted, however, that there was a significant scatter (a factor of 2 to 4) in the agglutinin and antibody titer in the complement fixation reaction for individual rabbits.

The second injection of the sorbed Flexner's bacteria antigen, according to the average data did not produce any significant increase in the reactions as compared with the results of the initial injection (the maximum agglutinin titers were 1:3500 and 1:3130 respectively; the compliment

fixing antibody titers were -- 1:70 and 1:100 respectively). It should be noted that the results after the second injection of antigen were not uniform for individual rabbits. Thus, in some rabbits the antibody titer by the seventh day after the second injection reached a maximum, exceeding the antibody titer after the first injection of antigen by a factor of 2 to 3, while for other rabbits the antibody titer was already lower than the value reached following the first injection of antigen. Some rabbits showed the same antibody titer seven days after the second injection as before the second injection.

The rabbits in the second group were inoculated twice by another method. In the primary immunization of this group (8 rabbits) a 3 mg dose of sorbed complete Flexner's bacteria antigen was also injected subcutaneously, but the second injection was made after a 20 day rest period, i.e., after a period which according to the data of a number of authors is optimum for this purpose (Zdrovskiy, Khalyapina, Vasil'yeva).

In addition, considering the anterotropicity of dysentery antigen which we have established and the literature indications on the expediency of subcutaneous dysentery immunization (Troitskiy), the second injection of this group of rabbits was conducted in a combined manner. A 1.5 mg dose of nonsorbed complete Flexner's bacteria antigen (since in the previous experiments some advantage of this was found with respect to the concentration of antibodies in the organs of the inoculated animals) was injected simultaneously with peroral injection of a 200 billion microbe dose of the corpuscular vaccine. The second immunization of this group of rabbits was conducted during the period when the concentration of antibodies in the blood serum after the initial immunization begins to lower. In response to the second injection of antigen increased antibody titer was produced in the blood of all animals, however, in the majority of

the animals it did not exceed the level which was reached after the primary innoculation.

To determine the reaction of the organ tissues on the double immunization by the combined method rabbits were sectioned five and ten days after the second injection of the antigen. The average data of the investigation of the concentration of antigen and antibodies in the organs and blood serum of the rabbits are given in Figure 4.

In comparing the data in Figure 2 and the corresponding data in Figure 1 it is apparent then that in the organ tissues after double inoculation of animals a significantly larger amount of antigen is contained than after a single inoculation, which is especially noticeable in the case of its presence in the walls of the gastrointestinal tract. With respect to the concentration of antibodies in the organ tissues there was no differnece in the singly and the doubly inoculated animals.

Keeping in mind the possibility of the suppression of the ability of the organism to respond to the second immunization due to the introduction of a large dose of antigen, we inoculated a small group of rabbits (4 animals) for the second time twenty days following the first inoculation with a factor of 2 smaller doses of vaccine (0.75 mg of the complete Flexner's bacteria antigen subcutaneously and 10 billion dead microbes perorally). It was noted that the organ tissues in rabbits, inoculated by this method contained a somewhat smaller amount of the dysentery antigen. Thus the change in the dose of the vaccine has practically no effect on the concentration of antibodies either in the blood serum or in the organs.

To produce a more active reaction to the introduced dysentery antigen, having its effect not only in the intensity of the absorption by the organ tissues, but also in the production of antibodies, we tested a procedure

involving the triple immunization of rabbits using different injection methods.

For this purpose the rabbits were preliminarily subjected to a double immunological subcutaneous injection of the complete Flexner's bacteria antigen (sorbed on aluminum hydroxide), and the additional (third) injection of 3 and 5mg doses. In this case one group of rabbits was inoculated subcutaneously with 1.5 mg dose of not sorbed complete Flexner's bacteria antigen, while the second group was inoculated with the heated corpuscular Flexner's vaccine in an amount equal to 200 billion per os and the third group was inoculated simultaneously with both, not sorbed complete antigen subcutaneously and the corpuscular vaccine per os in the above indicated doses.

Each group of rabbits (12 animals each) was divided into subgroups (6 rabbits each) of which one subgroup was inoculated for the third time following a twenty day interval between the inoculations, while the second group was inoculated after a two month waiting period. The rabbits were totally exsanguinated and dissected on the 5th, 10th and the 20th day after the third inoculation of the dysentery antigen. The results of the study of organ extracts from these animals for the concentration of dysentery antigen and antibodies are given in Figure 5.

It is first of all necessary to note that the nature of the distribution of dysentery antigen in the animal organisms was found to be independent of the number of injections. The tropism of the dysentery antigen to the intestinal tract was even more pronounced (Peyer's patches, cecum) after the third injection.

In comparing the data in Figures 4 and 5 one finds that the concentration of dysentery antigen in the organs is significantly different depending on the number of inoculations and the inoculation method. After triple

subcutaneous injection, the complete Flexner's bacteria antigen was found in the majority of organs in smaller amounts than after double subcutaneousperoral innoculation.

However, after using the peroral method for the third innoculation, the concentration of dysentery antigen in the majority of organs (the cecum and small intestines, mesentery lymphatic nodes, liver and kidneys) exceeded significantly its concentration after double immunization, and in certain organs it was even higher than after a triple inoculation using other antigen injection methods.

Thus, the method for the introduction of dysentery vaccine into the identically specifically prepared animal organism has an effect on the intensity of the absorption of the dysentery antigen by the organ tissues. It is possible that the preliminary inoculation of the sorbed complete antigen produces a sensitized state of the cell tissues, primirily the gastrointestinal tract, which results in the increased absorption of the antigen during the peroral introduction.

The results of the measurements of the concentration of antibodies in organs and the blood serum of the same rabbit are shown in Figure 6.

The cited data indicates that the antibody titer in the organs and blood serum of rabbits after a triple subcutaneous innoculation with the complete antigen and after subcutaneous-peroral introduction during the third inoculation was significantly higher than the titer after a single or double injection of the antigen. Only the peroral injection of the vaccine to the animals which were prepared in a similar manner did not produce the formation of antibodies.

In comparing the results of the subcutaneous and the combined subcutaneous-peroral method for the introduction of dysentery antigen in the third inoculation it is found that the latter is more advantageous. Thus, in the spleen

of animals, which on the third time, were inoculated subcutaneously with complete antigen and at the same time they received corpuscular vaccine per os, the average antibody titer was almost four times higher than for rabbits which were only inoculated subcutaneously; in mesentery nodes; the same titer was a factor of two higher, and even in the liver although the concentration of antibodies was lower than in other organs, it was still higher than in the case of other innoculation methods.

In the blood serum the antibody titer, obtained in the complement fixation reaction, after using the combined method of the introduction of antigen in the triple immunization proceedure was almost 3½ times higher than after subcutaneous inoculation. Analogous results were also obtained for the agglutinate.

It should also be pointed out that the experimental results, shown in Figures 1, 2, 5 and 6, indicate the absence of the direct relationship between the concentration of antigen and antibodies in the animal organism. Thus, after peroral introduction of antigens it exists in the organs in higher concentrations, while antibodies in these organs produce a smaller titer than with other methods of immunization.

The cause of this is unknown. It is possible to hypothesize that after peroral introduction of the antigen it undergoes severe decomposition in the intestine, as a result of which it loses the properties of the full strength stimulant.

Subsequently, the reaction of the organism (subjected to the identical, specific preparation by double subcutaneous injection of the sorbed dysentery antigen) to the third injection, made after a significantly longer period of time (2 months) was investigated. The corresponding data are shown in Figures 7 and 8.

It can be seen from the figures that the third inoculation made after a significantly longer interval between the second and the third inoculation, produces in the organs a lower concentration of not only antigen, but also of antibodies. Only in the blood serum is the average antibody titer after the combined subcutaneous-peroral inoculation relatively high.

Thus, using the double subcutaneous immunization with the absorbed complete Flexner's bacteria antigen with a ten-day interval between the first and the second inoculation, followed by the third subcutansous inoculation twenty days after the second with the same not sorbed antigen, and at the same time with corpuscular dose of vaccine per os, we obtain a definite shift in the reaction of animal organisms, manifested in the intensity of the absorption of antigen by the organ tissues and in the formation of antibodies.

The obtained data indicate that in this method of immunization the whole organism of the animal is more involved in the reaction to the introduced antigens, since the antibodies of relatively high titer were found in the blood serum, spleen, lungs and even the liver. The lymphoid elements of intestines also contained significant amounts of antigen and antibodies.

All of the above described data indicates that during the dysentery immunization, it is possible to obtain a definite shift in the reaction of the organism. In this study an attempt was made to achieve this by varying the method and increasing the number of inoculations of the antigen. It is possible that increase in the reaction of the organism to the introduced dysentery vaccine may also be achieved by using other methods, which are nonspecifically increasing the activity of the reticuloendothelial system of the organism. The study of this aspect of the problem is the subject of our subsequent investigation.

Conclusions

1. Dysentery Flexner's bacteria antigen is almost uniformly distributed in the organsim of rabbits regardless of the form of the vaccine (corpuscular vaccine or complete antigen) and the method of injection (subcutaneously or per os).

The largest concentration of dysentery antigen is found in the cecum, the Peyer's patches of the intestine, the kidneys; smaller amounts in the walls of other sections of the intestinal tract, lungs, mesentery lymphatic nodes and still smaller amounts in other organs.

2. After a single subcutaneous introduction of the complete Flexner's bacteria antigen or corpuscular vaccine, the antibodies in the blood serum as well as in the organ tissue extract, was found in low titers, while after peroral introduction of the vaccine they were almost not found at all.

3. The second subcutaneous injection of the sorbed complete Felxner's bacteria antigen, ten-days after the initial inoculation of a large dose of the same antigen did not cause, in the majority of rabbits, an increase in the concentration of antibodies in the blood serum in comparison with the results of the single inoculation.

4. The double inoculation with a twenty day interval between the two inoculations, when the first inoculation is made with the sorbed complete Flexner's bacteria antigen, while the second inoculation is made with unsorbed antigen subcutaneously and at the same time the corpuscular vaccine is introduced per os, produced a significant increase in the concentration of antigen in the organ tissues, however, the antibody titer both in the blood serum as well as in the organ tissue extracts, was practically the same as the maximum achieved as a result of the first inoculation.

5. The reaction of the organism of the rabbits to the third inoculation, made after the double inoculation of the sorbed Flexner's bacteria antigen with a ten day interval between them, varies as a function of time and the method of the introduction of antigen.

6. The increase in strength of the reaction of the organism of rabbits to the introduction of dysentery antigen, manifested in the presence of a large titer of antibodies both in the blood serum as well as in the organs of the animals, may be achieved by the triple inoculation with the use of the combined subcutaneous-peroral immunization twenty days after the double subcutaneous injection of the sorbed complete Flexner's bacteria antigen.

When the third inoculation is made two months after the second inoculation, a lowering of its effect is observed. The advantage of the combined subcutaneous-peroral method of immunization is still displayed in this case also.

7. Further investigations are necessary to find methods of increasing the immunological activity of the organism to the injected dysentery antigen.

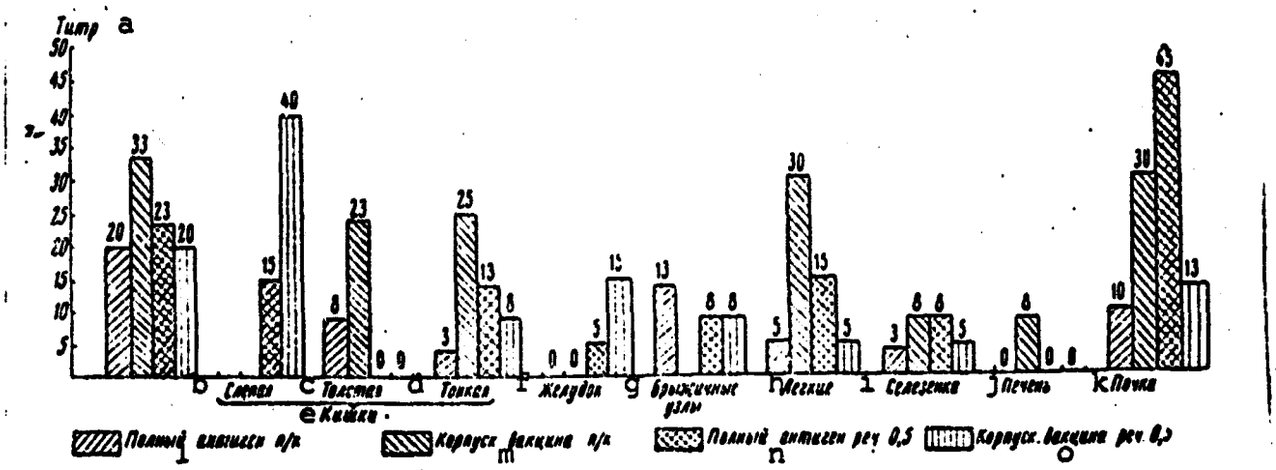

Figure 1. Dysentery antigen in rabbit organs after a single subcutaneous or peroral injection of the corpuscular vaccine and complete antigen.

Key:
a- Titer;
b- cecum;
c- large;
d- small;
e- intestines;
f- stomach;
g- mesenteric ganglions;
h- lungs;
i- spleen;
j- liver;
k- kidney;
l- complete antigen, subcutaneous;
m- corpuscular vaccine;
n- complete antigen peroral 0.5;
0- corpuscular vaccine peroral 0.5.

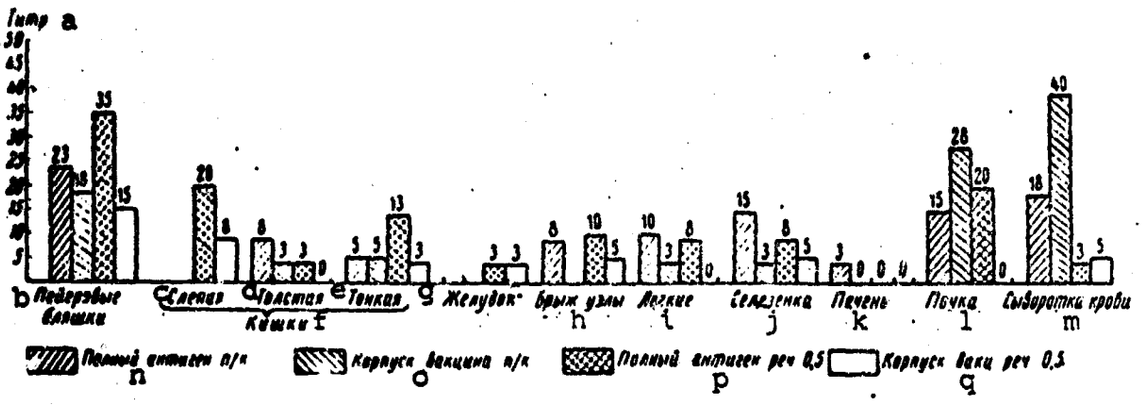

Figure 2. Antibodies in various organs of rabbits after a single subcutaneous or peroral introduction of the corpuscular vaccine and complete antigen of dysentery microbes.

Key:
a- Titer;
b- Peyer's patches;
c- cecum;
d- large;
e- small;
f- intestines;
g- stomach;
h- mesentery nodes;
i- lungs;
j- spleen;
k- liver;
l- kidneys;
m- blood serum;
n- total antigen, subcutaneous;
o- corpuscular vaccine, subcutaneous;
p- total antigen, peroral o.5;
q- corpuscular vaccine peroral 0.5.

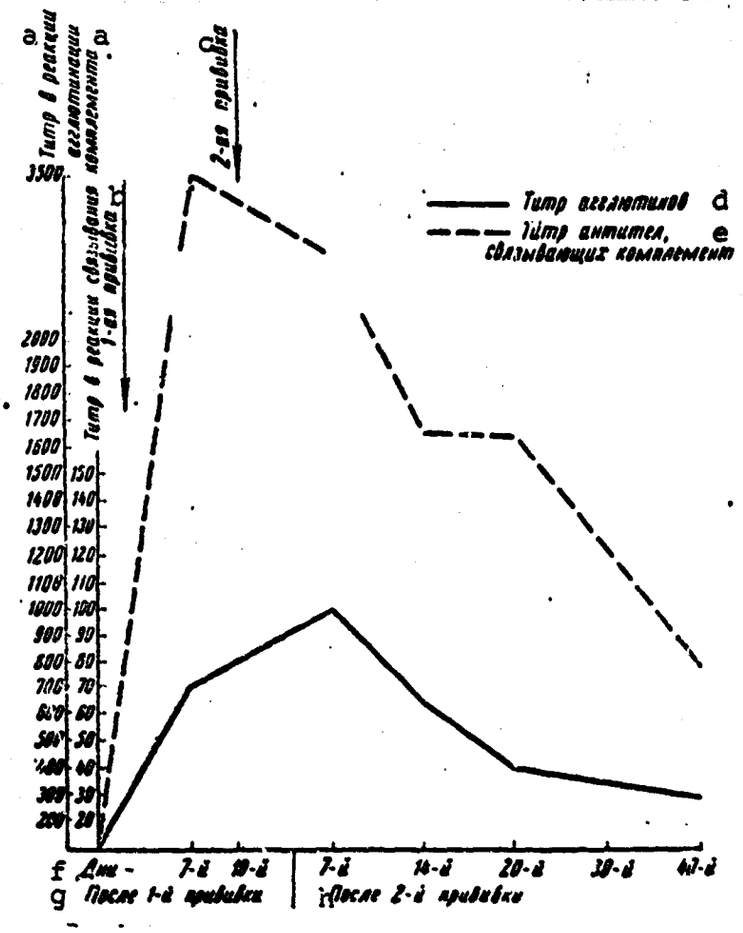

Figure 3. Antibodies in the blood serum after two injections of the sorbed antigen with a 10-day interval between the two injections

Key:
- a- Titer in the agglutination reaction, compliment fixation titer;
- b- first injection;
- c- second injection;
- d- agglutinin titer;
- e- antibody titer in the complement fixation reaction;
- f- days;
- g- after the first injection, 7th day, 10th day;
- h- days after the second injection, 7th, 14th 20th, 30th, 40th.

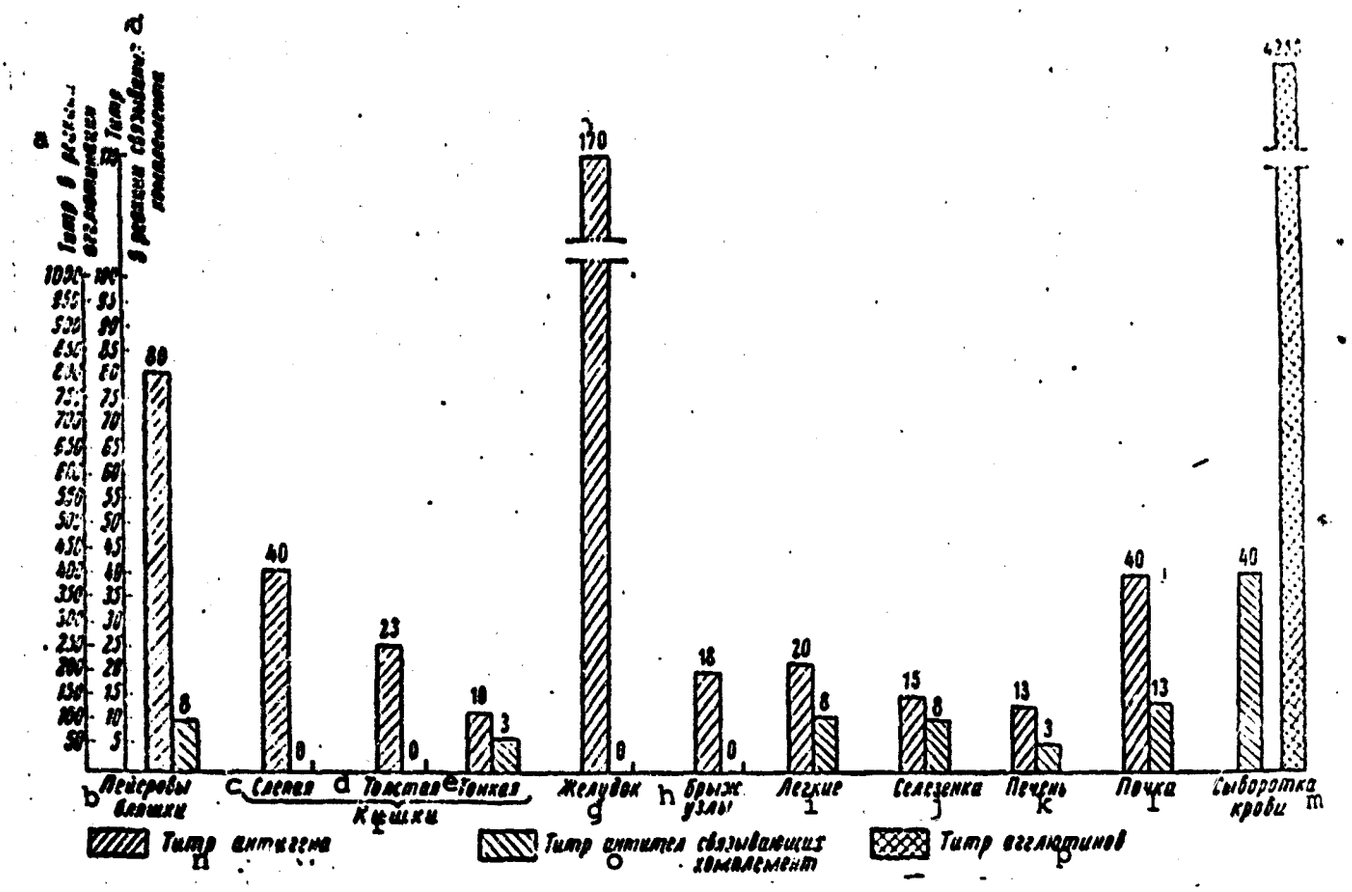

Figure 4. Antigen and antibodies in the organs and blood serum of rabbits after double immunization using the combined method of injection of the antigen.

Key:
- a- titer in the agglutination reaction, titer in the complement fixation reaction
- b- Peyer's patches;
- c- cecum;
- d- large;
- e- small;
- f- intestines;
- g- stomach;
- h- mesentery node;
- i- lungs;
- j- spleen;
- k- liver;
- l- kidney;
- m- blood serum;
- n- antigen titer;
- 0- antigen titer complement fixing;
- p- agglutination titer.

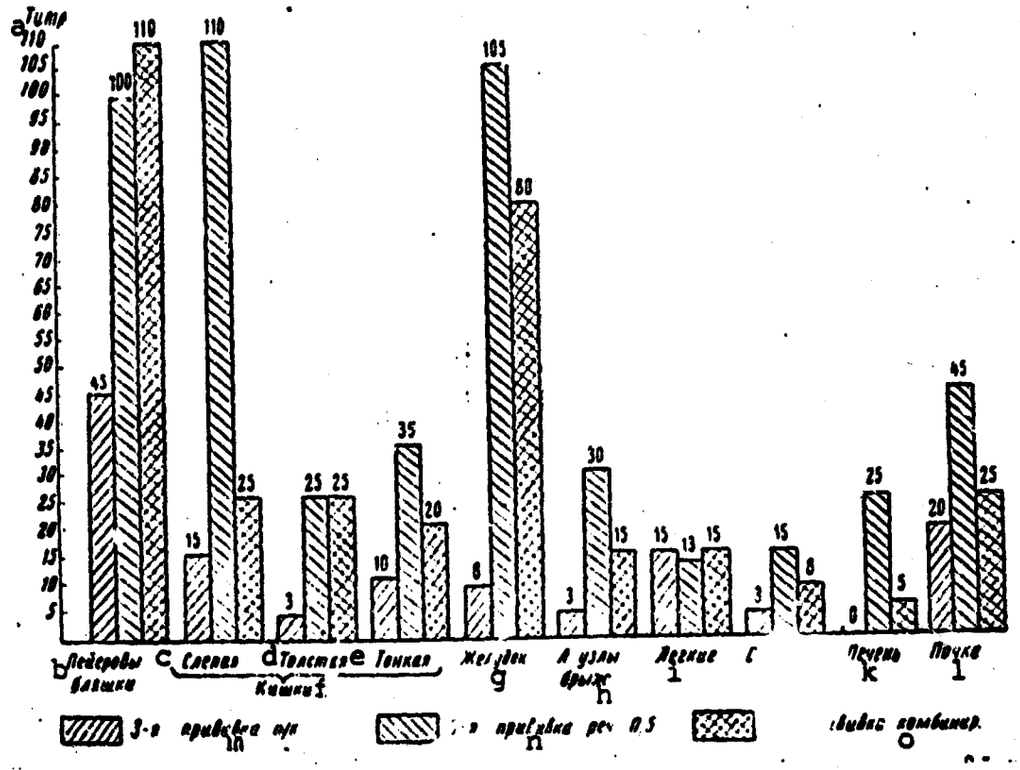

Figure 5. Antigen in the organs of rabbits after the triple inoculation of the vaccine (the interval between the second and the third inoculation was twenty days).

Key:
- a- Titer;
- b- Peyer's patches;
- c- cecum;
- d- large;
- e- small;
- f- intestines;
- g- stomach;
- h- mesentery nodes;
- i- lungs;
- j- spleen;
- k- liver;
- l- kidneys;
- m- third inoculation subcutaneously;
- n- third inoculation perorally 0.5;
- o- third inoculation combined.

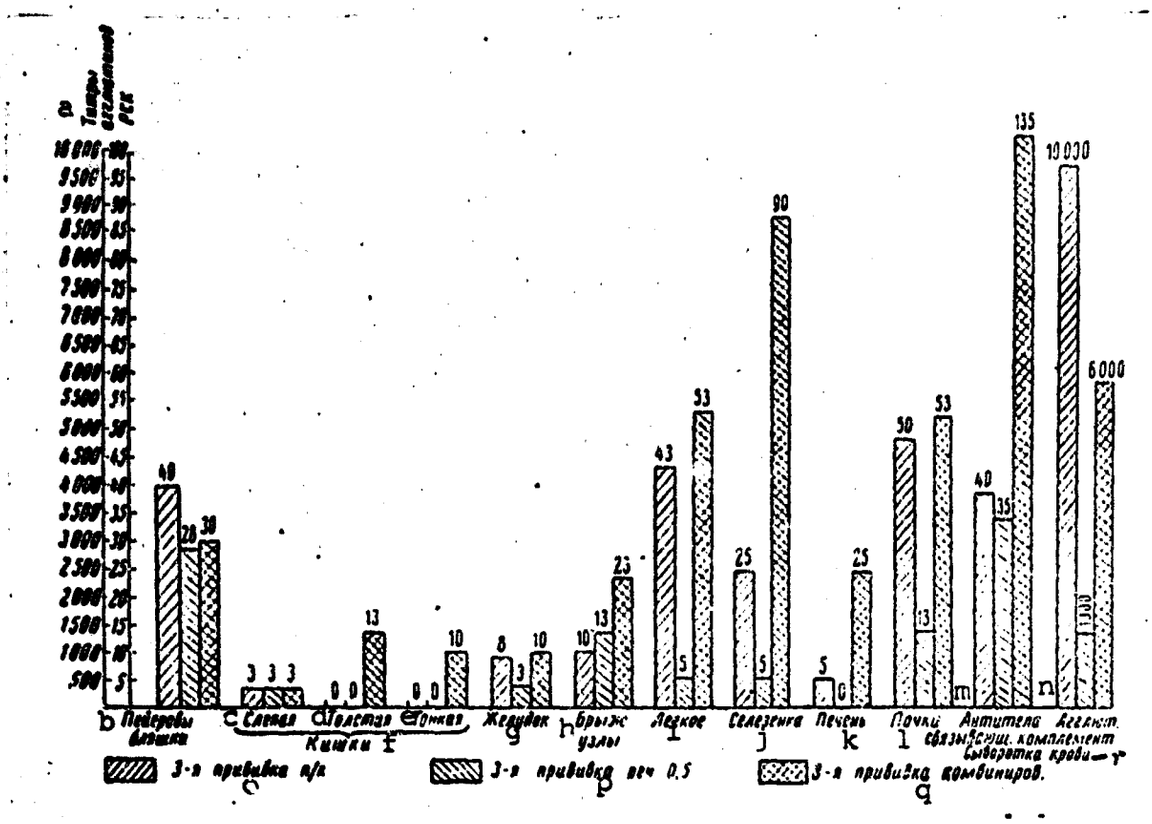

Figure 6. Antibodies in the organs and blood serum of rabbits following a triple inoculation with dysentery vaccine (the interval between the second and the third inoculation was twenty days.

Key:

a- Agglutinin titer, complement fixation reaction (RSK);
b- Peyer's patches;
c- Cecum;
d- large;
e- small;
f- intestines;
g- stomach;
h- mesentery nodes;
i- lungs;
j- spleen;
k- liver;
l- kidney;
m- antibodies determined in the complement fixation reaction;
n- agglutinin;
o- third subcutaneous inoculation;
p- third peroral inoculation 0.5;
q- third combined inoculation;
r- blood-serum.

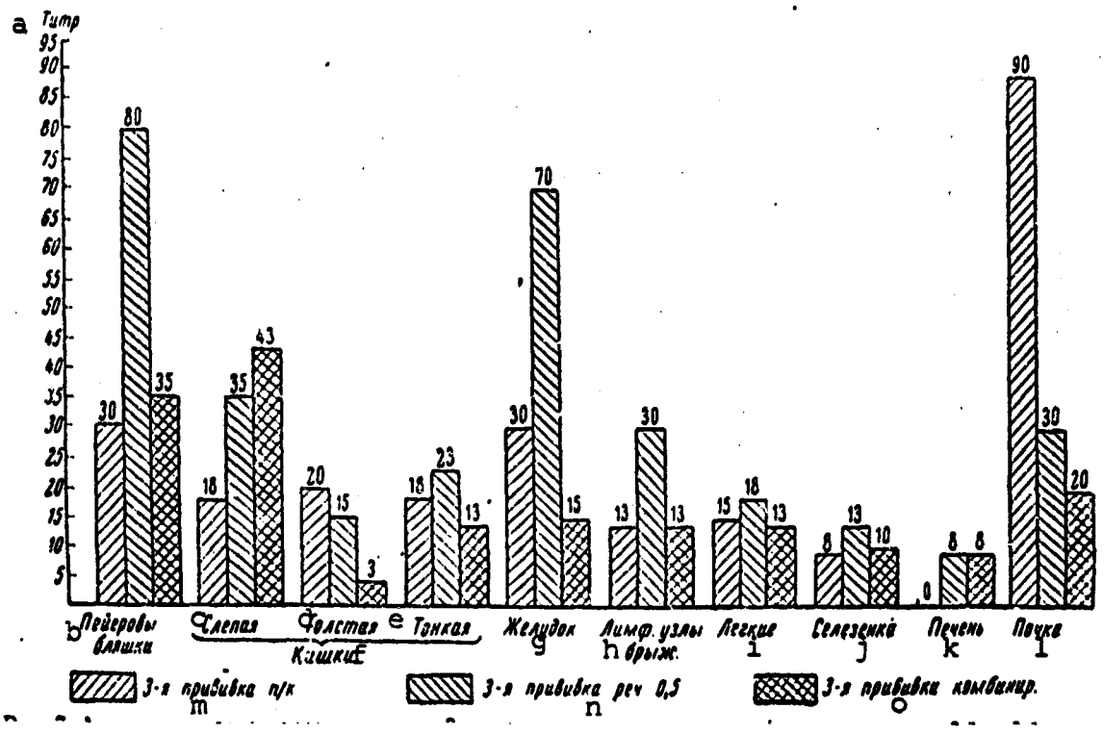

Figure 7. Antigen in the organs of rabbits after the third introduction of the vaccine (the interval between the second and the third inoculation, was 2 months)

Key:
a- Titer;
b- Peyer's patches;
c- cecum;
d- large;
e- small;
f- intestine;
g- stomacn;
h- mesentery lymphatic nodes;
i- lungs;
j- spleen;
k- liver;
l- kidneys;
m- third subcutaneous inoculation;
n- third peroral inoculation 0.5;
o- third combined inoculation.

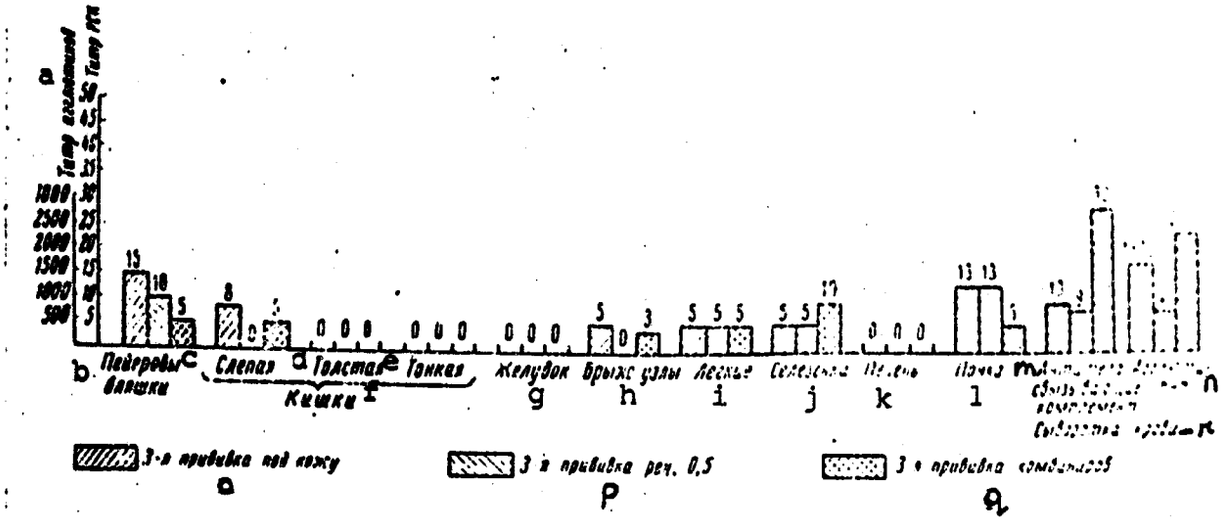

Figure 8. Antibodies in the organs and blood serum of rabbits after the third injection of the dysentery vaccine (the interval between the second and the third inoculations was 2 months).

Key:
- a- Agglutinin titer, complement fixation reaction titer;
- b- Peyer's patches;
- c- cecum;
- d- large;
- e- small;
- f- intestines;
- g- stomach;
- h- mesentery nodes;
- i- lungs;
- j- spleen;
- k- liver;
- l- kidney;
- m- antibodies determined by the complement fixation reaction;
- n- agglutinins;
- o- third subcutaneous inoculation;
- p- third peroral inoculation; 0.5
- q- third combined inoculation;
- r- blood serum.

EFFECT OF THE AGE FACTOR OF THE PROCESSES OF ABSORPTION OF ANTIGEN AND THE FORMATION OF ANTIBODIES IN RABBITS

by

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It is well known that young animals, which are born underdeveloped, are insensitive or display low sensitivity to the injection of many pathogenic microbes, and the infectious process in these animals proceeds in a unique manner.

At the same time there are indications that these animals have low resistance to the organisms causing septic processes and death as a result of the generalized infection (Arshavskiy, Sirotinin and others).

The same phenomenon is observed in human pathology, and it is manifested in the tendency of the newborn babies to the septic processes, while at the same time they have a high resistance to the casual organisms of a number of other diseases (exanthematous fever, scarlet fever, etc.) and the absence of the local reaction to the injected toxins.

The available observations indicate that phagocytosis in newborn babies and animals is generally low and retarded. This phenomena is associated both with lowering of the phagocytic activity of leukocytes, as well as with weak opsonocytophagic ability of the serum (Latysheva). The latter is increased with age and according to the data of Nuriyev it achieves a maximum for staphilocci in rabbits by the age of 3 to 3½ months.

The functional ability of the reticuloendothelial system in children and newborn animals is also significantly (approximately a factor of 3) lower than in adults (Litvak).

The permeability of the connective tissues in young animals is higher, which according to the opinion of Duran Reynolds results in much more severe cases of a number of infectious diseases and a tendency to the development of septic processes.

There are also indications that the permeability of the mucous membranes of the intestines in young animals is also higher, mice calves (Sinay, Korshakova and Dobronitskaya, Fradkina, Kirch, Khait and others). In the newborn animals bacteremia occured upon peroral injection of a dose of microbes (Flexner's bacteria and others), which did not produce any penetration of the bacteria beyond the bounds of the intestinal tract in adults. This is explained by the inability to destroy microbes in the intestinal tract due to immature and areactive tissues of the organisms of young animals.

The inability of the newborn animals to produce the reaction to the injected pathogenic casual organisms, which is characteristic of the sexually mature animals, is closely related to the absence of the ability of forming active immunity in the young. Thus, for example, Pletsityy has shown that active immunization of mice with tetanus antitoxin is effective only after the age of three weeks.

The literature data have shown that antibodies are not produced in young animals, which are born underdeveloped (Pletsityy, Khalyapina). Other investigators were able to obtain antibodies by the immunization of young animals, rats, rabbits, piglets. (Pille, Sokolova and Kolegayeva), however, their titer was lower than for the adult animal. The ability to reproduce antibodies increases with age and is the highest in the middle age. The experimental data also shows that guinea pigs, which are normally born more mature physiologically, do not display such age difference in the ability to produce antibodies (Pille, Khalyapina).

Berman shows that young animals, (mice, pigs, rabbits) are capable of not only producing antibodies (although in a smaller degree than the sexually mature animals), but also that they have a sharply developed barrier fixing function, and there is no reason to reject the possibility of immunological reconstruction in such animals after the injection of antigen. However, in view of the immature enzymatic systems of the organism of the newborn, Berman ascribes a significant effect to the property of the injected antigen and considers that the chemical vaccines for the young are much more effective than the corpuscular vaccines.

In view of the insufficient and partially contradictory literature data on the possibility of producing immunity in newborn animals we undertook this work in order to study certain aspects of the mechanism of inoculated immunity in animals which are born underdeveloped.

Earlier it was shown (Ginzburg-Khalinina and Vakarina), that the antigen of the typhus abdominalis microbe, as well as of the corresponding antibodies, may be found by complement fixation (at low temperature) in many organs of rabbits in the course of a more or less prolonged period of time after the injection of the vaccine.

It was of interest to detect the ability of cells of the organ tissues in newborn rabbits to absorb abdominalis antigen and the effect of the level of the physical development on this ability. At the same time the ability to form antibodies by determining their concentration both in the blood serum as well as in the internal organs, was determined.

The experimental young rabbits were those born to females which were not subjected to the experiments. After 1, 5, 10, or 15 days from their birth the rabbits were injected subcutaneously in the side with heated typhus

abdominalis vaccine from the T_{u2} strain culture. Accordingly, the newborn rabbits were subdivided into four experimental groups.

The injection of 1 ml of typhus abdominalis vaccine, containing 1 billion microbes, had no effect on the weight of the experimental rabbits in comparison with the control rabbits which were not inoculated. The above dose was therefore used in the experiments. The animals of each experimental group were killed by exsanguination 6 hours, 5 and 10 days after the injection of the vaccines, and for each of these time studies the organs and blood serum were taken from 3 to 5 rabbits.

The concentration of typhus abdominalis antigen and antibodies was determined in the spleen, liver, the walls of the small intestines, kidneys, lungs, tissue in the area of the injection of the vaccine, blood serum and in a number of cases in blood clots.

The method for obtaining the salt extract from the organs and the determination of the concentration of antigen and the complement fixing antibodies we described in our earlier publications.

To obtain the extracts from blood clots the latter, after separation from the serum, were covered with an equal volume of saline solution and extracted in a manner similar to that used with other organs.

It should be noted that the organs of non inoculated newborn control rabbits contained no traces of typhus abdominalis antigen or antibodies.

The data on the amount of antigen in the organs of newborn rabbits 6 hours after the injection of typhus abdominalis vaccine are given in Figure 1.

The same figure shows the results of the experiments on the concentration of typhus abdominalis antigen and antibodies in the organs and the blood serum of sexually mature rabbits (weighing 1700 to 1800 g).

In view of the significantly larger body weight of these rabbits, they were injected a correspondingly larger amount of typhus abdominalis antigen, equal to 7 billion microbes.

As it can be seen from the figure the increase in the development of the rabbits has a significant effect on the ability of the organ cell tissues to absorb typhus abdominalis antigen. The animals inoculated on the second day after birth, 6 hours after the injection of the vaccine did not contain antigen in the organs and it was detected only in the tissues in the vicinity of the injection. The rabbits which were injected with the vaccine 5 days after birth demonstrated somewhat greater absorption ability, since in addition to the tissues in the vicinity of the injection antigen was also found in the walls of the small intestines.

When the antigen was injected into rabbits 10 days after birth their absorption function was developed even stronger. The rabbits of this age group contained antigen in the organs in an amount comparable to that found in the organs of sexually mature animals and its distribution in the organism was also identical: antigen was found in the kidneys, the walls of the small intestines, the lungs, the spleen, the tissues in the vicinity of the injection, and was barely detected in the liver. The typhus abdominalis antigen in the organism of rabbits injected 15 days after birth was distributed in a similar manner.

In view of the inability to detect the subcutaneously injected antigens of typhus abdominalis microbes in the organs of newborn rabbits during the first few days of life we attempted to detect it in the circulating blood. For this purpose blood and saline extract of blood clots were studied by the complement fixation reaction. The results of the investigation of the concentration of typhus abdominalis antigen in the blood serum were always negative, however, in blood clot extracts we were able to detect antigens in

1:2 to 1:20 dilutions. It should be noted that all of our attempts to detect typhus abdominalis antigen in the blood clot extract of sexually mature rabbits after the injection of vaccine by different methods generally did not produce any results. Only in isolated cases (when the blood was taken soon after the injection of the antigen) positive results were obtained.

The possibility of detecting typhus abdominalis antigen in blood clot extracts of newborn rabbits, can be explained on one hand by the injection of a large amount of antigen with respect to their total volume of blood, and on the other hand, the underdeveloped reticuloendothelial system. This creates the condition for the circulation of a larger amount of antigen in the blood of newborn animals than in adults. However, even under these conditions the antigen is found in the extracts of blood clots and not in the serum. Apparently the large absorptivity of erythrocytes is very easily manifested with respect to typhus abdominalis antigen.

The data, represented in Figure 2, indicates that newborn rabbits, injected with typhus abdominalis vaccine during the first day after birth, had no detectable complement fixing antibodies 5 days after the injection of the vaccine either in the serum of in the organs. The injection of the antigen into the 5 day old animals produces detectable concentrations of antibodies only in the spleen and lungs. In the rabbits of the next test group, inoculated 10 days after birth, antibodies were found in the tissues in the vicinity of the injection, in the spleen and a small titer (1:10) in the lungs and kidneys.

The animals inoculated 15 days after birth contained the complement fixing antibodies in the majority of the investigated organs. They also contained the antibodies in the blood serum. However, neither in the organs nor in the blood serum of newborn rabbits did the antibody titer ever reach the same level as it did in sexually mature animals,

produced as a result of the injection of corresponding doses of vaccine.

Thus, it was found that the ability to absorb typhus abdominalis antigen by the cell tissues of organs in newborn rabbits is developed significantly earlier than the antibody forming function. The experimental data indicates that for the production of antibodies the presence of antigen in the organism and the presence of functional activity of the reticuloendothelial system is insufficient. Thus, for example, rabbits innoculated 10 days after birth while displaying a well developed absorption ability of the organ cell tissues and a significant concentration of antigen in them, contain only traces of antibodies in the organs. When the antigen is injected on the fifteenth day after birth, the rabbits after recovery and restoring of great vital activity display a certain increase in the concentration of antibodies in comparison with the rabbits of the previous group. Apparently, for the formation of antibodies of relatively high titer after the injection of antigens the existence of the ability of the cell tissue to absorb antigen is insufficient. The function controlling the formation of the antibodies is closely related to the age of the development of the animals and the state of reactivity of the organism, which, according to the opinion of many investigators is determined by the degree of maturity of the central nervous system.

Conclusions

1. Maturing of newborn rabbits affects the ability of the organ cell tissues to absorb typhus abdominalis antigen after its subcutaneous injection.

2. The ability of the organ cell tissues of the newborn rabbits to absorb typhus abdominalis antigen is developed completely by the tenth day after the birth of the animal.

3. The ability to form antibodies is practically absent in newborn rabbits.

4. The ability to produce antibodies, which is associated with the maturing of the central nervous system and the formation of the total reactivity, increases with the overall development of the newborn rabbits.

5. The ability to absorb antigen by the organ cell tissues in the newborn rabbits develops earlier than the antibody producing function.

Bibliography

[1] Arshavskiy, I.A. "The Reflex Principle in Infectious Pathology in the Light of the Data of the Physiology of Orthogenesis," Journal of Microbiology, Epidemology and Immunobiology (ZhMEI), No. 10, 104 to 110 (1955).

[2] Berman, V.M. "Immunoreactivity of the Developing Organism" Pediatrics, No. 5, 26 to 37 (1947).

[3] Vakarina, Ye.F. and Ginzburg-Kalinina, S.I. "Absorption of Antigen and the Formation of Antibodies in Vaccinated Animals." Principles of Immunology -- Transactions of the Inter-Institute Scientific Conference, Moscow, Vol. 3, 247 to 259 (1956).

[4] Latysheva, M.I. "Opsonizing ability of the Serum of Children and Adults" (ZhMEI), No. 4, 97 to 102 (1955).

[5] Litvak, R.V. "Certain Physiological Indices and Their Variations in Adult and Newborn Rabbits" ZhMEI, no. 12, 91 to 95 (1956).

[6] Nuriyeva, A.R. "Study of the Functional Properties of the Reticuloendothelial system in the Normal State in Laboratory Animals," Collection of Abstracts and Individual Articles of Epidemology, Microbiology and Hygiene, Joint Publishing House, Baku, (1956), 134 to 135.

[7] Pille, E.F. "Immunological Characteristics as a Function of the Maturity of the Experimental Group" ZhMEI, No. 1, 58 to 64 (1952).

[8] Pletsityy, D.F. "Some Aspects of the Theory of Immunity and Prevention of Infections by Inoculation" Soviet Medicine, No. 11, 5 to 11 (1951).

[9] Plitsityy, D.F. "Sepsis Problems in Light of the General Pathological Behavior" Transactions of the Academy of Medical Sciences of the USSR, Academy of Medical Sciences of the USSR Publishing House, Moscow, Vol. 19, (1952), 187 to 198.

[10] Sinay, F.Ya., Korshakov, A.S. and Dobronitskaya, S.V. "Permeability of the Intestinal Membrane of Flexner's Group Bacteria Under Experimental Conditions" ZhMEI, No. 4, 58 to 61 (1951).

[11] Sirotinin, N.N. "Evolution of the Reactivity of Organisms" Problems of Reactivity and Shock, Medical Publishing House, Moscow, (1952), 8 to 19.

[12] Skolova, Yu.V. and Kolegayeva, A.I. "Production of Antibodies in the Developing Organism," Problems of Immunology in Different Age Groups, Medical Publishing House, Leningrad, (1947) 97 to 107.

[13] Frankina, R.V., Kirch, N.L., Khait, S.L., Kogan, N.V., Sinay, G.Ya. and Neyshtadt, A.G. "Pneumoenteritis, Septic and Paratyphoid Disease in Newborn Calves" Soviet Veterinary Medicine, No. 1, 17 to 36 (1935).

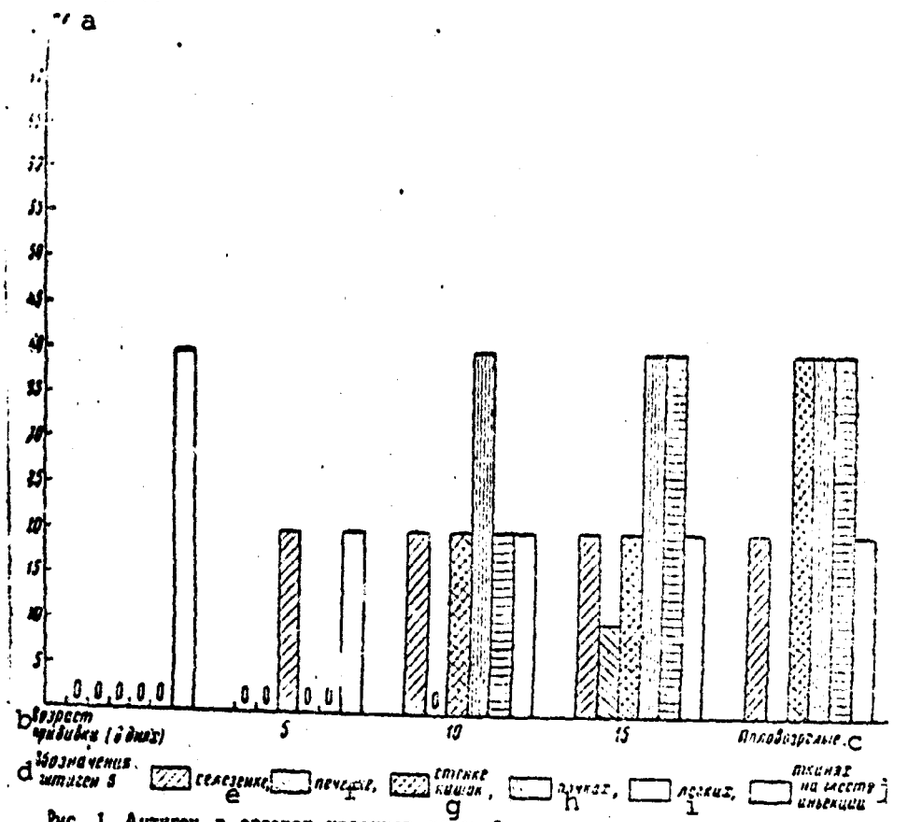

Рис. 1. Антиген в органах кроликов через 6 час. после введения брюшнотифозной вакцины

Figure 1. Antigen in organs of rabbits 6 hours after the injection of typhus abdominalis vaccine.

Key:
- a- Titer;
- b- age of inoculated rabbits (in days);
- c- sexually mature;
- d- designation for antigen in;
- e- spleen;
- f- liver;
- g- intestine walls;
- h- kidney;
- i- lungs;
- j- tissues in the vicinity of the injection.

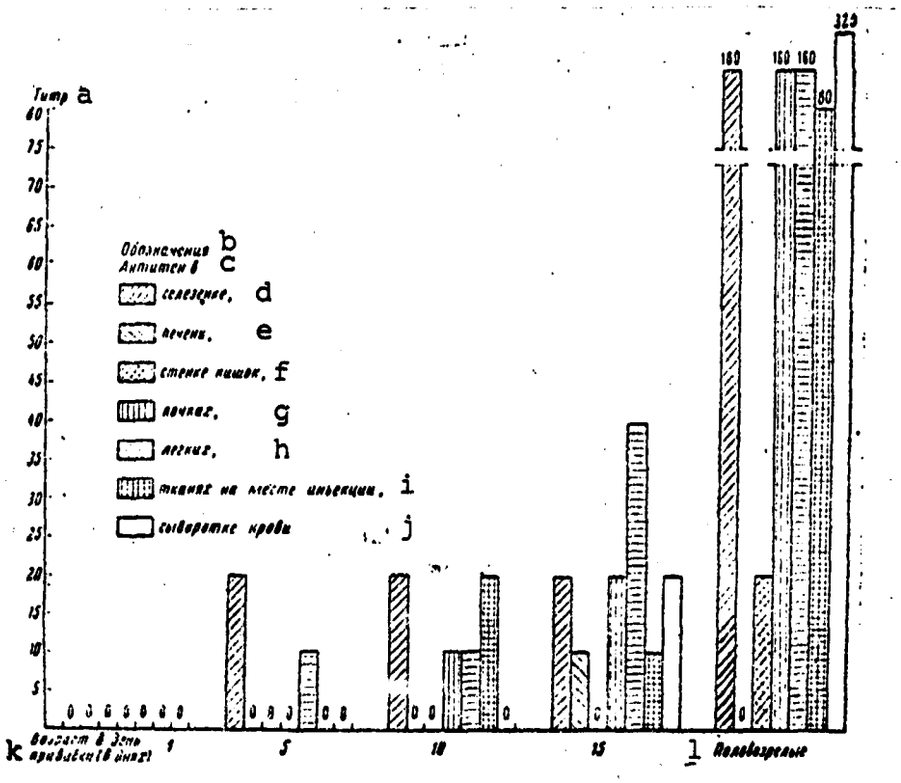

Figure 2. Antibodies in organs and blood serum of rabbits 5 days after introduction of typhus abdominalis vaccine.

Key:
a- Titer;
b- Legend;
c- Antibodies in;
d- spleen;
e- liver;
f- intestine walls;
g- kidneys;
h- lungs;
i- tissue in vicinity of injection;
j- blood serum;
k- age on day of inoculation;
l- sexually mature.

PROTECTIVE PROPERTIES OF ORGAN TISSUES AND BLOOD SERUM IN VACCINATED ANIMALS

by

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In the mechanism of inoculated immunity the role of the antibodies thus far has not been completely solved. Some investigators deny them any role, while others ascribe to them a significant effect in the protection from infection. It was established that antibodies change the state of the casual organism, aid in its phagocytosis by the tissue cells, and lysis. From this standpoint it is not sufficient to study antibodies in the blood serum, since the principal protective process (phagocytosis) and also the formation of antibodies occur in the organ cell tissues. According to the data of a number of authors the typhus abdominalis microbes entering the organism, penetrate very rapidly the cells of the parenchymatous organs, where they multiply. Therefore, the development of the infectious process depends to a significant extent on the presence of the absence of the protective factors in the cell tissues of the organs of the animal. The investigations of Ginzburg-Khalinina and Vakharina have shown that in addition to the blood serum antibodies are localized, primarily in the tissues of three organs in animals, where under the experimental infectious conditions, multiplication of the microbes takes place. Thus, in the case of immunization of rabbits with typhus abdominalis antigen, antibodies were discovered in many organs of the animal: the mesentery lymphatic nodes, the spleen, etc. After the immunization with dysentery antigen

the antibodies were more localized, primarily in the lymphatic apparatus of the intestines, and were found in much lower titers. A significant volume of data is available in literature of the protective properties of the blood serum and there is practically no experimental evidence of the presence of these properties in the tissues of organs of the immunized animals.

The purpose of this work was to determine whether the extracts of organs of the immunized animals have the protective properties with respect to the pathogenic bacteria and to what extent these properties are developed in the different organs. The work was carried out with rabbits. For immunization of the rabbits a formalin treated paratyphoid V vaccine was used. Seven billion microbes were injected into each rabbit subcutaneously into the hip. In the case of double subcutaneous immunization, the rabbits were also injected seven billion microbes with a ten day interval between each injection (3.5 billion x 2).

Following different time intervals after vaccination the rabbits were totally exsanguinated. The tissues from the organs (lungs, spleen, liver, kidneys, mesentery lymphatic nodes) after profuse washing with saline solution were macerated, ground in a mortar with quartz sand and covered with saline solution in a 1:5 ratio. The samples of organs were kept for extraction in the course of three days at 4 to 6°C temperature, after which the supernate liquid was pulled off by suction. The work was carried out under sterile conditions (preservative was not used).

The preventative properties of extracts from the tissues of the organs and the blood serum were tested in the experiments with mice, each of which 24 prior to infection was injected subcutaneously 0.3 ml of different dilutions of the blood serum and the tissue extracts from the rabbit organs (1:10 to 1:8000), extracted during different periods

after the rabbits were vaccinated. Twenty-four hours after the injection of the extract 1 Ds 1 of a wash of 24 hours paratyphoid bacteria V culture suspended in 0.4 per cent agar gel, was introduced into the mice intraperitoneally.

The determination of the antibody concentration was conducted by the quantitative complement fixation reaction at low temperatures. Breliminarily the preventative properties of the organ tissue extracts and the blood serum were determined on unvaccinated animals. The extract of tissues of the different organs of rabbits did not protect the mice from deadly infection with paratyphoid V culture and contained no antibodies for these bacteria. The blood serum of the control rabbits possessed weakly developing preventative properties with respect to the paratyphoid V bacteria (detected only in low dilutions -- 1:10 to 1:20) and low antibody titers. Therefore, in the serum of each animal prior to immunization the preventative properties and antibodies fixing the complement were determined. (fig. 1)

The obtained data are shown graphically, where in the determination of the degree of the development of the protective properties we based our judgement on the maximum dilution of the blood serum and extracts of organs which insured the 50% survival of the experimental mice, which received the lethal dose (1 Ds 1) of live culture.

It can be seen that, four days after the injection of the vaccine the protective properties of the blood serum were weakly expressed and were detected in the dilution not exceeding 1:30. The extracts of rabbit organs during these periods of time showed no signs of the development of protective properties.

Seven days after immunization the protective properties of the blood serum were three times higher: under the same conditions of the experiment the serum protected mice in 1:100 dilution.

By this time extracts of a number of organ tissues also developed certain protective properties (extracts from the mesentery lymphatic nodes, spleen, kidneys in 1:50 dilution, the lungs in 1:100 dilution). Liver extracts had practically no protective properties, in 1:10 dilution they protected only 25 percent of the mice from death.

On the fourteenth day after immunization, the protective properties both of the blood serum as well as of the extracts of organ tissues reached approximately the same level.

Rabbits, seven days after double subcutaneous injection of the vaccine with a ten day interval between injections, developed an increased amount of protective properties in the tissues of certain organs (mesenterial lymphatic nodes, lungs, spleen). The development of the protective properties in the liver did not increase. It should be notes, that the protective properties of the mesentery lymphatic nodes extracts and blood serum of doubly immunized rabbits were identical (titer 1:100).

Thus, after a single or a double subcutaneous injection of vaccine the extracts from a number of organs of rabbits (lungs, mesentery lymphatic nodes, kidneys, spleen) possess protective properties with respect to paratyphoid V bacteria, while in the liver extract these properties were significantly lower.

In order to increase the protective properties of the blood serum and organ tissues triple intravenous immunization of rabbits was carried out with paratyphoid V vaccine (500 million, 1 billion, 1 million). The results are shown in Figure 2.

As it can be seen from the figure, 7 days after the triple vaccination the protective properties of the blood serum were well expressed and appeared in dilution up to 1: 600.

Fourteen days after the injection of the vaccine the protective properties of the blood serum, as well as of certain other organs (lungs, mesentery lymphatic nodes, spleen, kidneys) increase somewhat (1:700 for serum and 1:160 to 1:125 for the organs) and only the liver extract had the lowest protective indices (1:70). In the study of the materials obtained from rabbits, disected on the 20th day after immunization, it was found that the protective property of their blood serum was somewhat lower than from rabbits killed on the 14th day (titer 1:500). The extracts from a majority of organs also protected the mice only in 1:100 to 1:60 dilutions.

After a longer period of time following the vaccination we observed even further lowering of the preventative properties both of the blood serum and organ extracts, and on the forty-fifth day the preventative properties were satisfactory only in 1:25 to 1:10 dilutions.

As a result of the experiments with repeated intravenous immunization of rabbits with paratyphoid V vaccine, it was found that the same regularities which were observed in subcutaneous immunization also apply here.

Thus, the protective properties against paratyphoid V casual organisms were present not only in the blood serum of the vaccinated animals, but also in their organ tissues. In the mesentery lymphatic nodes, spleen, lungs, and kidneys these properties are much more developed than in the liver.

The maximum protective action both of the blood serum as well as of the organ tissues upon three fold intravenous immunization were observed on the seventh through twentieth day after the last vaccination. Subsequently, they begin to decrease and 45 days after the vaccination the protective properties of the blood serum and of the organ tissues are almost insignificant.

It is of interest to compare the obtained data on the preventative properties in the blood serum and organ tissues

with the concentration of antibodies in them. For this purpose in the blood serum and organ tissue extracts of rabbits in addition to determining the protective properties on the same days after the vaccination, using the complement fixation reaction we determined the concentration of antibodies. Figure 3 shows the corresponding data, obtained after triple intravenous immunization of rabbits.

It is necessary to note that in the study of the blood serum and organ tissue extracts, obtained on different days after subcutaneous or intravenous immunization, the preventative properties under the conditions of our experiments were found in higher dilutions than the antibodies fixing the complement. Thus 7 days after immunization the preventative properties in the blood serum were found in very high dilutions, 1:600, while the antibodies were reaching 1:100 titer. The preventative properties of organ tissue extracts at the same time were also well expressed in 1:150 to 1:100 dilutions and the antibodies were found in 1:50 and 1:30 titers. In the liver extracts the preventative properties were weakly expressed and the antibody titer was negligible.

The animals killed on the 14th day after immunization showed only slightly increased preventative properties in the blood serum (titer 1:700), while the antibody titer was greatly increased (1:160). At the same time a certain increase in the preventative properties of organ tissue extracts were noted (1:160 to 1:125) along with the increase of antibodies in them (1:80 to 1:60). It should be noted that in the liver, which contained lower preventative properties (1:70), an insignificant concentration of antibodies was found (1:20). Twenty days after vaccination the preventative properties of blood serum and organ tissues were found to be slightly smaller. The antibody titer was also lower. On the forty-fifth day after immunization both the preventative properties as well as the antibody titer in the blood serum and in organ extracts were found to be lower

(1:25 to 1:10 dilution).

The presented data do not show a total parallelism between the antibodies, found in the complement fixation reaction, and the preventative properties, however, one cannot deny the presence of a correlation between them.

Subsequently, on the basis of the characteristics of the pathogensis and immunogenesis of dysentery, it was of interest to determine the preventative properties in the organs of rabbits after immunization with dysentery vaccine. The foundation for these experiments was laid by the observations of Ginzburg-Khalinina and Vakharina on the distribution of antigen and antibodies in animal organisms after the injection of dysentery vaccine.

It was shown, that the dysentery bacteria antigen, in comparison with typhus abdominalis bacteria antigen, is much more localized. Thus, dysentery antigen and antibodies were found, primarily in the lymphoid elements of the intestinal tract of rabbits, while typhus abdominalis antigen and antibodies were found in large amounts in the tissues of a number of organs (spleen, lungs, lymphatic nodes). The antibodies both in the blood serum as well as in the organ tissues after the injection of typhus abdominalis vaccine, were much more pronounced than due to dysentery antigen immunization.

The study of the preventative properties of tissues of different organs in the blood serum of rabbits were conducted after a triple intravenous immunization with Flexner's type S dysentery vaccine. The immunization method and the study of the preventative properties were the same as in the above described experiments. Figure 4 shows the data of the investigation of the preventative properties of the blood serum and the organ tissues of animals after the injection of dysentery vaccine. Along with this for comparison the results are given which were conducted under

the same conditions with respect to dosage and date, etc., but with the use of paratyphoid V vaccine.

As it can be seen from the figure that on the seventh day after the triple intravenous immunization of the rabbits with dysentery vaccine the preventative properties of the blood serum were found only in 1:100 dilution. In organ tissues they were even weaker, among which the highest preventative property was displayed by the Peyer's patches and the walls of the vermiform process of the cecum (1:50), the mesentery nodes (1:40), and the lung tissues, spleen, and kidneys had the lowest preventative properties (1:25 to 1:20). It should be noted that the preventative properties of the liver were somewhat higher than those of the lungs and spleen (1:35). For rabbits, killed on the 20th day after immunization with dysentery vaccine, the level of the preventative properties both in the blood serum as well as in the organ tissue extracts, was significantly lower than for animals on the seventh day. Thus, in the blood serum it was threefold lower (1:35). Along with this it should be noted that the Peyer's patches of the intestinal tract and the walls of the cecum had the same preventative properties as the blood serum (1:35). The tissues of other organs had lower preventative effecitveness (1:15).

In comparing tnese data with the results obtained in the study of the preventative properties of blood serums, and organ tissues of rabbits after the injection of paratyphoid vaccine, it can be clearly seen that the level is lower following the immunization with dysentery vaccine. Thus, on the seventh day after the injection of the dysentery vaccine the preventative properties of the blood serum were sixfold and the tissue of the lungs, spleen, mesentery lymphatic nodes were 5 to 6 times lower than in the experiments with paratyphoid vaccine. An even greater difference was detected on the 20th day after immunization.

Thus, after the immunization with the paratyphoid vaccine the preventative properties of the blood serum during this period were high (1:500), while after the injection of dysentery vaccine they were 15 times lower (1:35). The preventative properties of organ tissues were also on the average 5 to 7 times lower.

These data again support the opinion that the immunigenesis of dysentery antigen in comparison with the salmonella group microbe antigens is lower.

As a result of the conducted investigation it was established, that in the tissues of a number of organs of animals after immunization with paratyphoid V vaccine, protective properties were manifested against the paratyphoid bacteria. It was shown that in the tissues of different organs this ability is not expressed to the same extent. The protective properties of organ tissues and blood serum in animals are to a certain extent associated with the presence of antibodies in them.

After the immunization of rabbits with dysentery vaccine, the preventative properties of organ tissues and blood serum were lower in comparison with the results of the injection of paratyphoid antigen. This corresponds to the data on the lower effectiveness of antidysenterial vaccination.

On should note the well expressed protective properties of the lymphatic apparatus of the intestines, which is significant in the mechanism of antidysentery immunity. The presence of preventative properties of organ tissues of immunized animals with respect to the corresponding casual organisms cannot be inconsequential to the state of microbes which enter the organism during infection and absorption by the macrophagal cells of a number of organs. Apparently, this must have a significant effect on the preventative immunity.

Conclusions

1. After subcutaneous and intravenous immunization of rabbits with paratyphoid V vaccine, the organ tissues of animals acquire protective properties against the appropriate casual organism.

2. The protective properties in animals immunized with paratyphoid V vaccine wer the highest in the mesentery lymphatic nodes, lungs, spleen, and kidneys, and least pronounced in the liver.

3. The highest development of the protective properties in the tissues of organs of animals after a triple intravenous immunization with paratyphoid antigen was observed during the period of time between the seventh and twentieth days after the immunization. Forty-five days after the immunization the protective properties of organs were found in lower dilutions.

4. The blood serum and organ tissues of animals, immunized with paratyphoid V vaccine, and having the protective properties at the same time, contained antibodies which were determinable by the complement fixation reaction.

5. In comparison with the results obtained during the introduction of paratyphoid and dysentery vaccine into the organisms of the animals, it was found that the blood serum and organ tissues of animals immunized with the dysentery vaccine, have lower preventative properties. After the injection of dysentery vaccine the preventative properties are best manifested in the lymphatic tissues of the intestinal walls.

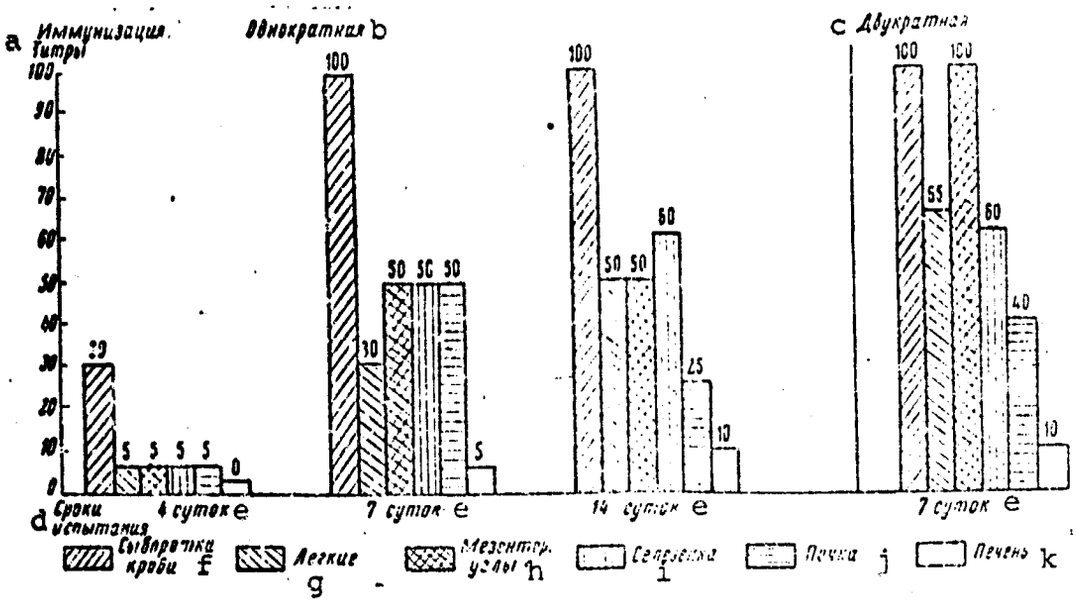

Figure 1. Preventative properties of blood serum and extracts from organs following a single and double subcutaneous immunization with paratyphoid vaccine.

Key:
a- Immunization titers;
b- single;
c- double;
d- test periods;
e- days;
f- blood serum;
g- lungs;
h- mesentery nodes;
i- spleen;
j- kidneys;
k- liver.

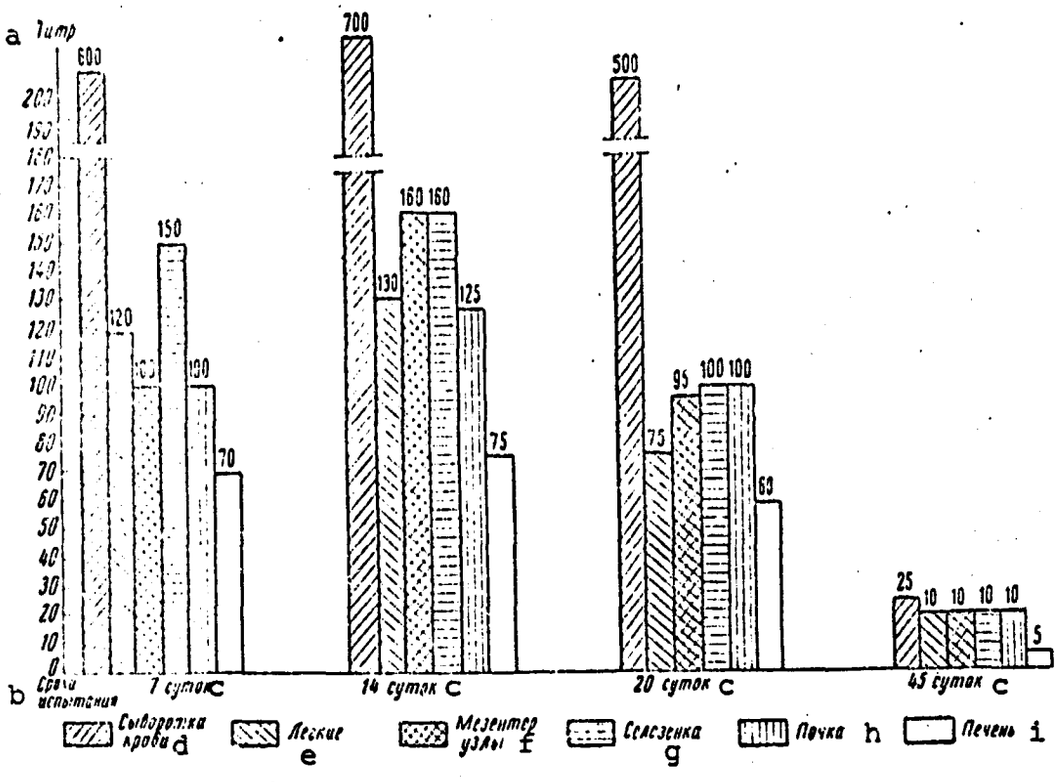

Figure 2. Preventative properties of blood serum and extracts from organs of rabbits after a triple immunization.

Key:
a- titer;
b- test period;
c- days;
d- blood serum;
e- lungs;
f- mesenterynodes;
g- spleen;
h- kidneys;
i- liver.

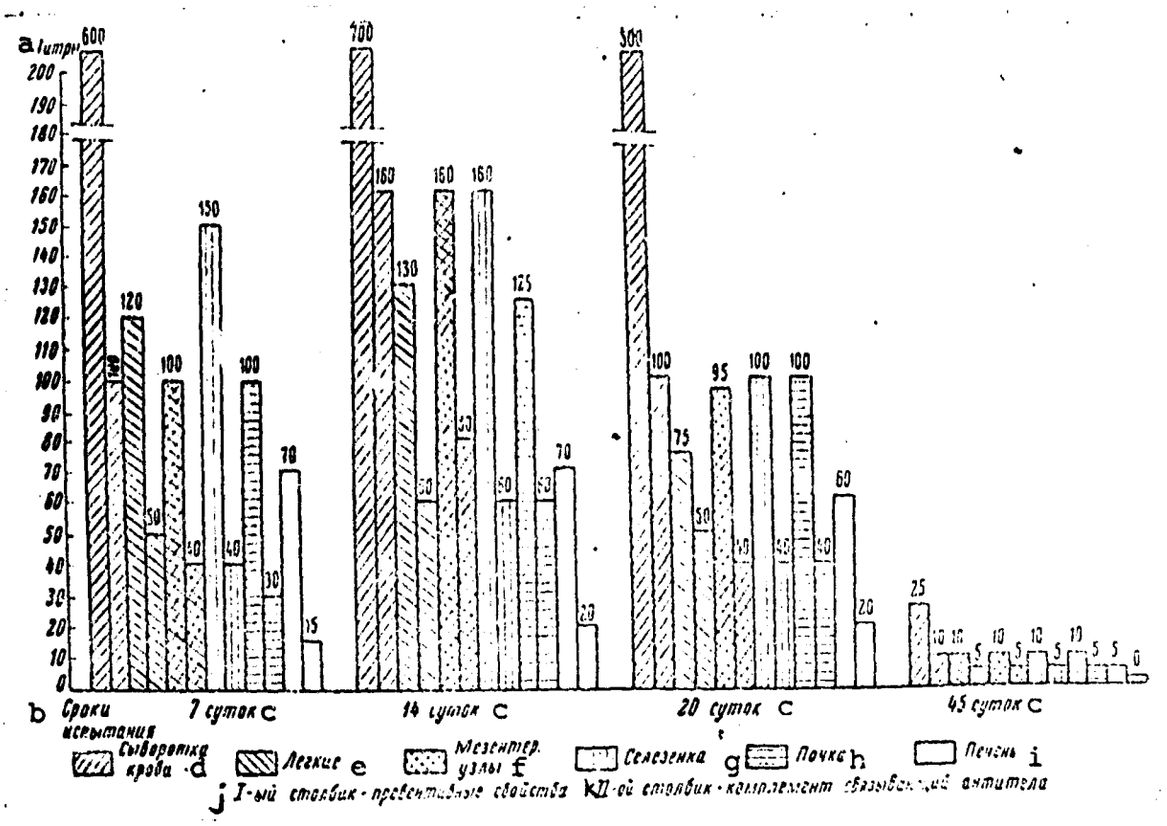

Figure 3. Preventative properties and antibody concentration, determined by the complement fixation reaction, in the blood serum and organ tissues after triple intravenous immunization of rabbits with paratyphoid V. vaccine.

Key:

a- titer;
b- test period;
c- days;
d- blood serum;
e- lungs;
f- mesentery nodes;
g- spleen;
h- kidney;
i- liver;
j- the first column represents the preventative property and
k- the second column the complement fixing antibodies.

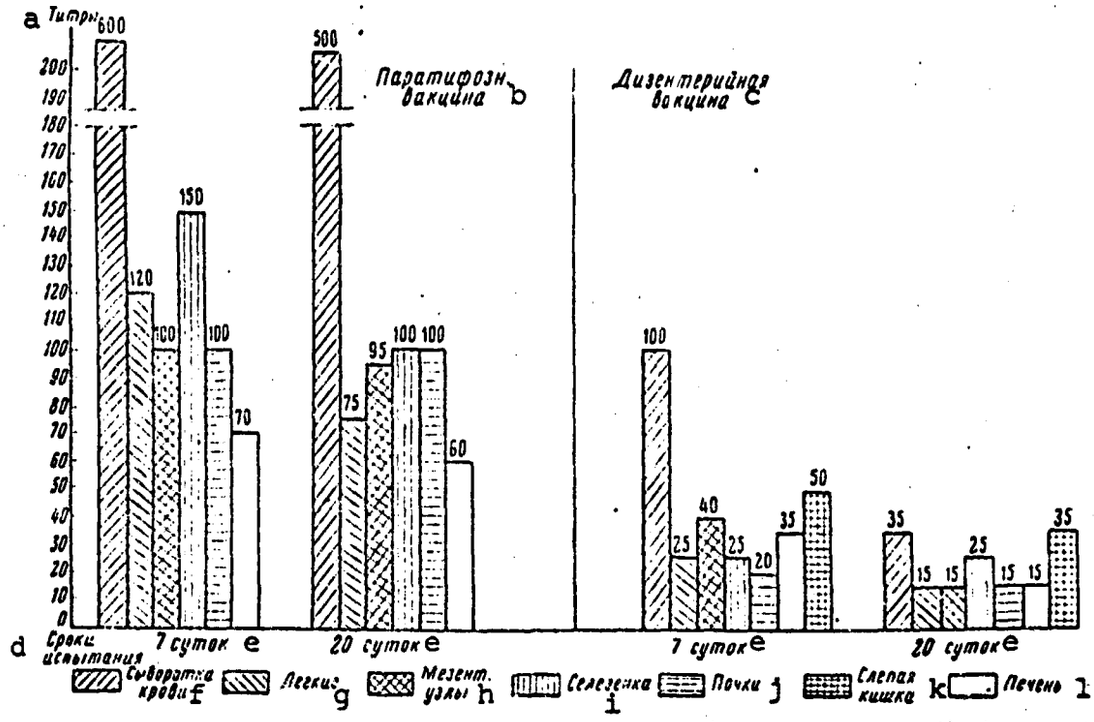

Figure 4. Comparison of the preventative properties of blood serum and organ tissue extracts of rabbits after immunization with paratyphoid and dysentery vaccines.

Key:
- a- titer;
- b- paratyphoid vaccines;
- c- dysentery vaccine;
- d- test periods;
- e- days;
- f- blood serum;
- g- lungs;
- h- mesentery nodes;
- i- spleen;
- j- kidney;
- k- cecum;
- l- liver.

THE EFFECT OF ADRENALECTOMY ON THE FORMATION OF THE INOCULATED IMMUNITY TO INFECTION IN WHITE RATS

by

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A great deal has been published on the role of hormones in immunological reactivity. It had thus been conclusively shown that:

a) the introduction of large doses of glucocorticoids (cortisone type hormones) is accompanied by lowering of the congential resistance of animals to infection and in the retardation of the production of antibodies; b) the existing physiological antagonism between different hormones is also found in the case of immunity phenomena; c) the removal of the adrenal glands in animals is accompanied by a sharp lowering of their resistance to infection and intoxication; d) introduction of glucocorticoids to animals following adrenolectomy restores partially or totally their resistance to infection or intoxication.

To determine the role of different endocrine glands in the immunity phenomena it is necessary to study not only the pharmacological, but also the physiological effects of the various hormones from these glands on the difrerent immunity manifestations, and first of all on the naturally acquired immunity (resistance to infection).

The work with animals in which the endocrine glands were removed and those in which hormones were used in substitution therepy are of great interest in connection with the above described problems.

The results of the multiple use of hormones in large doses (4 to 8 mg/100 g of weight per day) do not explain their functional (physiological) effects in the immunity

phenomena, since concentrations of this magnitude are not found under natural conditions. The data of such investigations contradict the favorable results which are obtained with the use of hormones in clinical practice in treating different infectious diseases (Smadel and co-workers, 1951; Ducci and Katz, 1952 and others) and the beneficial results obtained with the multiple use of small doses (0.01 mg/100 g of weight per day) of corticosteroids in the experiments (Van Cauwenberge, Fazio, Lato, and Ceccarelli, 1956, and others).

Thus, from our viewpoint the most suitable models for the study of the role of the adrenal hormones in immunity are the animals in which adrenal glands were surgically removed, or animals which were subjected to this operation but received supplementary doses of these hormones.

Using this model a number of investigations have been carried out in which the role of the adrenal glands in the formation of antibodies was studied (Roberts and White, 1951 and others) or in the natural resistance to exotoxins (Lewis and Page, 1948; Tonutti, 1952 and others) and endotoxins (Chedid, 1957; Ivanov, 1960a and others).

Since the most reliable index of immunity is the stability of the immunized animal to infection, it is natural that the investigators are interested in the effect of the removal of the adrenal gland and injection of hormones into these animals on the formation of inoculated immunity.

Unfortunately, we were unable to find detailed studies of this problem in the available publications.

The purpose of this work was to study the effect of a bilateral adrenalectomy on the formation of inoculated immunity to Gärtner's bacilli infection [salomenella enderitides.]

Method

In this study 64 normal and 64 adrenalectomized male white rats weighing from 180 to 200 g were used. The

bilateral adrenalectomy was done under ether anesthesia in two stages (the second adrenal gland was removed three days after the removal of the first) through a lumbar incision.

A false adrenalectomy was performed on the control animals.

The immunization of animals with formaldehyde vaccine, prepared from Gärtners bacilli (laboratory strain 466) began seven days after the removal of the second adrenal gland. Each animal was given a single injection of 1 ml of the suspension, containing varying amounts of microbes.

The changes in the resistance to infection under the influence of vaccine immunization were studied according to the following schemes.

Experiment 1. The first vaccination - 1.56×10^7 microbes/ml; the second vaccination (one day after the first) - 1.56×10^7 microbes/ml; the third vaccination (two days after the second vaccination) - 6.24×10^7 microbes/ml. The vaccination in all cases was conducted intraperitoneally.

Experiment 2. The first vaccination - 1.56×10^7 microbes/ml, subcutaneously; second vaccination (one day after the first) - 1.56×10^7 microbes/ml, intraperitoneally; third vaccination (two days after the second) - 2.5×10^8 microbes/ml, subcutaneously; fourth vaccination (three days after the third) - 1.25×10^8 microbes/ml. In this experiment the vaccination was started with a subcutaneous injection because after the primary intraperitoneal injection of 1.56×10^7 microbes/ml part of the animals died (the low resistance of the adrenalectomized animals to the toxic effects of the vaccine becomes very apparent). The single injection of the indicated dose subcutaneously produces a certain tolerance to the subsequent injection of the vaccine.

The infection of animals with different doses (for the determination of the stability from the magnitude LD_{50} according to the method of Rid and Minch, 1938) was conducted

ten days after the completion of the immunization cycle. For infection the same culture was used as for the preparation of vaccine. The death of the animals was evaluated in the course of ten days from the moment of infection.

Experimental Results

In the previous investigations (Ivanov, 1960) it was shown that neither adrenalectomy not the injection of small doses of cortisone (1 ml/100 g. of body weight) to the normal or adrenalectomized animals have any significant effect on the increase of the antibody titer (agglutinins) in the blood serum of immunized animals.

Since the presence of antibodies in the blood serum is not an absolute index of the state of true immunity (i.e., resistance fo infection), then in order to determine the role of hormones of the adrenal glands in the development of innoculated immunity it was necessary to study the effect of the removal of the adrenal glands on the changes in the resistance to infection under the influence of immunization (i.e. on the effectiveness of the immunization on the basis of the resistance to infection).

The indices of the resistance to infection were:
1) the magnitude of the LD_{50} to the live culture and
2) the percent lethality. Two similar experiments were conducted the results of which do not quite coincide (Tables 1 and 2).

Table 1.

The resistance of rats to infection (intraperitoneal immunization).

a Показатель устойчивости	e Число животных			
	f нормальные		g адреналэктомированные	
	неиммунизированные h	иммунизированные i	неиммунизированные h	иммунизированные i
b Число животных	16	16	16	16
c Величина LD_{50} (число микробных тел в 1 мл)	$1,15\times10^{9}$	4×10^{3}	$0,017\times10^{3}$	$0,062\times10^{9}$
d Летальность (в %)	25	14,3	71,4	50

Key: a- resistance to animals; b- number of animals; c- LD_{50} magnitude (number of microbes per ml); d- lethality; e- number of animals; f- normal; g- adrenalectomized; h- not immunized; i- immunized.

To obtain data indicate that adrenalectomy is accompanied by a sharp decrease of the inherited resistance of rats to the infection caused by Gärtner's bacillus (LD_{50} magnitude for adrenalectomized rats was almost 70 times lower than for normal rats, and the mortality was significantly higher: 71.4 and 25.1 per cent respectively). Approximately the same effect was obtained during adrenalectomy in the immunized animal group.

The effects of adrenalectomy on the inoculated immunity may be characterized by comparing the data on the effectiveness of immunization in groups of normal and adrenalectomized animals. The concept of the effectiveness of immunization may be obtained by comparing the resistance (from the magnitude of LD_{50}) of the immunized animals with the resistance of nonimmunized animals.

Such a comparison shows that the resistance of normal immunized animals is 3.48 times higher than the resistance

of normal immunized animals is 3.48 times higher than the resistance of normal nonimmunized animals, while the resistance of adrenalectomized immunized animals is 3.5 times higher than the resistance of adrenalectomized nonimmunized animals (See Table 1.)

Thus, these data show that the removal of the adrenal glands does not have any affect on the formation of inoculated immunity, but it is accompanied by a sharp decrease in the inherited resistance to infection.

Having determined that the removal of the adrenal glands does not have any effect on the formation of inoculated immunity, we naturally were interested in repeating the experiment (see Table 2).

Table 2.

Resistance of rats to infection (mixed immunization)

a Показатель устойчивости	e Число животных			
			Адреналэктомированные h	
	невакцинированные f	вакцинированные g	невакцинированные f	вакцинированные g
b Число животных	16	16	16	16
c Величина LD_{50} (число микробных тел в 1 мл)	$0{,}61\times10^{9}$	$2\cdot10^{9}$	$0{,}0032\times10^{9}$	$0{,}19\times10^{9}$
d Летальность (в %)	32	21,4	82,1	42,8

Key: a- resistance index; b- number of animals; c- magnitude of LD_{50} (number of microbes per ml); d- mortality (in per cent); e- number of animals; f- nonvaccinated; g- vaccinated; h- adrenalectomized.

In the second investigation the scheme of immunization was changed, as it was desired to increase the duration and the intensity of the antigen stimulation (due to the increase of the dose of the third inoculation and the combination of intraperitoneal inoculations with subcutaneous inoculations).

One can say that in a group of normal animals immunization was not any more effective in comparison with an analog-

ous group in experiment one (3.3 and 3.48 respectively). However, in the adrenalectomized group of animals a sharp increase in the effectiveness of immunization was noted. While in experiment number 1 the effectiveness index was equal to 3.5, in experiment number 2 it was equal to 60 (see Table 2).

Thus, the data of experiments numbers 1 and 2 indicate that the removal of the adrenal glands does not affect the development of the inoculated immunity to the same extent as it does in normal rats.

In the evaluation of the obtained results it should be stressed that 24 hours after the removal of both adrenal glands a total exhaustion of the corticosteroids in the organism is noted (Opsal)*.

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* Cited from Dougherti (1952, p. 328)

Consequently, in the experiments we dealt with animals the organism of which had a drastically low corticosteroid level. This is verified by a sharp lowering of the inherited resistance of the adrenalectomized rats to the killed (during the vaccination period) and the live Gärtner's bacilli (see Tables 1 and 2).

This fact is in agreement with the multiple data on the lowering of the inherited resistance to different injurious effects as a result of the removal of hypophysis and adrenal glands.

The significance of the deficiency of glucocorticoids in the lowering of the resistance of the organism of the animal. In the case of adrenalectomized animals it was a primary while in the case of hypophysectomized animals, it was a secondary difficiency, i.e., due to the absence of the stimulation of the adrenal cortex by adrenocorticotropic hormone (Lewis and Page, 1948; Tonutti, 1952; Ivanov 1957, 1959, and others).

Thus the presented data shows, unquestionably the effect of adrenal hormones on the inherited resistance of animals to pathogenic effects.

Apparently, the situation is totally different with the resistance obtained dur to immunization. The obtained data show that in the case of adrenalectomized animals the immunity under the effect of vaccination develops to the same extent (in any case not lower) as in normal animals. This conclusion is supported by the data on the constancy of the antibody titer (agglutinins) in the blood of immunized animals under the influence of adrenalectomy and the influence of the injection of small doses of cortisone to the adrenalectomized and normal animals (Ivanov, 1960b).

It is interesting to note that the injection of the indicated doses of cortisone to adrenalectomized rats restores totally the inherited resistance of rats to the toxic effect of the vaccine, prepared from Gärtner's culture (Ivanov, 1960a).

Conclusions

1. The bilateral adrenalectomy is accompanied by a sharp decrease of the inherited resistance of rats to infection.

2. The absence of the adrenal glands does not affect the development of the acquired (under the influence of vaccination) immunity to the Gärtner's culture infection.

3. In the adrenalectomized rats the mixed (subcutaneous and intraperitoneal) vaccination is more effective than intraperitoneal vaccination alone.

Bibliography

[1] Ivanov, V.M. "The Role of Hypophysis in the Resistance of rats to Diptheria Toxin" Bulletin of Experimental

Biology and Medicine, No.9, 56 to 57 (1957).

[2] Ivanov, V.M. "On the Mechanism of the Natural Resistance of Rats to Diptheria Toxin" Second Conference of Young Scientists, Moscow, pages 27 to 29 (1957) (Second Moscow Medical Institute).

[3] Ivanov, V.M. "The Neurohumoral Effects on the Outcome of Diptheria Intoxication in White Rats" Journal of Microbiology, Epidemology and Immunobiology (ZhMEI), No.11, 90 to 94 (1959).

[4] Ivanov, V.M. "The effect of Certain Hormones on the Resistance of Animals to the Toxic Effects of Gärtner's Bacillus Vaccine" ZhMEI, No.6, 113 (1960).

[5] Ivanov, V.M. "The Mechanism of the Effect of Corticorteroids on Infection and Immunity." Problems of Bacteriology, Virology and Immunity" Transactions of the Moscow Scientific Institute of Vaccines and Serums im. I.I.Mechnikov. Moscow, Vol. 14, 45 to 62 (1960).

[6] Meshalova, A.N. "Effects of Cortisone on the Immunogenesis Processes." Communication No. 2. Immunological Shift Under the Influence of Cortisone and inimmunized rabbits" ZhMEI, No. 1, 11 to 13 (1959).

[7] Berglund, K. Acta pathol. et microbiol. scand. 1956a, 383, 311; 156b, 403; 1956c, 329.

[8] Berglund, K. Proc. Soc. Exper. Biol, and Med., 1956b, 91, 592.

[9] Chedid, L. Bordeaux chirurg., 1957, 3, 158.

[10] Ducci, H. and Katz, R. Gastroenterol., 1952, 21, 357.

[11] Dougherti, T.F. Rec. Prog. in Hormone Res., 1952, 7, 307.

[12] Lato, M. and Ceccarelli, A.Aggiorn, pediatr., 1956, 7, 371.

[13] Moll, T. Am. J. Veterin Res., 1956, 17, 786.

[14] Nicol, T. and Snell, R.S. Nature, 1956, 177, 430.
[15] Pelocchino, A.M. et al. Giorn. batteriol. e. immunol., 1956, 49, 227.
[16] Roberts, S. and White, A. Endocrinol., 1951, 48, 741.
[17] Scheifferth, F. Acta Allergol., 1958, 12, Suppl. 5, 309 to 318.
[18] Schmidt, H. et al. Brit. J. Veneral Dis., 1957, 33, 259.
[19] Schwartzman, G. and Fisher, A. J. Exper. med. 1952, 79, 347.
[20] Smadel, J.E. et al. Ann. Internal. med. 1951, 34, 1 to 9.
[21] Thatcher, et al, Endocrinol., 1948, 43, 330.
[22] Tonutti, E. Z. mikrosk. anat. Forsch., 1942, 52, 32.
[23] Tonutti, E. Arzneimittel-Forsch. 1952, 2, 97.
[24] Van Cauwenberge, H. Acta clin. belg., 1956, 11, 389.

IMMUNOLGICAL, MORPHOLOGICAL AND HISTOCHEMICAL REACTIONS OF THE ORGANS OF RABBITS TO POLYVACCINE INJECTIONS

by

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In spite of its great theoretical significance the relationship of the antigen absorption process to the formation of antibodies in different cell systmes is still unclear.

During the infectious process a number of changes in the morphological structures and biochemical reactions of the organism take place. Analogously, one must assume that after the injection of vaccine into the organism changes in its ordinary structures and functions must also occur.

In spite of the significant volume of experimental data a great deal remains unclear in the interpretation of the cell reactions observed in the organ tissues in response to the injection of antigen. The participation of the individual organs in the synthesis of antibodies and their role in various processes is still insufficiently well elucidated.

The majority of the investigators indicate the significance of plasmatic cells in the production of antibodies (Bing and Plum, Koons and co-workers, Fagreus) and the cells of the macrophagal-endothelial system (Mechnikov, Sebin). On the other hand, there are data on the participation of lymphocytes in the formation of antibodies (Harris and co-workers, Daugherty, Chez and White).

After the acceptance of the protein nature of antibodies many of the investigators established the significance of the intensity of protein metabolism in cells for the formation of antibodies. Individual investigations show that

the increasing basophilia and increased concentration of ribonuclei acid may be used as the indices of the biological activity of cells (Brashe, Fagreus, Rappoport).

The investigations of Kartashova and Bodisko established the relationship between the increased amount of ribonucleic acid and the formation of antibodies in rats, innoculated with Gayskiy's tularemia vaccine. The obtained data are based on the comparison of the concentration of ribonuclei acid in the organ tissue cells, and antibodies in the blood serum. On the other hand the work of Ginzburg-Khalinina and Vakarinina has shown that after the injection of typhus abdominalis microbe antigen into rabbits both, the antigen as well as the antibodies may be found in the tissues of a number of organs in the course of a long period of time.

Therefore, it is of interest, to expand the above-described observations. In this connection the work had been carried out, the purpose of which was:

1) to determine the relationship between the concentration of antigen in the different organs and the morphological changes resulting from its presence;

2) to determine the effect of antigen in the organs on their activity in the antibody formation processes. This is partially manifested by the activation of reticuloendothelia the occurence of plasmatic cells and the increase in the concentration of ribonucleic acid.

This work was carried out with chinchilla rabbits weighing from 2.4 to 2.7 kg. Polyvaccine series 361, precipitated with calcium phosphate and containing antigens of typhus abdominalis, paratyphoid A and B, Flexner's and Zonne dysentery, cholera and tetanus antitoxin, was injected.

The polyvaccine was injected in a single 2 ml dose subcutaneously on the inner part of the hip. The rabbits were totally exsanguinated after different periods of time had elapsed, since the injection of the vaccine (1, 2, 7, 10, 30 and 45 days).

The internal organs of the animals (liver, spleen, mesentery lymphatic nodes, small intestine walls with Peyer's patches, lungs, kidneys) were studied by serological reactions to the concentration of the most active typhus abdominalis antigen in the polyvaccine and the corresponding antibodies. The morphological studies involved the determination of the presence of the active reticuloendothelial and plasmatic cells. Histochemical studies were conducted to determine the presence of ribonucleic acid.

For serological investigations saline solution extracts of the organs were prepared in a 1:4 ratio.

The concentration of typhus abdominalis antigen was determined in the quantitative complement fixation reaction at low temperature with the corresponding hyperimmune rabbit serum. Antibodies which fixed the complement, were determined in the extracts of organs and blood serum by the complement fixation reaction with typhus abdominalis antigen.

One day after the injection of the polyvaccine, insignificant amounts of the antigen of typhus abdominalis microbes were found in the spleen. Two days after the injection it was not determinable at all. However, in the period starting with the seventh day, through to the forty-fifth day after the injection, an almost identical low concentration of antigen was found (the titer was 1:10 to 1:15) (Fig. 1).

Low titers of antibodies were found in the spleen (up to 1:20) after one day. Two days after the injection, their concentration was lower, and subsequently the antibody titers were increasing (1:25 to 1:30) and only after 30 days the lowering was again observed (1:10 on the 45th day).

The amount of ribonucleic acid was increasing moderately on the first day after the immunization and increased significantly by the seventh day. On the tenth day it began to decrease slightly and remained at that sufficiently high level until the end of the experiment.

The activation of cells of the reticuloendothelial system was noted one day after the injection of antigen and continued through the seventh day. Later the number of activated cells decreased, since by this time, the majority of them were converted to plasmatic cells. However, on the 30th and the 45th day, the number of activated reticulendothelial cells in the spleen again increased. It is believed that this phenomenon is associated with the recirculation of the antigen which is gradually resorbed from the depot.

One day following the injection of the vaccine, the number of immature plasmatic cells in the spleen doubled and by the seventh day, it tripled in comparison with their content in control rabbits. It remained at this level until the tenth day and then decreased by the thirtieth day. On the fourth to fifth day after the immunization, the number of immature plasmatic cells again increased similarly to the behavior of the reticuloendothelium. By the seventh day after the injection of the vaccine, the number of mature plasmatic cells in the spleen varied, but it was generally somewhat higher than in the control rabbits. After the tenth day simultaneously with lowering of the number of immature cells, the number of mature cells increased, i.e., the plasmatic cells matured. In the period from the 30th to the 45th day the number of mature plasmatic cells remained constant, although it was significantly higher than in the non-innoculated animals.

In comparing the above curve it is found that the period of maximum concentration of antibodies in the spleen (the tenth day after immunization) is preceded by the accumulation of the greatest amount of ribonucleic acid, activated reticuloendothelial cells and immature plasmatic cells in its tissues.

The period of lowering of the antibody titer (the 13th day after the injection of the vaccine) coincided with the

occurrence of the multiple mature plasmatic cells in the tissues, the level of which remained constant up to the forty-fifth day.

The mesentery lymphatic nodes of rabbits produce an intense reaction to the injected antigens. Immunologically this is determined by the absorption of antigen and the presence of antibodies in the lymphatic node fibers (Figure 2). After the injection of polyvaccine, analogously to spleen data, the typhus abdominalis antigen was found in the lymphatic nodes on the first day. Subsequently, on the second and seventh day the antigen was not found. However, by the tenth day it was again found in a relatively larger titer (1:30) and remained high through the 45th day (1:15 titer). The antibodies were found in the lymphatic nodes of individual rabbits after 1 day in insignificant amounts (average titer 1:5), but by the tenth day the titer increased (1:20) and only by the 45th day it began to decrease (1:15).

Ribonucleic acid was found in the mesentery lymphatic nodes also in the control rabbits (those which were not innoculated) in multiple cells of the activated reticuloendothelium and immature plasmatic cells.

After the immunization of rabbits the concentration of ribonucleic acid and activated reticuloendothelial cells increase significantly, reaching a maximum on the seventh through the tenth day. By the 30th day lowering of the corresponding curve was observed, and on the 45th day, the amount of ribonucleic acid and activated reticulendothelial cells again increased.

The number of immature plasmatic cells increased somewhat during the first day after immunization. Subsequently, it lowered and only after the tenth day the increase in their concentration was again noted, reaching a maximum by the 30th day. It remained at the same level on the 45th day after immunization.

The mature plasmatic cells were almost totally absent

in the mesentery nodes of the control rabbits. In the case of the immunized animals their concentration by the tenth day remained at the same level until the 30th day, after which from the 30th to the 45th day it was decreased, yet it was still higher than the level in the uninoculated animals.

Subsequently, we investigated the walls of the small intestines, taking those sections of the stomach walls which contained Peyer's patches.

The typhus abdominalis microbe antigen was detected in the small intestine walls during the seventh, through the tenth day period after the injection of polyvaccine (titer up to 1:35). Antibodies were detected rarely and in a low titer (up to 1:20) (See Figure 3).

During the histochemical study of the walls of the small intestines, it was found that in immunized rabbits, the amount of ribonucleic acid increased in the Peyer's patches, ribonucleic acid was also found in the Peyer's patches of the nonimmunized control animals, but in smaller amounts. The enrichment of the Peyer's patches with the ribonucleic acid is primarily associated with the increase of the amount of activated reticularendothelial cells, where the increase in the plasmatic cells is practically unobserved.

On the investigation of all of the indices in the dynamics of immunization it was found that the period of the greatest concentration of antigen in the intestine walls (by the seventh day after immunization' coincides with the period of greatest activation of the cells of the reticulendothelial system and the accumulation of ribonucleic acid in them.

After the seventh day the amount of ribonucleic acid and the number of activated recticuloendothelial cells decreases. On the 45th day after the injection of the polyvaccine the concentration of the latter again increases, but the amount of ribonucleic acid remains at the same, moderately high level.

After the injection of polyvaccine the liver of the

animal did not contain typhus abdominalis antigen, but the antibodies were found on occasion in different rabbits in a low titer (Figure 4).

As nearly as it is possible to detect, by means of histochemical, Brache method the injection of the polyvaccine affects the concentration of ribonucleic acid only in the cells of Glisson's sac of the liver, and partially in the reticuloendothelial cells, located along the capillaries. Even here the effect is not so regular as in the case of the lymphatic organs. The concentration of ribonucleic acid in the liver cells was unaffected by the injection of polyvaccine.

Only in isolated cases, the activated reticuloendothelial cells were found in the liver. Apparently the activation process of reticuloendothelial cells in the liver, i.e., histocytes of Glisson's sac occurs unnoticeably.

The number of immature plasmatic cells and the amount of ribonucleic acid one day after the injection of polyvaccine increased in the liver by approximately a factor or two. After the seventh day, the number of immature plasmatic cells began to decrease, while the number of mature cells was increasing up to the 10th day. After the tenth day the lowering of the number of mature plasmatic cells was noted as well as lowering of the concentration of ribonucleic acid. By the 45th day, a slight increase in the amount of ribonucleic acid and the plasmatic cells was again noted.

The typhus abdominalis antigen and the corresponding antibodies were discovered in the lungs after the injection of the vaccine throughout the whole investigated period. The antigen titer was low (1:10:15) while the antibody titer was higher than in other organs (Figure 5).

In the histochemical investigations it was found that the lungs of the control animals (not inoculated) contained small amounts of ribonucleic acid. Small numbers of immature and mature plasmatic cells, localized in the accumulation of

the lung septa, primarily near large blood vessals, and the bronchial tubes were found. In immunized rabbits, the amount of ribonucleic acid was increased moderately, primarily due to the increase of the number of immature and later mature plasmatic cells. The activation of reticuloendothelial cells both in the lungs as well as in the liver was rarely found. Later the number of plasmatic cells and the amount of ribonucleic acid decreased gradually, and by the 45th day, it increased again.

Totally different data were obtained in the study of the kidneys of rabbits. Both typhus abdominalis antigen and the corresponding antibodies were discovered in the kidneys of rabbits innoculated with polyvaccine in titers greater than in a number of other organs, in the course of the whole 45 day investigation period. However, the cell tissues of the kidneys did not react, in the same manner as other organs, to the concentration of antigen either through the elements of the endothelial system or through the development of plasmatic cells or by the increase of the concentration of ribonucleic acid (Figure 6).

Here it should be remembered, that in the cells of the kidney tissues, as well as tissues of other organs with high physiological activity (for example muscles), it is not possible to determine the presence of ribonucleic acid by means of the chemical method.

In order to make a better visual comparison, the average data are given below on the concentration of the typhus abdominalis antigen and antibodies in different organs in the course of the 45 day period after immunization. These data are arithmetic means of the antigen and antibody titers throughout the whole indicated period. Cytological shifts, determined as indicated above, by the activation of the reticuloendothelium elements, the presence of plasmatic cells, both mature and immature, and also by the increase

of the concentration of ribonucleic acid in the organ tissues are given along with the average antigen and antibody titers. (Figure 7).

The reactions of the tissues of different organs to the injection of polyvaccine were not the same. Thus, for example, the presence of the antigen of the typhus abdominalis microbe in the spleen, mesentery lymphatic nodes and the small intestine walls produced the activation of the reticuloendothelial system elements. In the liver, on the other hand, where the antigen was not found, the cell reaction was weak and sluggish.

In the kidneys, where the antigen was found in amounts exceeding its concentration in other organs, the activity of the cell reaction to its presence was not noticed. In the lungs also the presence of antigen caused only a weak reaction on the part of the reticuloendothelial system.

It was of interest to determine how the presence of antibodies in different organs affects the concentration of the plasmatic cells in them.

In the majority of organs (spleen, lings, mesentery lymphatic nodes) the presence of antibodies in relatively high titers causes an increase in the number of the plasmatic cells.

It was also noted that this period of increase of antibody titer was asscciated with the increase of the number of immature plasmatic cells, while the period of lowering of titer preceded or coincided with relative increase of the number of mature plasmatic cells.

In the cells of the majority of organs, containing antibodies (lymphatic nodes, spleen, lungs) the amount of ribonucleic acid was also increased.

The presented observations support the opinion of many authors (Planel'yes and Forshter; Mac Neil, Fagreus, Rice, Martins and Erick) regarding the role of the plasmatic cells in the production of antibodies and indicate the participation

of a number of organs in the process.

The data of this work shows that in contrast to other organs the kidneys of rabbits do not react to the presence of antigen by changing their histological structure. Apparently, the antigen is captured by the cells of the epithelial kidney channels, which have a tendency to capture foreign substances, of nonantigen nature, designated for excreation from the organism.

Keeping in mind the literature indications of the ability of the organ tissues to absorb antibodies (Robers, Adams, and White), it is possible to assume that antibodies were not produced in the kidney's and they were only absorbed from the blood by the kidney tissue cells.

It is possible that in the lungs, where the activation process of the reticuloendothelial system elements after the injection of antigen is taking place rapidly, its concentration was partially associated with the function of the freeing of the organism from foreign principle by oxidation of the cell elements which contain it (Anichkov). However, in view of the pronounced cell reaction of the lung tissues to the presence of antigen, manifesting in the increase of the number of plasmatic cells, as well as in the concentration of ribonucleic acid, one could assume that the lung tissues participate to some extent in the production of antibodies.

On the basis of the data of this investigation one may assume that the absorption of antigen by the cells of the reticuloendothelial system produces activation of these elements, which is observed in the spleen, mesentery lymphatic nodes and in the walls of the intestines. In the spleen, and lymphatic nodes one observes increased metabolic processes (increased amount of ribonucleic acid), the occurrence of plasmatic cells and antibodies.

The significance of the cellular reaction of the intestine walls (Peyer's patches) to the introduced typhus abdominalis antigen required further investigation. It

is only possible to hypothesize that the concentration of antigen in them is associated with the tropism of the typhus abdominalis microbe to the walls of the small intestine and depends in this case on the chemical properties of the antigen.

The role of the liver in the reaction of the organism to the introduced polyvaccine is not clear. Although the reaction of the liver, determined by the concentration of typhus abdominalis antigen and antibodies in comparison with other organs was weak and less consistent. In the tissues of Glisson's sac of the liver a small increase of the ribonucleic acid and the number of plasmatic cells were onserved.

Conclusions

1. In a number of organs, containing typhus abdominalis antigen of the polyvaccine (spleen, mesentery lymphatic nodes, Peyer's patches, small intestine walls), activation of the cells of the reticuloendothelial system was observed.

2. During the period of the increased concentration of antibodies in response to the introduced antigen increase of the concentration of immature plasmatic cells in a number of organs was observed. The periods of lowering of the antibody titer preceeded or coincided with the relative increase of the number of mature plasmatic cells.

3. The concentration of ribonucleic acid in a number of organs (lymphatic nodes, spleen lungs,) increased in parallel to the increase of the antibody titer.

4. In rabbit kidneys after the injection of polyvaccine along with the increased concentration of typhus abdominalis antigen and antibody no morphological changes were observed, which supports the role of the excretory function of the kidneys in immunogenesis.

5. In the walls of the small intestines (Peyer's patches) after the injection of polyvaccine along with a significant concentration of antigen and the activation phenomenon of

of the cells of the reticuloendothelial system, plasmatic cells did not occur. Antibodies were discovered for a short period of time in low titer.

6. The typhus abdominalis antigen of the polyvaccine was not found in the liver. Its antibodies were found irregularly and in insignificant titer. The histological changes were weakly expressed and were not consistent.

7. The increase in the number of plasmatic cells, primarily, immature cells and ribonucleic acid during the period of increase of the concentration of antibodies in the spleen, lymphatic nodes and lungs indicates the participation of these organs in the production of antibodies.

Bibliography

[1] Anichkov, N.N. "Certain Characteristics of the Accumultion of Substances in the Reticulendothelial System" Physiological Journal of the USSR, Vol.17, No.3, 422 to 429.

[2] Brashe, Zh. "Localization and Probable Role of Nucleic Acids in the Cell Embryo" Progress in Modern Biology Vol.29, No.1, 140 to 144 (1950).

[3] Vakarina, Ye.F. and Ginzburg-Khalinina, S.I. "Absorption of Antigen and Formation of Antibodies in Vaccinated Animals. Principles of Immunity" Transactions of the Moscow Scientific Research Institute im.Mechnikov, Moscow, 247 to 259 (1956) USSR Ministry of Health, Bureau of Institutes of Vaccines and Serums.

[4] Kartasheva, V.N., Bodisko, V.P. and Gorbynova, L.A. "Study of the Concentration of Nucleic acids in the Reticuloendothelial System and the State of Mesoglia in White Rats, Immunized with Gayskiy Strain of Tularemia. Problems of Bacteriology, Virology and Immunology." Transactions of the Moscow Scientific Institute im. Mechnikov., Moscow, Vol.12, 177 to 194 (1958).

[5] Mechnikov, I.I. Insusceptibility in Infectious Disease State Medical Publishing House, 1947.

[6] Planel'yes, Kh. Kh. and Forshter, KH. K. :Pathogenesis and Immunological Reactions During Experimental Typhus Abdominalis Infection of White Mice" Journal of Medical Biology, Epidemology and Immunobiology, No.1, 10 to 23 (1947).

[7] Rapoport, Ya. L. "Morphological Basis of Immunologenesis" Pathology Archives, No.2, 3 to 19 (1957).

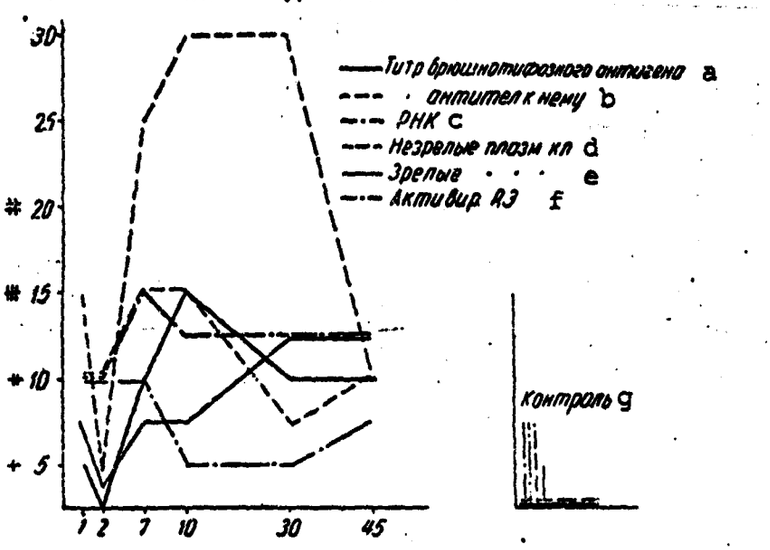

Figure 1. The reaction of the spleen to the injection of polyvaccine.

Key:
a- Typhus abdominalis antigen titer;
b- antibody titer of typhus abdominalis;
c- RNA;
d- Immature plasma cells;
e- Mature plasma cells;
f- activated reticuloendothelial cells;
g- control.

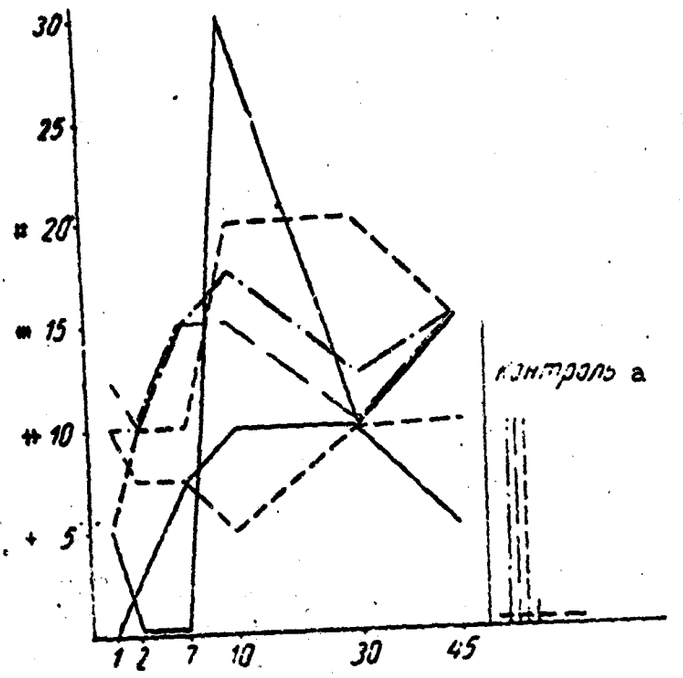

Figure 2. The reaction of the mesentery nodes to the injected polyvaccine.

Key: а- control.

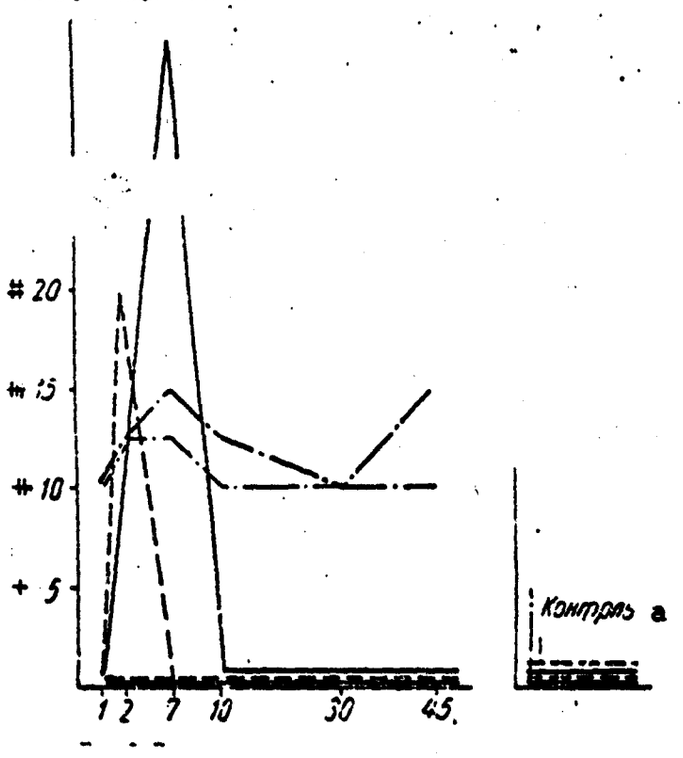

Figure 3. Reaction of the small intestinal walls to the injection of polyvaccine.

Key: a- Control.

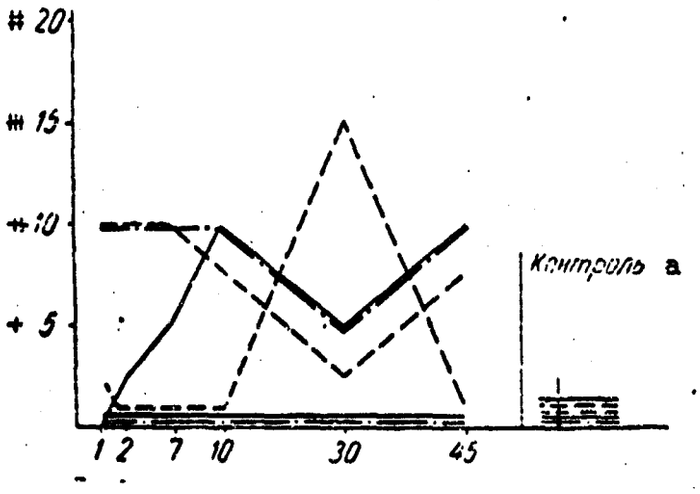

Figure 4. The reaction of the liver to the injection of polyvaccine.

Key: a- Control.

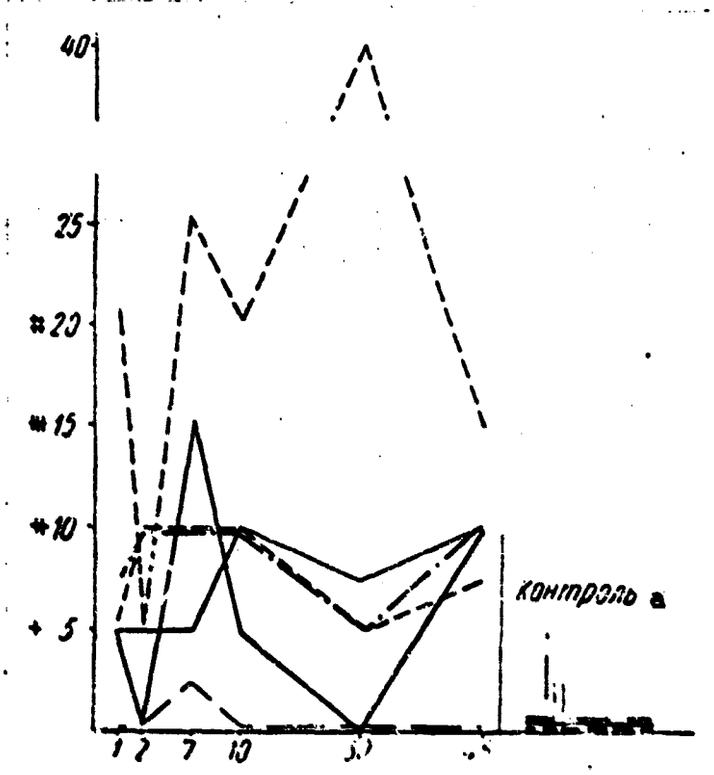

Figure 5. The reaction of the lungs to the injection of polyvaccine.

Key: a- Control.

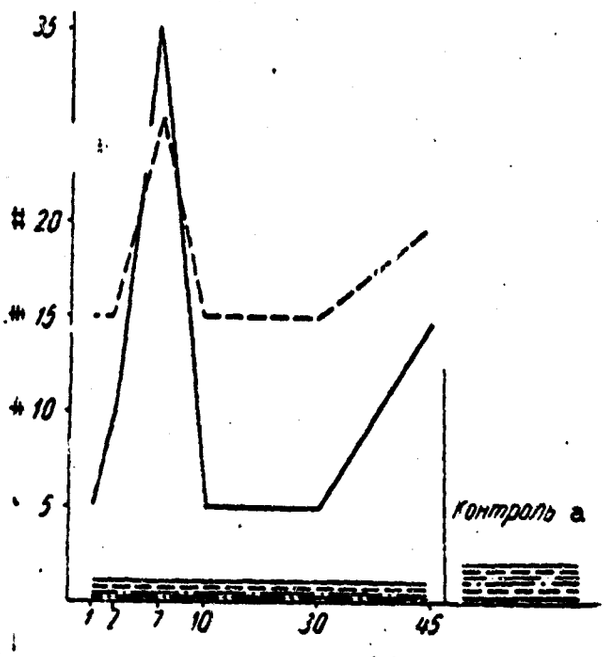

Figure 6. The reaction of the kidneys to the injection of polyvaccine.

Key: a- Control.

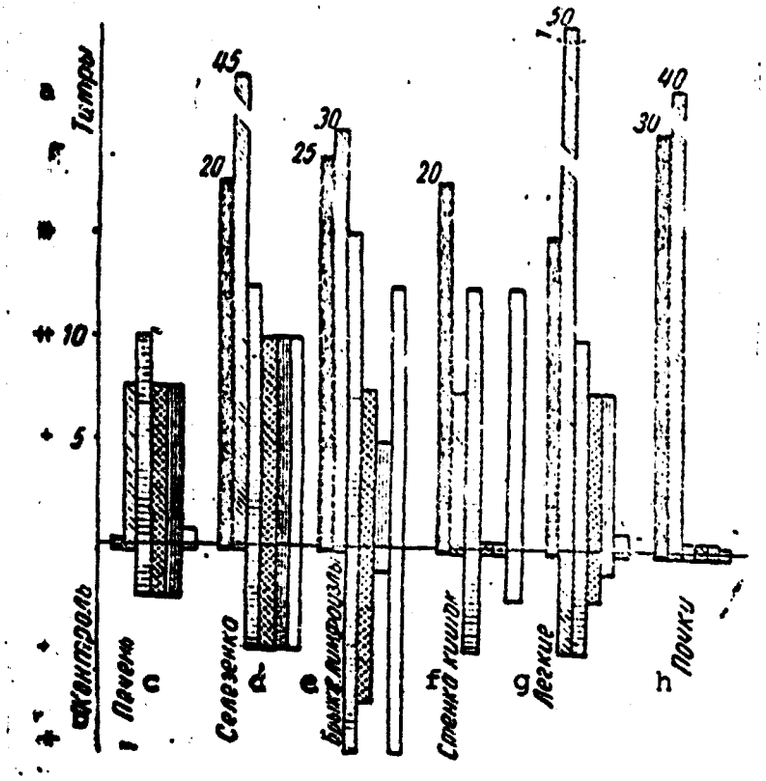

Figure 7. Averaged indices of the concentration of typhus abdominalis antigen, antibodies, ribonucleic acid and cytological changes in the organs of rabbits in the course of the 45 day period after the injection of polyvaccine.

Key:
a- Titers;
b- Control;
c- liver;
d- spleen;
e- mesentery lymphatic nodes;
f- intestinal walls;
g- lungs;
h- kidneys.

MORPHOLOGICAL CHANGES IN THE ORGAN TISSUES OF RABBITS AFTER THE INJECTION OF ADSORBED AND NONADSORBED POLYVACCINE ANTIGENS

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The method of immunization with sorbed antigen is based on the fact that after the subcutaneous injection these antigens cause, in the surrounding tissues, stronger and more widely spread reactions than nonsorbed antigens. During a prolonged period of time encapsulation and organization of the sorbed antigen processes develop, in the course of which they are only slowly and gradually absorbed from the depot formed in the place of the injection. At the same time the duration of the action of antigens on the organism is increased.

The introduction of large particles of substance with the antigen sorbed on their surface causes a local inflammation reaction. The inflammation reaction is associated with the activation of local elements of the connective tissue, the increased absorption ability of the histocytes and an increase in the rate of the metabolic processes in the tissue cells. It was, therefore, noted that the inflammatory reaction after the injection of vaccines has a prolonged effect on the formation of immunity.

On the other hand, some investigators believe that sorbing substances (similar to nonseptic stimulants) may increase the stimulation of the nerve endings and help the development of a number of reactions of the organism, which has a significant effect on immunity (for example, phagocytosis,

the state of tropic functions, etc.).

In spite of the broad applicability of aorbed antigens in the practice of innoculation up to the present time there are practically no observations of the cytological characteristics of the reactions of the organisms to their injection. The existing data refers only to the study of local reactions of the tissue to the injection of the sorbed antigens.

Consequently, this investigation was undertaken in order to determine the nature and the dynamics of the development of the cellular reactions of the animal organisms after the injection of polyvaccine antigens in the sorbed state in comparison with the results of inoculation with the same antigens but not in the sorbed state.

At the same time the state of the immunological reactivity of the animals was determined by the complement fixation titer of typhus abdominalis, antigen agglutinins, and antibodies in the blood serum.

The work was conducted with male chinchilla rabbits weighing from 2.3 to 2.7 kg. The rabbits were injected with commercial polyvaccine series 361, which contained 0.05 mg/ml. of typhus abdominalis microbe antigens, paratyphoid A and B and Flexner's dysentery (0.125 mg each), Zonne dysentery (0.05 mg) and cholera (0.25 mg).

Calcium chloride (20% solution) and sodium phosphate (20% solution) were added to the polyvaccine as sorbents in the amounts equal to 2.5 ml/100 ml and 7.5 ml/100 ml of diluted polyvaccine respectively.

A parallel experiment was conducted with rabbits which were also injected with the same polyvaccine, but without the addition of sorbents.

The polyvaccine was injected into the rabbits subcutaneously on the inner part of the right hip. Following different periods of time after immunization (1,2,7,10,30 and 45 days) the rabbits were exsanguinated. Their organs and tissues at the site of the injection of the antigen were subjected

to morphological and histochemical investigations (ribonucleic acid was determined by the Brache method with staining according to Goldmann's method and with hemtaosilin-eosin). The concentration of antibodies in the agglutin complement fixation reaction was determined in the blood serum of the rabbits.

For each time interval after the injection data were obtained from at least two rabbits, immunized with sorbed or unsorbed polyvaccine.

To determine the changes which occur in the tissues after the injection of polyvaccine data obtained from six control rabbits (i.e., uninoculated) were investigated at the same time.

Results of the Investigation of the Immunization of Rabbits with Unsorbed Polyvaccine

In the tissues, at the site of the injection of unsorbed polyvaccine, one day following immunization a center of acute phlegmonous inflammation in the region of the accumulation of the mass of the antigen was observed. Later, on the boundry of such an accumulation, a narrow band of necrosis of the adjacent tissues was occasionally detected. Subsequently, the process of the resorption of the antigen mass was accompanied by the development of a rather moderate amount of granulation tissue, sometimes containing a limited number of fine gigantic resorption cells. The granulation tissue matured and became mature connecting tissue. The excess connecting tissue was gradually reduced. By the 45th day the tissue in the place of injection was generally normal and only in isolated cases was it possible to detect the small growth of mature connecting tissue in the deep part of the derma. The lymphatic nodes, mainly the regional node near the place of the injection of the

vaccine, and also the spleen increased somewhat in volume by the second through the tenth day after immunization. During these days histological investigations generally revealed hyperplasia of the reactive centers of the follicles of the lymphatic nodes, and occasionally of the spleen and Peyer's patches. Later this hyperplasia decreased, and then disappeared totally. On different days after immunization (1 day to 45 days) a well expressed myeloid metaplasia of pulp was developed in the spleen of certain rabbits.

Frequently, predominantly during the first few days after immunization, along the liver capillaries an insignificant microfocal proliferation of fine cells of the reticuloendothelium could be observed. In the region of the liver capillaries, small stoppers were occasionally formed from these cells.

In the study of the concentration of ribonucleic acid in the tissues it was found that even one day after immunization certain organs contained insignificant amounts of ribonucleic acid. Two days after immunization, this phenomena was very clearly expressed and reached a maximum 7 to 10 days after immunization. Subsequently, its intensity gradually decreased, but even 45 days after the immunization, the amount of ribonucleic acid did not always return to its normal level, and not everywhere.

Increase in the amount of ribonucleic acid was most noticeable in the spleen, Peyer's patches and the lymphatic nodes. It should be noted that it was observed the earliest and most significantly in the lymphatic nodes, close to the region of the injection of the antigen, i.e., in the right inguinal lymphatic node, as well as in the mesentery lymphatic nodes, where this phenomena began somewhat later, but it was more intense.

A very small, but regularly observable increase in the amount of ribonucleic acid was noted in the thyroid gland and lungs -- the organs which in control (nonimmunized)

rabbits contained low amounts of ribonucleic acid. The increase of the ribonucleic acid concentration was observed less regularly and in insignificant amounts in the Glisson's sac of the liver.

The increase in the amount of ribonucleic acid occurred predominantly due to the occurrence of multiple activated reticuloendothelial cells, immature and later mature plasmatic cells. In the reactive centers undergoing hyperplasia ribonucleic acid was noted in large and medium lymphocytes.

In the spleen of the control (nonimmunized) rabbits, a significant amount of ribonucleic acid was also obtained in the cytoplasm of the medium lymphocytes, in the region surrounding the follicles. The immunization process affects the amount of ribonucleic acid in this region only to a small extent.

The activation process of reticuloendothelial cells occurs as follows: the ordinary reticuloendothelial cells in the midst of a number of small size reticuloendothelial cells, normally not rich in ribonucleic acid, begins to enrich in it, which is manifested in the occurrence of ribonucleic acid containing granules and lumps, gradually increasing in the number and dimensions. The nucleus of the tissue cell increases in size and becomes also enriched in ribonucleic acid. The longitudinal thin processes of the cell begin to shorten, thicken and the cell acquires the form of an irregular polygon. These cells we call activated reticuloendothelial cells, since they are activated with respect to the synthesis of protein. Subsequently, these cells are very frequently converted to plasmatic cells. In this case the body of the cell acquires a spherical shape, decreases in its dimensions and becomes richer and richer in ribonucleic acid. The cell nucleus also decreases in dimensions and finally becomes unnoticeable. The nucleus gradually recesses to the periphery and responds more intensely to the basic dye stain. Near the nucleus at the

place of the cell center and the sphere, a light spot develops and the cell is converted into a typical plasmatic cell.

In the control (uninoculated) animals the activated cells of the reticuloendothelium were regularly found in a very small amount in the cortex and medullar matter of the lymphatic nodes, in the reactive centers of the lymphatic node follicles, in the spleen and Peyer's patches, in the spleen pulp, in the Peyer's parches -- outside of the reactive centers, in the bone marrow -- in the form of single cells and occasionally in the thyroid gland.

The liver and the lungs of the control animals, contained no activated reticuloendothelial cells, while in the immunized animals they were found only occasionally.

The conversion of activated reticuloendothelial cells into plasmatic cells is not mandatory. In the reactive centers of lymphatic follicules undergoing hyperplasia the number of activated reticuloendothelial cells always increases, but there they are converted into large and medium lymphocytes.

A relatively insignificant enrichment of the thyroid gland with ribonucleic acid, predominantly its substantia corticalis occurred primarily at the expense of an increased number of large and medium lymphocytes, and only to a small extent at the expense of cells of the activated reticuloendothelium, immature and mature plasmatic cells.

Since the cells of the activated reticuloendothelium are converted to plasmatic cells, it becomes quite clear why on the first days after the immunization in the tissues it is primarily the number of activated reticuloendothelial cells which is increased, while seven to ten days after immunization a large increase in the plasmatic cells is observed. Initially, it is the immature, and later mature plasmatic cells which are predominant.

The immunization process may cause a slight increase in the number of activated reticuloendothelial, immature and mature plasmatic cells in the bone marrow of rabbits.

In the bone marrow of nonimmunized (control) rabbits the amount of these elements is insignificant.

After the injection of polyvaccine the process involving the splitting of the cytoplasm, enriched with ribonucleic acid, from mature and immature plasmatic cells of the activated reticuloendothelium increases. This phenomenon can be observed two days after the injection of the polyvaccine, but it becomes extremely pronounced seven to ten days after the immunization. This coincides with the occurrence of the highest antibody titer in the blood serum of the immunized animals. One can assume that the cytoplasm particles, rich in ribonucleic acid, are utilized in some fashion in the process of the formation of antibodies. In the course of the subsequent days the intensity of the cytoplasm splitting process rapidly diminishes. It is generally difficult to detect the cytoplasm splitting process in the liver and the lungs, but in isolated cases it was observed on much later dates after immunization (ten to thirty days following the immunization).

During the initial days after immunization, particularly on the second day, the vacuolization of the cytoplasm of the reticuloendothelium of the lymphatic node sinuses, (predominantly the node which is reactive to the place of the injection of antigen) increases. Some authors associate this phenomenon with the processing of antigen by the cells of the reticuloendothelium.

In the lymphatic nodes of immunized rabbits, and less frequently in the spleen, an increased number of Russel's bodies, formed in the plasmatic cell cytoplasm are detected. Sometimes, this is observed seven days after the injection of antigen. On later dates the number of Russel's corpuscles increased and the dimensions of the corpuscles themselves also increased.

The Results of the Investigation of the Immunization of Rabbits with Polyvaccine Sorbed on Calcium Phosphate or Hydrated Aluminum Oxide

The local resection of tissues to local injection of polyvaccine, precipitated with calcium phosphate or hydrous aluminum oxide, was manifested in the intense exudation and proliferation processes with the organization and resorption of the bulk of the deposited antigen. On the forty-fifth day, primarily in the place of the injection of the antigen, only a samll amount of the connecting scar tissue is found. In contrast to changes during the immunization with nonsorbed antigen we were unable to observe the development of necrosis of the adjecent tissue at the boundary with the accumulations of the antigen, but at the place of the injection a large inflammation site developes which was liquidated much slower.*

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* M.S.Didukh. "Study of Local Histological Reactions During Subcutaneous Injection of Vaccines and Serums of Deposited Antigen" Problems of Bacteriology, Virology and Immunology, Transactions of the Moscow Scientific Research Institute im. Mechnikov. Moscow, 1958, Vol.12 pages 37 to 48.

Protecting histological structures, similar to those which were described earlier for rabbits immunized with non-sorbed antigens were developing in the internal organs of rabbits immunized with sorbed polyvaccine. However, during the study of the obtained data the following differences were noted.

The reactive centers of the lymphatic follicules in rabbits, immunized with the sorbed antigen, remained in hyperplasia state in the course of a much longer period of time, than after the injection of nonsorbed vaccine. Occasionally, even after forty-five days they did not return to their initial state.

On different days (first through the tenth day after immunization) moderately pronounced myeloid metaplasia of the pulp of the spleen was developing of the same nature as in rabbits immunized with not sorbed antigen. However, the increase in the number of myeloid elements in the capillary region of the internal organs in rabbits which received sorbed polyvaccine was much more significant.

Proliferation reaction on the part of the reticuloendothelium more widespread and more frequently developed.

An occasional occurrence of small polynuclear giant cells in polyferrides, and the development of small areas of light reticuloendothelial cells in the lymphatic nodes and the Peyer's patches was observed only after an injection of sorbed antigens.

Both after the injection of sorbed as well as nonsorbed antigens of polyvaccines the amount of ribonucleic acid in the tissue was increased. Its level reached a maximum on the seventh to the tenth day after the injection. However, after the injection of sorbed antigen, a significantly larger amount of ribonucleic acid was synthesized in the tissue cells than after the injection of nonsorbed polyvaccine. Correspondingly, during the indicated period of time the tissues contained a larger number of cell elements with ribonucleic acid, i.e. cells of the activated reticuloendothelium and mature and immature plasmatic cells.

In contrast to the rabbits which were injected with nonsorbed antigen, in the animals immunized with sorbed vaccine the amount of ribonucleic acid did not decrease by the tenth day, but was preserved for long periods of time and at a relatively high level.

After the injection of sorbed antigen into the course of a much longer period of time the presence of reticuloendothelium cells containing ribonucleic acid was observed in the tissues, i.e., the cells of the activated reticuloendothelium and immature plasmatic cells. The mature plasmatic cells in

in the tissues of these rabbits were also numerous (Figure 1 to 10)[sic]

The reaction of the rabbits mesentery nodes and the Peyer's patches to the injection antigens of polyvaccine was unique.

Although the mesentery nodes reacted to the injection of polyvaccine somewhat later than the regional lymphatic nodes, their reaction was much more pronounced and the amount of ribonucleic acid accumulated in them was large. However, the maturing of the plasmatic cells in the mesentery lymphatic nodes of rabbits occurred very unenergetically and the mature plasmatic cells were rarely found, but the cells of the activated reticuloendothelium with the admixture of immature plasmatic cells were predominant.

The generalizations which we have thus far expressed, are not always completely valid with respect to the mesentery lymphatic nodes and the Peyer's patches. It is possible that the cause for this is the closely located other medium (the intestinal tract region).

The process of splitting of cytoplasm from the body of reticuloendothelial and plasmatic cells, rich in ribonucleic acid, is especially significant seven to ten days after the injection of both sorbed and nonsorbed antigen. However, during the latter dates after an injection, the undulatory increase of this process was observed which was not noted in rabbits innoculated with polyvaccine without adsorbing substances. This probably results from the delivery of new portions of antigen from the depot at the place of the injection.

The vacuolization of reticuloendothelial sinuses of the lymphatic node in rabbits, immunized with sorbed antigen, was observed not only in the course of the first two days after immunization, but also later, frequently as long as forty-five days after immunization.

The number of Russel's bodies in the rabbit tissues

immunized with sorbed polyvaccine was also larger than after the injection of nonsorbed antigens.

In order to clarify the relationship between the described morphological changes with the production of antibodies in rabbits, immunized with polyvaccine (native and sorbed), comparisons were made between the average indices of the concentration of ribonucleic acid in the tissue, the number of reticuloendothelial cells, (enriched in ribonucleic acid), the number of plasmatic cells (both mature and immature) on one hand and the average antibody titers with respect to typhus abdominalis antigen of polyvaccine in the blood serum on the other.

The average indices of morphological changes were determined in those organs which on the basis of the available data appear to participate in the antibody formation process -- the spleen and lymphatic nodes (regional, further removed and mesentery).

The average agglutinin and antibody titers were determined in the blood serum by the complement fixation reaction (Figure 11)[sic]

On the seventh day after the injection of both sorbed and unsorbed polyvaccine, high agglutinin titers were found in the blood serum. However, in rabbits which received nonsorbed polyvaccine, the average titer was higher (1:200), and by the tenth day, it increased even further (1:320). On the thirtieth day, agglutinin was no longer found in the blood serum of these rabbits. The dynamics of the agglutinin titer in the blood serum of rabbits immunized with sorbed polyvaccine was somewhat different. After the maximum titer on the seventh day, a slight decrease of the titer was observed by the tenth day (1:50). However, agglutinins were found in the blood serum of rabbits of this group through the forty-fifth day (the end of the observations) with some variations in the titer (1:40 to 1:60).

The antibody titer curve, (complement fixation reaction) was resembling primarily the agglutinin concentration curve. The maximum titer of these antibodies in the rabbits, immunized with nonsorbed antigen (1:60) was observed also on the tenth day but it was not decreasing as sharply as the agglutinin titer. In rabbits which received sorbed antigen injections, the concentration of complement fixing antibodies reached the highest level on the seventh day (titer -- 1:50). On the tenth day the titer of these antibodies was significantly lower (1:15). On subsequent days, on the thirtieth and forty-fifth days, the complement fixing antibodies were found in the blood serum in the same titer as on the seventh day after immunization (1:50). The concentration of activated reticuloendothelium in the tissues of rabbits, immunized with nonsorbed polyvaccine, increases by the second day after immunization, remains the same up to the seventh day and by the tenth day decreases and reaches a norm by the forty-fifth day (Figure 12).

In rabbits vaccinated with sorbed antigens, the concentration of the activated reticuloendothelium curve began to increase after one day. After an insignificant decrease on the seventh day, by the tenth day this curve again reached the previous level where it remained up to the thirtieth day. On the forty-fifth day after the immunization, it decreased somewhat, but still remained at a much higher level than after the injection of nonsorbed antigens.

The curve for the concentration of immature plasmatic cells in rabbits, vaccinated with nonsorbed antigens, increased on the second through seventh day after the injection, then began to decrease, and on the forty-fifth day, reached a lower level than in the control animals, which were not inoculated.

The general level of the same curve for rabbits immunized with sorbed antigen was higher than for animals which were injected with nonsorbed vaccine. The natural increase of this

curve was also observed on the seventh day but it was not decreasing on the tenth day. On the thirtieth and the forty-fifth days, this curve remained at the same lower level. It was the same level, however as in the rabbits injected with nonsorbed antigens during their period of maximum increase (i.e., the seventh day).

The curve of indices of the concentration of mature plasmatic cells in rabbits immunized with nonsorbed vaccine increased by the tenth day after their injection, remained at that level through the thirtieth day after which it decreased to the normal level on the forty-fifth day.

After the injection of sorbed polyvaccine this curve increased after the second day to a level significantly higher than in rabbits inoculated with nonsorbed antigen, and only on the forty-fifth day it decreased somewhat to a level which is maximum for rabbits immunized with polyvaccine without sorbents.

The curve for the concentration of ribonucleic acid after the injection of polyvaccine without sorbents increased after one day, remained at this level through the seventh day, and by the tenth day it began to decrease a level in non-immunized control animals.

As a result of the injection of sorbed polyvaccine the ribonucleic acid concentration curve, began to increase from the first day to the tenth day after immunization. The general level of this curve was higher in comparison with the corresponding curve for the injection of nonsorbed polyvaccine. By the thirtieth day, the level of the curves for the concentration of ribonucleic acid decreased to the highest level of its concentration in rabbits inoculated with nonsorbed vaccine, and remained there until the end of the experiment (to the forty-fifth day).

Thus, in comparing the cited indices in rabbits, immunized with native and sorbed polyvaccine, it is apparent that in animals immunized with sorbed polyvaccine the concentration

curves for ribonucleic acid, activated reticuloendothelium, immature and mature plasmatic cells remain at a higher level until the end of the experiment than in the rabbits inoculated with nonsorbed antigen. The concentration of ribonucleic acid and immature plasmatic cells from the thirtieth through the forty-fifth days stabilized in the rabbits at the same elevated level. The amount of the activated reticuloendothelium in the course of the thirty days after the immunization remained almost identical.

The curves for the concentration of antibodies in the blood serum of rabbits immunized with sorbed antigen in the latter days were observed on the thirtieth through the forty-fifth days after immunization anf found at a higher level than the corresponding curves for the injection of nonsorbed vaccine.

In comparing the curves for the concentration of antibodies in the blood serum, indices of morphological shifts and the presence of ribonucleic acid in the organ tissues of rabbits, one may note that the antibody, ribonucleic acid and immature plasmatic cell concentration curves are dissimilar in their dynamics.

Due to the rapid assimilation of the nonsorbed antigen the activation process of the reticuloendothelial cells of the system is characterized by a short duration after injection.

After the injection of antigens with substances which serve as the depot in the organism, the activation processes of the reticuloendothelial cells, enriched in ribonucleic acid, the development of their derivative (plasmatic cells) and splitting of cytoplasm particles from the bodies of these cells, enriched in ribonucleic acid occurred over a long period of time. One may assume that the cause of this phenomenon is prolonged delivery of fractional doses of antigen into general circulation from the depot in the place of the injection.

Conclusions

1. After the immunization of rabbits with polyvaccine, the following was observed in the tissues of their organs:

a) proliferation of reticuloendothelial cells of the internal organs;

b) increase of the number of cells of activated reticuloendothelium and plasmatic cells (both mature and immature);

c) increase of the concentration of ribonucleic acid in the tissues.

2. In comparing the results of histological and histochemical investigations of the organ tissues of rabbits after the injection of native and calcium phosphate sorbed vaccine, it was found that in the rabbits injected with sorbed vaccine the indicated changes were more pronounced and persisted over longer periods of time than in rabbits which were injected with nonsorbed polyvaccine.

3. The maximum antibody and agglutinin titers in the complement fixation reaction, determined for blood serum of rabbits injected with sorbed polyvaccine without sorbents. However, after the injection of the sorbed polyvaccine, antibodies were found in the blood serum in the course of a significantly longer period of time.

4. One may assume that the cause of the indicated differences in the action of sorbed and nonsorbed antigens is the prolonged stimulation of the protective mechanisms of the organism as a result of retarded arrival of antigen from the depot, formed at the site of the injection of the sorbed vaccine.

LOCALIZATION OF WHOOPING COUGH ANTIGEN AND ANTIBODIES IN THE ANIMAL ORGANISMS

by

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In spite of the significance which the investigators ascribed to the antigen as a factor which aids in the formation of insusceptibility, the literature data on the problem of localization of antigens, introduced artificially into the organism, are still insufficient.

Specifically, in the available literature, there are no corresponding data on the localization and the duration of the existence of whooping cough antigen in the organism. This data, however, is very significant for the determination of the nature of immunity for this illness.

According to the data of the majority of investigators (Palant, Borde-Zhangu, Voyno-Yasenetskiy, Ioffe and others), in the case of experimentally induced whooping cough infection the principal lesion is local in character. After the death of the animal the whooping cough microbes are found in the trachea and bronchi. It should be noted that microbes are found deep in the mucous membranes. On very rare occasions one finds whooping cough microbes in other organs.

Mice were experimentally infected by Ioffe and co-workers with whooping cough, by aspirating microbes under a bell jar. The whooping cough bacilli were ejected from the lungs of the mice on the sixth day after infection. The maximum number of bacteria were discovered by the twentieth day, and subsequently, their concentration in the lungs was decreasing significantly. The concentration of the antigen in the blood serum was determined by the complement fixation reaction at low temperature, and was found to be at a maximum by the

fourth day after the infection, after which it began to decrease and by the twentieth day the antigen was not detected in the blood serum. At the same time the curve for the concentration of the complement fixing antibodies in the blood serum increased significantly by the twentieth day.

In the experiments of Boyno-Yasenetskiy and Khay after intranasal injection of 100 million whooping cough bacteria into mice, an accumulation of leukocytes around the bronchi was detected after three to four hours. It was noted that the pathological processes were developing in the lung tissue itself.

During the 96 day investigations of experimental whooping cough infection in mice, Orlov, Malysheva and Dvizhkov observed the dynamics of the development of the process and established the following pathological characteristics.

Eight to ten days after intranasal introduction of the emulsion of the live whooping cough culture (250 thousand microbes in each nostril) hypermia and pulmonary endema developed.

By the eleventh through the twenty-fifth days, microscopic changes in the lungs were observed -- pulmonary endema and small indurations. By the end of the observations, the infectious process terminated.

During the investigation of the effectiveness of intranasal and subcutaneous immunization of mice with a suspension of whooping cough microbes, Marchenko observed localized pneumonia with multiplication of the bacteria on the surface of the epithelium of the bronchi. This led to the belief that the respitory tract and lungs must have a significant effect in the mechanism of the formation of antiwhooping cough immunity.

In the investigations conducted earlier on another model in the study of the distribution of typhus abdominalis and dysentery antigens and antibodies in the animal organism, some relationship was established between the localization

of microbes during the infectious process (Ginzburg-Khalinina and Vakarina).

It was of interest to study the localization of antigens of the infection stimulants in the organism with the use of another method of transmission, namely the drip method, in order to establish a relationship between the localization of the artificially introduced antigen, and the causal organisms during the infectious process.

It was of interest to determine the localization of antigens of the whooping cough microbes and antibodies in the organs of animals after the use of the intranasal method for the injection of the vaccine. This method closely resembles the natural method by which people contact infection. For this purpose the whooping cough vaccine, forty billion microbes two ml in volume was introduced into the nose of the rabbit (one ml in each nostril) by means of a Pasteur pipette. During this operation the rabbits were lightly anesthetized with ether. Subsequently, the rabbits were killed and exsanguinated on the same days after the injection of the vaccine as after the subcutaneous introduction. Their organs were investigated by the above-described method. Figure 3 shows the results of these investigations. On the basis of the presented data it is apparent that just as in the case of subcutaneous injection of the vaccine antigen first of all (on the first day) it is found in the lungs and kidneys, then in slightly lower amounts in the bronchial nodes, spleen and small intestines. On the twentieth day its titer decreased. In the liver and groin lymphatic nodes as well as in the blood serum the antigen was absent or was found in very small amounts. The data on the concentration of antibodies in the organs and the blood serum of these rabbits are shown in Figure 4.

The experiments were conducted with chinchilla rabbits weighing from 1,5 to 2 kg, which were injected subcutaneously

with seven billion microbes of whooping cough vaccine. The animals were killed after different periods of time subsequent to the injection of the vaccine (1, 2, 5, 10, 20, and 30 days). From the organs of the dissected animals (lungs, kidneys, spleen, small intestines, liver, bronchi, and groin lymphatic nodes) physiological solution suspensions were prepared in a 1:5 ratio. For the extraction the suspension of organs was kept for three days at 4 to 6°C in a refrigerator.

The concentration of antigen was determined by means of the quantitative complement fixation reaction at low temperature using antiwhooping cough serum of high titer. The antigen represents the greatest dilution of the extract with respect to the unit weight of the organ, which produced a positive reaction in the fixing of the complement with antiwhooping cough serum, taken in a 1:20 dilution for the experiment.

Figure 1 shows the results of the investigations of the concentration of antigen in the organisms of animals on different days after the injection of whooping cough vaccine. As it can be seen, the antigen was found in the greatest amount (titer 1:80) in the lungs after twenty-four to forty-eight hours. It was found in a lower titer (1:50), in the kidneys, in the bronchial nodes (1:20) and in the small intestinal walls (1:40). On the tenth day, the antigen titer in the organs decreased significantly and only in the lungs and bronchial lymphatic nodes was it found in small amounts (titer 1:20) on the twentieth to the thirtieth day.

Antibodies were also determined in the quantitative the complement fixing reaction (at low temperature) with whooping cough antigen. The data on the concentration of antibodies in the blood serum and in the organs of animals are shown in Figure 2.

As it can be seen from the figure, the maximum antibody titer was found in the blood serum, lungs and bronchial nodes of rabbits beginning with the fifth day after the

injection of the antigen.

In the blood serum, the high titer (1igo) remained through the thirtieth day (termination of observations) and in the lungs also through the thirtieth (titer 1:40). In the bronchial nodes the increase of the antibody titer was observed on the fifth and the twentieth days (titer 1:50 and 1:60), after which the titer again decreased.

In the remaining organs (small intestine, liver, spleen and groin lymphatic nodes) the antibody titer was insignificant.

After the intranasal injection, just as in the case of subcutaneous injection, antibodies were found in the lungs, kidneys, and spleen. However, they appear there somewhat later than after the subcutaneous injection of the vaccine and in smaller amounts (titer 1:20 to 1:30).

Antibodies were not found in the other organs (in the kidney and groin lymphatic nodes). In the blood serum antibodies were found on the fifth through the twentieth days after the immunization in a titer equal to 1:60.

In comparing the results of the subcutaneous and intranasal injection of whooping cough vaccine one should note that antibodies are localized in the same organs, in both methods of injection. However, after the intranasal injection of the vaccine, their titers are lower.

Thus, using any method for the injection of whooping cough vaccine, the antigen is found primarily in the lungs, bronchial nodes and kidneys, but the formation of antibodies occur more intensly in the case of subcutaneous vaccination.

Conclusions

1. After subcutaneous injection of whooping cough vaccine into rabbits, the antigen is localized primarily in the lungs and bronchial nodes, i.e., in the tissues of those organs where multiplication of microbes for this particular infection takes place (lungs, bronchial nodes) or in those

organs from which the antigen is excreated (kidneys).

2. Antibodies, produced in the reaction to the injected whooping cough antigen, are found primarily in the blood serum and in organs containing the antigen (lungs, bronchial lymphatic nodes).

3. The results of intranasal immunization against whooping cough, using sufficiently large doses of the vaccine antigen, show that the distribution is approximately the same as for subcutaneous vaccination, but the ability to produce antibodies in the former case is less intense.

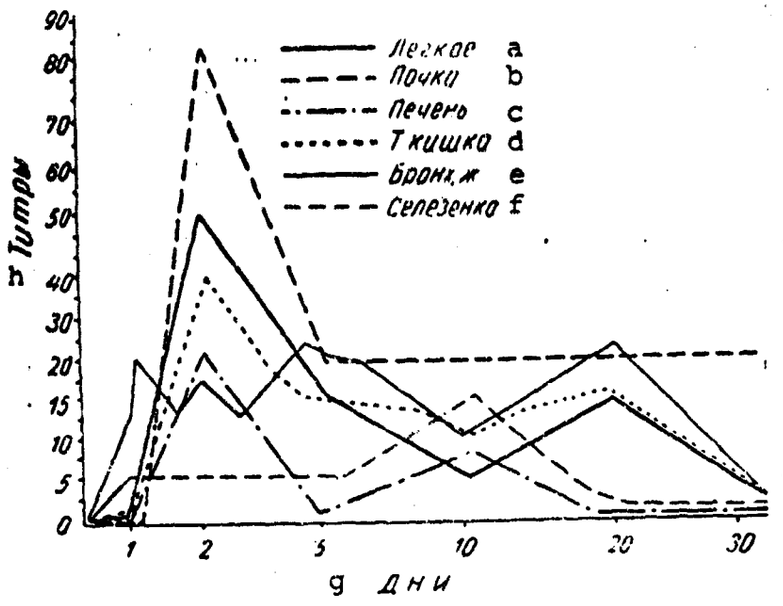

Figure 1. Concentration of antigen in organs after subcutaneous injection of whooping cough vaccine.

Key:
a- lungs;
b- kidneys;
c- liver;
d- small intestine;
e- bronchial gland;
f- spleen;
g- days;
h- titer.

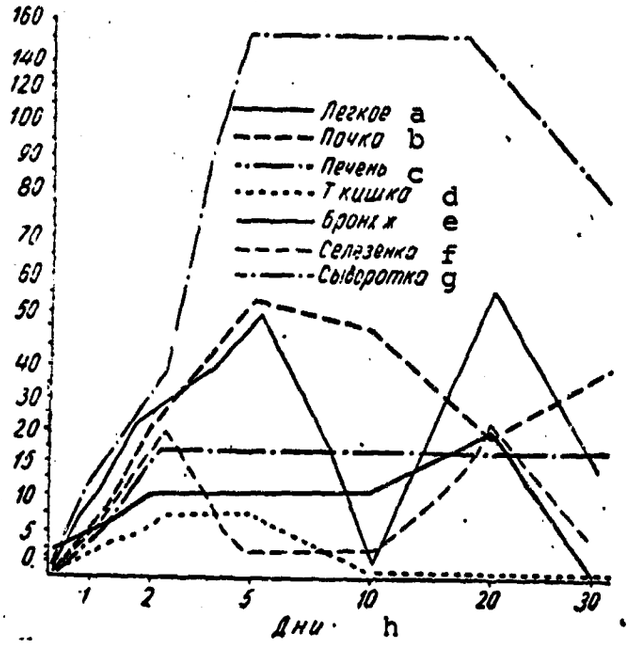

Figure 2. Complement fixing antibodies found in organs after subcutaneous injection of whooping cough vaccine.

Key:
a- lungs;
b- kidneys;
c- liver;
d- small intestine;
e- bronchial glands;
f- spleen;
g- serum;
h- days.

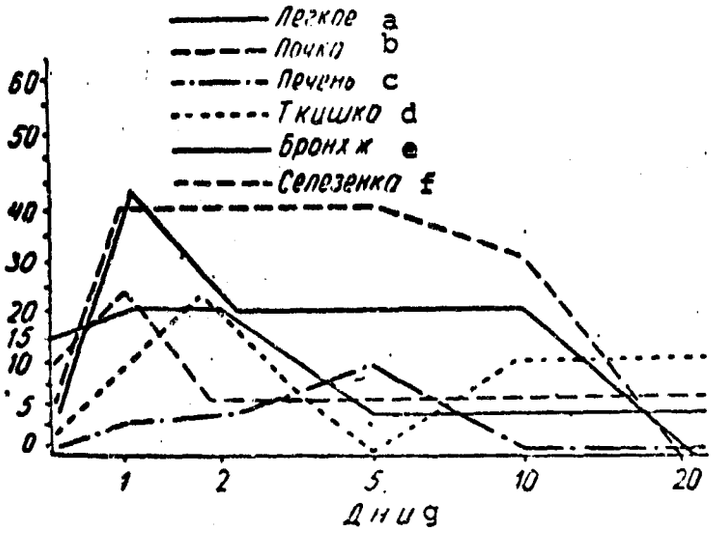

Figure 3. Antigen titer after intranasal injection of whooping cough vaccine.

Key:

a- lungs;
b- kidney;
c- liver;
d- small intestine;
e- bronchial glands;
f- spleen;
g- days.

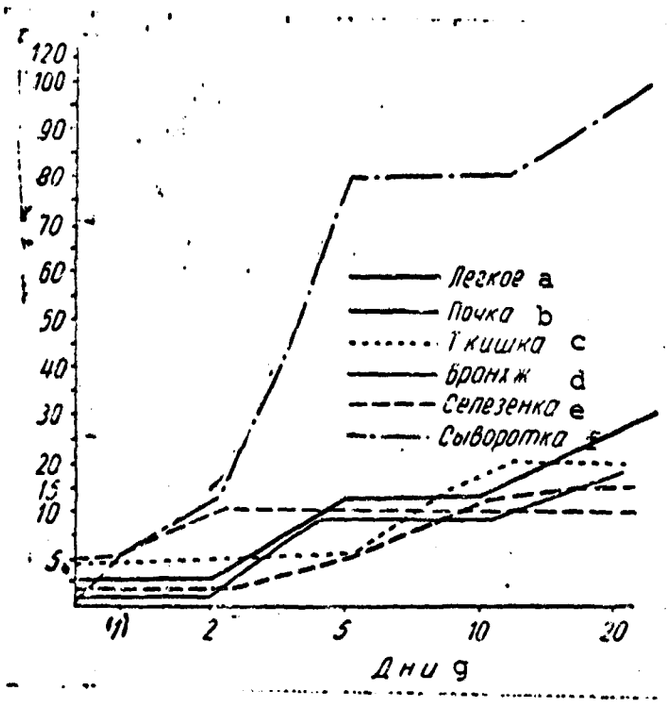

Figure 4. Complement fixing antibodies after intranasal injection of whooping cough vaccine.

Key:
a- lungs;
b- kidneys;
c- small intestine;
d- bronchial gland;
e- spleen;
f- blood serum;
g- days.

LOCALIZATION AND THE DURATION OF THE PRESENCE OF ANTIBODIES IN THE ANIMAL ORGANISM AFTER THE INJECTION OF WHOOPING COUGH GAMMA GLOBULIN

by

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While the role of antibodies in the mechanism of active development of immunity is thus far not totally clear, in the case of passive immunity antibodies play a main and deciding role.

The effectiveness of the injection of antiserum depends to a significant extent on the conditions which insure close and prolonged contact of the antibodies with the causal organisms of the corresponding illness in the organism. Therefore, the localization and the duration of the existence of antibodies in the organ tissues and blood serum of animals after the injection of antiserum is of great significance.

At present it has been established that antibodies are modified globulins of serum. Therefore, the fate of the antibodies depends to a great extent on the distribution of the globulin of the introduced antiserum in the organism of the animal.

The investigations of Ginzburg-Khalinina and Shereshevskaya have shown that after the subcutaneous injection of equine serum into rabbits the equine serum protein can be found in the blood serum of animals starting within the first few hours after the injection and until twelve or fourteen days after the injection, while in the organ tissues (lungs, spleen, kidneys, lymphatic nodes, and also at the place of the injection) it is retained through the twentieth day and beyond.

Koons, Leduke and Connoly after intravenous injection of foreign albumen into mice (chicken egg albumen, bovine

plasma and human gamma globulin, labeled with fluorestsin) during microscopic examinations discovered that these proteins are found in the reticuloendothelial cells of the spleen, lymphatic nodes and also in the epithelium of the kidney tubules. The duration of the time over which foreign proteins were discovered in the organs of animals, according to the data of these authors were different: egg albumen -- 1 day, bovine plasma -- 2 days, human gamma globulin -- 8 days or more, and it also depended on the amount of the introduced albumen.

Because of the wide use of gamma globulin in the medical practice for both prevention and treatment of a number of infectious diseases, it was of interest to study the localization and the duration of the existence of globulin fractions of the immune serum in the organisms of animals, and also of the specific antibodies which were introduced with this serum.

The experiments were conducted with rabbits and guinea pigs. The rabbits, weighing 1.8 to 2 kg, were injected subcutaneously with 5 ml of gamma globulin of bovine, anti whooping cough serum, after which, on different days (in the course of twenty-five to thirdy days) the distribution of gamma globulin and whooping cough antibodies were investigated in the organ tissues and the blood serum of the animal. The determination of the concentration of bovine gamma globulin in the organ tissues and the blood serum of rabbits was conducted by means of the quantitative complement fixation reaction at low temperatures with rabbit blood serum, multiply immunized with bovine gamma globulin (titer 1:640). The determination of the whooping cough antibodies in the blood serum and organs of animals was conducted by the complement fixation reaction with whooping cough vaccine (Table 1.)

Bovine blood gamma globulin was found in significant amounts in the tissues of a number of organs of the animal -- lungs, kidneys, spleen, liver, lymphatic nodes and the walls

of the small intestines. In the blood serum it was found in a significantaly smaller amount. The injected gamma globulin was preserved in the organ tissues in the course of a prolonged period of time. Even on the twentieth day it was possible to detect it in the lungs, kidneys, spleen and other organs. It was not detected in the blood serum during this period of time.

Table 1.

Protein antigens of organ tissues and blood serum of rabbits after a single subcutaneous injection of bovine whooping cough, gamma globulin.

a Орган	i Титр белкового антигена: j день после введения гамма-глобулина				
	1-й	2-й	5-й	10-й	20-й
b Легкие	80	80	80	80	40
c Почки	80	80	80	80	40
d Селезенка	60	80	60	60	20
e Бронхиальные лимфатические узлы	80	80	60	80	20
f Печень	60	80	80	80	20
g Тонкая кишка	40	40	40	40	20
h Сыворотка	10	10	10	10	0

Key: a- Organ; b- lungs; c- kidneys; d- spleen; e- bronchial lymphatic nodes; f- liver; g- small intestine; h- blood serum; i- protein antigen titer; j- days after the injection of gamma globulin.

The specific antibodies, passively injected with whooping cough gamma globulin, were also detected in the tissues of the same organs where the gamma globulin was detected.

Table 2.

Specific antibodies in the organ tissues and blood serum of rabbits after a single subcutaneous injection of bovine whooping cough gamma globulin.

a Орган	i Титр антител: j день после введения гамма-глобулина				
	1-й	2-й	5-й	10-й	20-й
b Легкие	10	10	20	20	10
c Почки	10	10	10	10	10
d Селезенка	10	20	10	20	0
e Бронхиальные лимфатические узлы	10	20	10	20	10
f Печень	10	10	10	20	0
g Тонкая кишка	10	10	10	0	0
h Сыворотка крови	20	20	10	10	0

Key: a- organs; b- lungs; c- kidneys; d- spleen; e- bronchial lymphatic nodes; f- liver; g- small intestine; h- blood serum; i- antibody titer; j- days after the injection of gamma globulin.

The duration of time over which whooping cough antibodies were preserved in the organs tissues reached fifteen to twenty days, but on the twentieth day in the lungs, bronchial lymphatic nodes and kidneys antibodies were found in insignificant titers.

It should be notes, however, that the titer of the injected passive antibody both in the organ tissues as well as in the blood serum was not high. This is explained by the relatively low specific antibody titer (1:80) of the whooping cough gamma globulin which was used in the experiments.

In the case of double increased dose, intravenous injection with a brief interval (forty-eight hours) between the two injections, the blood serum contains this gamma globulin

in a significantly larger amount, than in the case of a single subcutaneous injection (Figure 1).

The bovine gamma globulin was found in the blood serum of rabbits in high titers beginning with the first through the twentieth day after the injection, and only on the twenty-fifth day was it beyond detection.

In the case of a given method of the injection of gamma globulin the whooping cough antibodies were found in low titers, just as after a single injection of gamma globulin in small doses. The duration of the detectability of the whooping cough antibodies in the blood serums was fifteen days.

Subsequently, taking into account the low whooping cough antibody titer of bovine gamma globulin, we obtained a globulin from the rabbit antiwhooping cough serum, characterized by a high whooping cough antibody titer (1:320).

After a subcutaneous injection 5 ml of the indicated globulin to guinea pigs, the specific whooping cough antibodies were found in the blood serum of the animals in a sufficiently high titer (Figure 2).

The duration of the existence of antibodies in the blood serum of animals reached fifteen days, and on the twentieth day they were not detected.

It should ne noted that during the injection of only 4 ml of globulin with specific antibody titer, given to guinea pigs,was equal to 1:320 the antibody titer in the blood serum of the animal reached 1:110, and for some individual animals it was as high as 1:160.

If one takes into account the total amount of blood in a guinea pig and the amount of the injected globulin, it becomes apparent that this titer is incomparably high.

A similar noncorrespondence between the amount of the injected antibodies and the antibody titer discovered in the blood serum of animals was also noted by Takey (1955). He even observed the phenomenon of the increase of antibody titer in the serum of recipients in comparison with the

titer of the injected antibodies. It should be noted that this author assumed that the antibody titer in the blood serum of animals is proportional not to the absolute amount of the introduced molecules, but to the number of molecules combined into groups. The number of the latter depends on the conditions of the distribution of antibodies in the organism. During the injection of rabbit antityphoid serum globulin into guinea pigs similar results were obtained.

Thus, whooping cough antibodies, passively injected with gamma globulin, are localized in the organ tissues and the blood serum of animals within the first few hours after their injection and may be found there until the fifteenth day.

At the same time along with the antibodies the organ tissues and the blood serum retain the protein component of the immune serum in the form of gamma globulin or globulin.

The duration of the existence of the specific antibodies and the investigated globulin in the organs and blood serum are similar. This indicates the relationship between the antibodies and globulin fractions of the serum. However, the data and their identity are still insufficient, since the protein component is discovered in the organ tissues over a much longer period of time than the specific antibody.

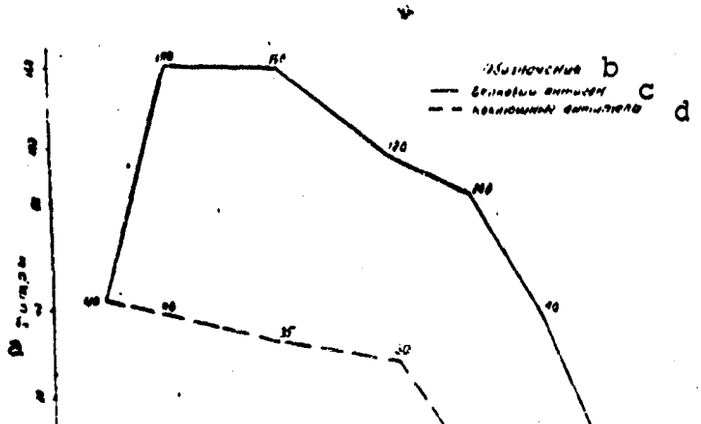

Figure 1. Specific whooping cough antibodies and protein antigen after intravenous injection of whooping cough bovine gamma globulin in the blood serum or rabbits.

Key:
a- titer;
b- designations;
c- protein antigen;
d- whooping cough antibodies;
e- days.

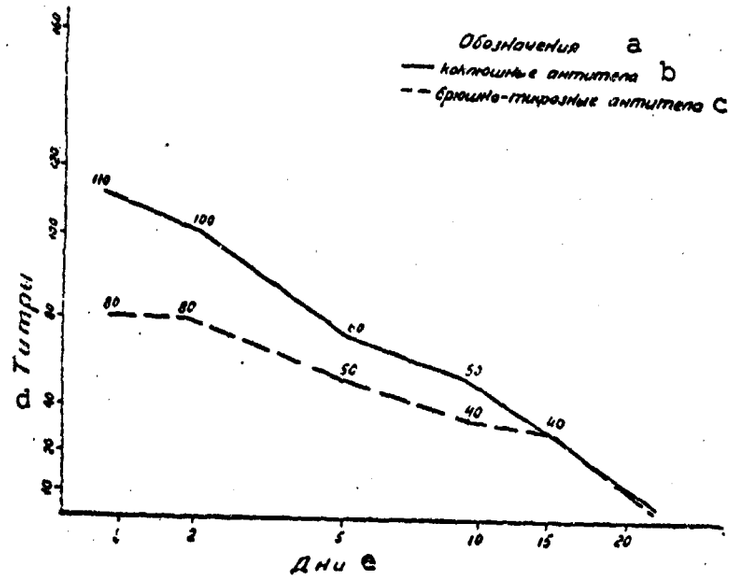

Figure 2.

Key:
a- designations;
b- whooping cough antibodies;
c- typhus abdominalis antibodies;
d- titers;
e- days.

EXPERIMENTAL EVALUATION OF THE EFFECT OF THE NUMBER OF INJECTIONS OF POLYVACCINE AND VI-ANTIGEN OF ESCHERICHIA COLI ON THE DEVELOPMENT OF IMMUNOLOGICAL INDICES

by

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In the previous work it was shown that there is no positive effect of Vi-antigen of E. coli microbes on the intensification of the immunological reaction with respect to typhus abdominalis infection both in the experiments on the development of agglutinins, as well as in the development of the preventative properties in the serums of the singly innoculated rabbits with calcium phosphate deposited polyvaccine 2.

The doubling of Flexner's dysentery microbe antigen dose caused an increase of the level of the development of agglutinins to the corresponding microbes. The increase of the dysentery antigen had no effect on the development of the preventative properties.

The introduction of purified and concentrated tetanus antitoxin into polyvaccine had a significant effect on the development of higher tetanus antitoxic immunity.

In regard to the effect of a number of injections of polyvaccine, deposited with calcium phosphate, it was found that in the case of one and two month intervals after the second injection the increase of the antibody level and the period of its lowering did not differ from that observed after a single immunization.

The increase of the interval to six months produced better results in the development of preventative properties in the serum with respect to typhus abdominalis infection in animals, innoculated with polyvaccine, not containing Vi-antigen of E. coli. The same was noted with respect to the development of antitetanus immunity.

The data of these investigations did not resolve completely. The question of the significance of Vi-antigen of E. coli in the development of typhus abdominalis immunity. These data differed from the data on the effectiveness of cross immunization with Vi-antigen of typhus abdominalis microbes of E. coli (strain 5396/38) (Zamukhovskaya), which necessitated continuance of this work, but using aluminum hydroxide as the sorbent. It is a better sorbing substance for the purified tetanus antitoxin than calcium phosphate.

In view of the posed problems we investigated two types of polyvaccine. One of the vaccines was polyvaccine 1, containing a double dose of the typhus abdominalis antigen. The second polyvaccine, number 2, containing a single dose of the same typhus abdominalis antigen and the dose of Vi-antigen of E. coli.

The Flexner's dysentery antigen type c in both vaccines was doubled similarily as it was done in polyvaccine 2 in the 1956 investigations. The tetanus antitoxin, purified and concentrated according to the Starcheus method, in both preparations contained 100 EC/ml. The sorbent was aluminum hydroxide.

The dynamics of the immunological reaction were investigated on eight groups of rabbits -- four groups for each polyvaccine. These experiments were grouped according to the intervals between injections.

The investigations were concerned with the dynamics of the development of Vi- and O-agglutinins and also in the preventative properties with respect to small doses of microbe cultures of typhus abdominalis and Flexner's type c dysentery. In addition, the tetanus antitoxin titer was determined in these serums. These investigations were conducted with a mixture of serums of the corresponding experiments.

The data on the dynamics of the development of O-agglutinins to the typhus abdominalis microbe during the immunization with its polyvaccine, containing Vi-antigen of E. coli strain 5396/38, in addition to the typhus antigen, both after a single as well as after a double immunization with three weeks, two months, and six month intervals between inoculations did not differ from the data obtained in the immunization with polyvaccine containing no E. coli Vi-antigen. In the case of revaccination following a ten month interval of animals singly inoculated with polyvaccine without this antigen the development of agglutinins occurred at a higher level.

In the case of immunization with polyvaccines containing the same antigens in the same amount deposited or not deposited with calcium phosphate, agglutinins to Flexner's type c dysentery microbe, were found at identical high levels (Figure 1).

The preventative properties of the serums were investigated with respect to the protection of white mice against a lethal dose of Vi-culture of typhus abdominalis (strain 4446) and Flexner's dysentery culture (strain 170), emulsified in 0.4% agar gel. The serums were injected in 0.2 ml doses subcutaneously, while the cultures were introduced intraperitoneally 22 to 24 hours after the injection of the serum. Each experiment for testing the serum was conducted on twenty mice. A same number of mice not injected, were used as a control. Prior to immunization, the serums of rabbits protected two out of thirty mice against a lethal dose of typhus abdominalis culture and three out of thirty mice against Flexner's dysentery culture.

After a single and also after a double inoculation (with a two month interval between the innoculations) with polyvaccine, not containing E. coli antigen, the development of the preventative properties with respect to typhus abdominalis culture took place after two weeks, further increase

of these properties continued during the course of four months, and in one experiment, even six months after a single immunization, preventative properties of the serum were at a high level. The development of these properties during the inoculation of polyvaccine with E. coli antigen occurred at a lower level. The same characteristics were noted also after revaccination both after six as well as after ten months. In the case of a ten month interval between re-inoculation, better effectiveness was observed, for the polyvaccine without E. coli antigen.

Only in the case of double inoculations with a short time interval between the inoculations (three weeks) there was no difference between the investigated polyvaccines.

As far as the development of the preventative properties with respect to Flexner's dysentery culture is concerned, their development proceeded at a sufficiently high level and did not differ significantly, as witnessed by the fact that the concentration of antigen of this type was identical in both polyvaccines.

The conducted investigations in this experiment showed not only the absence of the positive effect of E. coli Vi-antigen on the development of immunological reactions with respect to the typhus abdominalis infection, but in fact they showed the existence of inhibition of this development (Figure 2).

The development of antitetanus antitoxic immunity, both after a single injection of polyvaccine, conatining Vi-antigen of E. coli, as well as after the revaccination with six and ten month intervals between the inoculations occurred at a lower level than in the case of polyvaccine without this antigen. This was manifested both in the level of increase as well as, of the lowering of the antitoxin in the serums in the course of a six month observation period.

In the case of dual inoculation with polyvaccines containing the E. coli antigen with a three week interval

between the inoculations the antitoxin titer exceeded the titer during immunization with polyvaccine not containing this antigen by a factor of two. At the same time the lowering of the titer after three months also doubled.

In the case of double inoculations with a two month interval, the increase of the antitoxin level in the serum after a month reached identical levels for both polyvaccines (5 AE/ml), but after three months its level reached 1 AE/ml, and after six months ½ AE/ml when the polyvaccine with Vi-antigen of E. coli was used, while in the case of polyvaccine without this antigen the lowering of the antitoxin titer terminated at a level exceeding ½ AE/ml and remained there throughout the six month observation period (Figure 3).

Thus, the introduction of Vi-antigen of E. coli into the vaccine has a hindering effect on the development of antitetanus antitoxic immunity, apparently resulting from the toxic effect of this antigen which increases the reactogenic properties of the preparation.

Conclusions

1. The results of the conducted investigation verify the conclusions made in previous communications not only regarding the absence of the favorable effect of E. coli antigen, but also on the inhibition of the development of immunological reactions to typhus abdominalis, by this antigen.

2. The use of double immunization with a short time interval does not increase the development of the immunological reaction to typhus abdominalis, not to dysentery infection.

3. Revaccination with long time intervals between each vaccination (up to ten months) increases the level of both agglutinins as well as of the preventative properties of the serums.

4. High development of antitetanus immunity was noted during the immunization with polyvaccine, sorbed on aluminum hydroxide, containing purified tetanus anatoxin.

5. The double immunization with a two month interval between each immunization and particularly the revaccination after longer intervals of time (up to ten months) leads to a high development of the antitetanus immunity. Vi-antigen of E. coli also inhibits the development of antitetanus immunity.

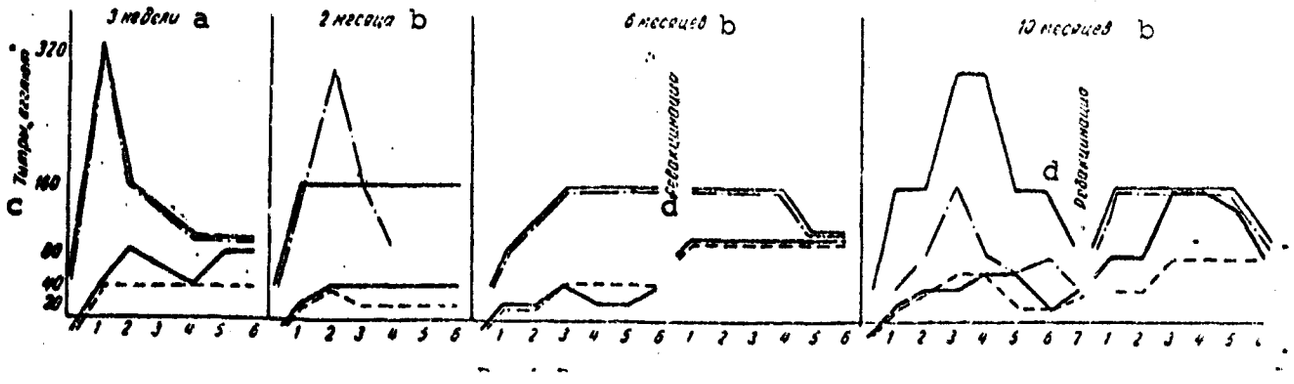

Figure 1. Development of agglutinins

Key:
a- three weeks;
b- months;
c- agglutinin titers;
d- revaccination.

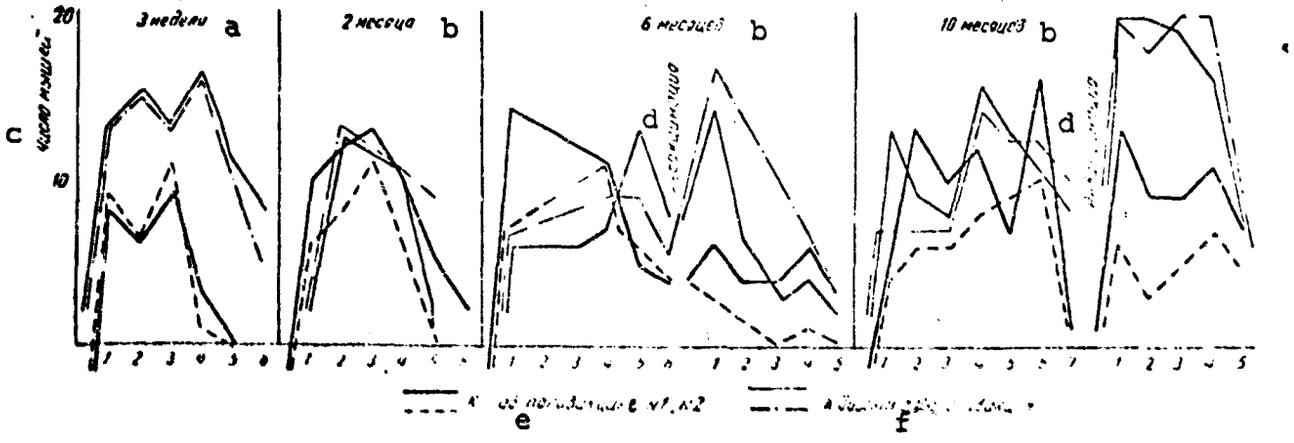

Figure 2. Preventative properties of serum.

Key:
a- three weeks;
b- months;
c- number of mice;
d- revaccination;
e- ab polyvaccine No. 1 and No. 2;
f- Flexner's dysentery polyvaccine.

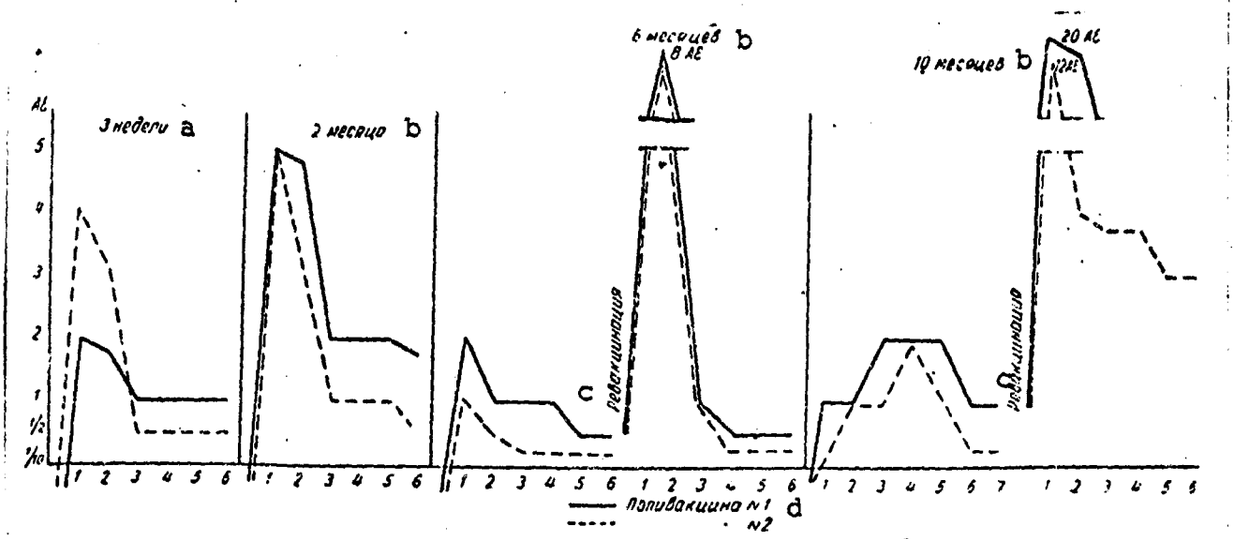

Figure 3. The development of antitetanus immunity.

Key:
a- weeks;
b- months;
c- revaccination;
d- polyvaccine No. 1 and No. 2.

STUDY OF THE COMPLETE ANTIGEN OF THE INTESTINAL GROUP OF MICROBES BY THE DIFFERENT LABORATORY METHODS

by

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In controlling intestinal infections, a large effort is directed towards the specific prevention which is realized through different vaccine preparations, including NIISI (Scientific Research Institute of Sanitary Engineering) polyvaccine.

In spite of the fact that there exist a number of methods for the determination of the quality of the vaccine for intestinal infections, the generally accepted method for testing of the active immunity is vaccination of mice followed by the injection of a lethal dose of the corresponding live culture.

Attempts are being made for quantitative determination of the immunogenic properties of intestinal vaccines and their standardization. Griffits (1944) proposed the immunization of mice by a single constant dose of the vaccine, and testing of the immunity with three different doses of live culture. Luippol'd (1954) proposed a method of double immunization of mice with different doses of vaccine varying by a factor of ten, with subsequent testing of the immunity by infecting the mice with 100 to 1000 Dlm in mucin. Troitskiy and Kovaleva (1947), Gekker and Zhgutova (1947), Gekker and Yakovleva (1949) worked on methods for the determination of the quality of typhus abdominalis and dysentery vaccine by determining the index of the effectiveness and the minimum immunization dose. Shvartsman and Sidorova (1955), and Lesnyak (1952) propose a method for the quantitative evaluation of typhus abdominalis and dysentery vaccine by

determining the minimum immunization dose. Grabaret (1955) evaluates the quality of typhus abdominalis vaccine by determining the preventative strength of rabbit serums on chick embryos immunized with the investigated vaccine. Grasset (1956) argues that the method for the determination of the quality of the vaccine from the results of the active protection of immunized mice is not as accurate as the method of determining their quality from the strength of the preventative properties of the serums. Olitskiy (1960) proposed the use of a sublethal infectious doses and the determination of the quality of microbes in the organs of immunized mice as a method for determining the strength of the vaccine. Greichter (1960) considered that the protective strength of rabbit serum, immunized with the investigated preparation, may serve as a sufficiently objective criterion for the evaluation of the quality of the preparation.

In this work we present the data on the study of complete antigens of the intestinal group of microbes, which are included in the polyvaccine, by different laboratory methods in vitro in vivo.

The serological properties of the complete antigen were studied in precipitation reactions, complement fixation reactions and adsorption of agglutinins. The immunogenic and antigenic properties of the antigens were determined in the immunization experiments on mice and the determination of the presence of agglutinins and preventative properties in serums of the immunized rabbits (Table 1).

It is apparent from the presented data that the highest and similar type of indices were obtained in all of the serological reactions for typhus abdominalis and Zonne dysentery complete antigens. The lowest indices were obtained for paratyphoid A. The indices for Flexner's type c dysentery and paratyphoid B complete antigens were less uniform, but average or high in magnitude.

Table 1.

Complete antigens of the intestinal group of microbes in serological reactions.

a Вид антигена	h Всего антигенов	Титр в реакции преципитации i			Титр в реакции связывания комплемента j			Реакция адсорбции агглютининов k		
		1:512000 — 1:128000	1:64000 — 1:32000	1:16000 — 1:4000	1:1600 — 1:400	1:200 — 1:100	<1:100	100—75%	50%	<50%
b Брюшнотифозный	7	3	4	—	6	1	—	7	—	—
c Паратифозный *A*	6	—	—	6	—	—	6	—	2	4
d Паратифозный *B*	16	6	7	3	8	6	2	13	3	—
e Дизентерийный Флекснера типа *c*	15	9	3	3	6	9	—	14	1	—
f Дизентерийный Зонне	7	7	—	—	6	1	—	7	—	—
g Всего	51	25	14	12	26	17	8	41	6	4

Key:
- a- type of antigen;
- b- typhus abdominalis;
- c- paratyphoid A;
- d- patatyphoid B;
- e- Flexner's type c dysentery;
- f- Zonne dysentery;
- g- total;
- h- total antigens;
- i- titer in percipitation reactions;
- j- titer in complement fixation reactions;
- k- reaction of the adsorption of agglutinins.

The presented data indicates the coincidence of the indices of the activity of whole antigens in the precipitation reaction and complement fixation reaction as well as certain discrepancies between the values of indices of these reactions with the indices of agglutinin adsorption reactions.

A significant number of identical results were obtained in the study of complete antigens in the precipitation reaction and the complement fixation reaction. This was expected, in the light of our concept of the uniqueness of antibodies, the view of which the majority of investigators adhere at present.

In regard to the agglutinin adsorption reaction, it is based on the Castellani principle. Apparantly this reaction reflects some other unknown properties of complete antigens.

The study of typhus abdominalis complete antigen in the precipitation reactions with three Vi-serums has shown the presence of Vi-antigens in them in titers equal to 1:16000 to 1:64000.

Thus, the study of the 51 industrial production series of whole antigens of the intestinal group of microbes, which are used in polyvaccine, have shown that they all, are active antigens in serological reactions with the exception of paratyphoid A. Moreover, typhus abdominalis antigens contained Vi-antigens.

Subsequently, comparisons of the immunogenic properties of the complete antigens with their serological activity were conducted. The determination of the immunogenic properties of whole antigens was conducted in the Laboratory of Polyvaccines of the Institute in the experiments on mice with 1 to 2 Dcl of the corresponding bacteria in 0.4 % agar.

Table 2 shows the summary data of the relationship of the precipitating properties and the ability of whole antigens to adsorb agglutinins with their immunological properties.

Table 2.

The relationship between serological and immunogenic properties of whole antigens.

a Число антигенов	Всего антигенов e	d Выживаемость мышей (в %) 70—100	50—60	<50	Всего антигенов e	70—100	50—60	<50	Всего антигенов e	70—100	50—60	<50
		f Титр 1:128000—1:512000				f Титр 1:32000—1:64000				f Титр 1:4000—1:16000		
b В реакции преципитации 54	23	22	1	—	14	10	4	—	17	6	4	7
		75—100%				50%				<50%		
c В реакции адсорбции агглютининов 56	43	42	—	1	9	—	9	—	4	—	—	4

Key:
- a- Number of antigens;
- b- in the precipitation adsorption reaction 54;
- c- in the agglutinin adsorption reaction 56;
- d- survival of mice (in %);
- e- total antigen;
- f- titer.

It is apparent from the presented data that in the group of antigens of high precipitating activity (1:128000 to 1:512000) there is almost a total coincidence of the precipitation index with immunogenic activity.

Thus, in a group of antigens with both high and weak precipitating activity there was a more significant coincidence of this index with immunogenic activity as compared with antigens of the average precipitating acitvity.

The significant coincidence of indices of the precipitating and specifically adsorbing agglutinin properties of whole antigens with their immunological properties indicate the ability of whole antigens to enter in serological

reactions, which in the absence of haptenes characterize their antigen properties, and may serve as some criterion of their quality.

Thus, in the investigation of a large number of industrial whole antigens in three serological reactions the high serological activity of the antigen for all representatives of the intestinal group which are used in polyvaccine, with the exception of paratyphoid A was found. It should be noted that a technically very simple method of the adsorption of agglutinins produced the results which are in the best agreement with the data of the study of the whole antigen properties.

The Castellani reaction serves as the foundation for the agglutinin adsorption reaction. The index of the quality of whole antigens is the magnitude of their adsorption ability. This quantity is measured by the degree of adsorption of agglutinins from the agglutinating serum by the investigated whole antigen. The index of the adsorption ability is the difference in the agglutinin titer of the starting agglutinating serum and the adsorbed serum. It may be expressed in percent. Generally, 1:50 dilution of the serum is used for this reaction, while the investigated whole antigen is used in 1:1000 dilution.

Subsequently, the ability of whole antigens of different serological activity to cause the formation of agglutinins in rabbits immunized with these antigens was investigated, as well as the preventative properties of serums of these rabbits in the experiments with the passive protection of mice.

In animal experiments, two series of typhus abdominalis, paratyphoid B, Flexner's type c dysentery and Zonne dysentery whole antigens, producing high and weak serological reactions were taken. In addition, three series of polyvaccine were taken, containing whole antigens with different serological

indices: series 3, prepared from antigens with high serological indices, series 4, consisting of antigens with lower serological indices with the exception of the Zonne dysentery component, and series 17 -- experimental vaccine (polyvaccine 2) containing whole antigens with high serological indices. It should be pointed out that Vi-antigen of E. coli strain 5396 was additionally introduced into the last vaccine and the amount of Flexner's type c dysentery component was doubled. The individual complete antigens were sorbed on calcium phosphate.

The rabbits were immunized by a single 2 ml subcutaneous injection of polyvaccine or by the innoculation dose of the whole antigen in the same volume. Each polyvaccine series of the whole antigen series was injected into three rabbits.

The serum of the rabbits was studied both before the injection of the preparation as well as after 1, 2, 3, 4, 5, and 6 months subsequent to the injection. The serum of the majority of rabbits prior to the injection contained Flexner's type c dysentery and typhus abdominalis O-agglutinins in titers equal to 1:10 to 1:40.

Upon immunization of rabbits with two typhus abdominalis antigens of different serological activity (series 293 a and 423) four months subsequent to the injection, the increase in the titer of O-agglutinins was observed up to 1:160 and its lowering by the third to fourth and sixth months (termination of the experiment).

During the immunization of rabbits with three series of polyvaccine (3, 4, and 17), containing different serologically active typhus abdominalis antigens, the highest increase of agglutinin titer (up to 1:160) was observed in all cases later, after two months, just as in the case of injection of individual typhus abdominalis whole antigens. The agglutinin titer rapidly decreased after three, four and six months (the termination of observations). Vi-agglutinin

were found primarily in 1:10, 1:20 and 1:40 titers and only very rarely in 1:80 titer. It was not possible to determine the characteristic dependence of the high agglutinin titer on the serological activity of typhus abdominalis complete antigens.

After a single injection of Flexner's type c whole dysentery antigens into rabbits and upon the injection of the polyvaccine the dynamics of the formation of agglutinins was similar to that observed during the injection of typhus abdominalis antigen. The maximum agglutinin titers (1:160) and the period during which they occurred were also one - two and three months. They began to lower after four and six months.

After rabbits were injected two series of dysentery antigens (382 and 82) with identical serological indices (precipitation reaction titer 1:64000) the curves for the formation of agglutinins were almost parallel.

During the immunization of rabbits with series 3 polyvaccine, containing highly serologically active dysentery antigen 326 Flexner's type c (the titer in the precipitation reaction was 1:256000) the agglutinin titer increased to 1:160 dilution, while upon the injection of series 4, containing less serologically active antigen 126 (the titer in the precipitation reaction was 1:32000) the maximum agglutin titer was equal to 1:80.

Thus, the Flexner's type c dysentery whole antigens are capable of causing the formation of agglutinins after a single injection into rabbits. It was noted that the size of the agglutinin titer is somewhat dependent on the serological activity of the antigens which were taken for the experiment.

When the rabbits wer immunized with a different series of polyvaccine, and also with paratyphoid A and B and Zonne dysentery antigens the agglutinins were found only in low titers (1:10, 1:20) and sometimes they were not found at all.

Th. preventative properties of the serums were studied prior to immunization and 1, 2, 3, 4, 5, and 6 months following the immunization. In these experiments the serum of each group of rabbits was mixed in equal volumes. The investigated 0.25 ml specimens of serum were injected subcutaneously into five mice weighing 14 to 16 g and following twenty-four hours the mice were injected intraperitoneally with 1 Dcl of the corresponding eighteen hour live culture in 0.4 percent agar. The observations of the mice were conducted for three days.

The preventative properties of the serums of rabbits immunized with three series of polyvaccines, displayed identical dynamics of the protective properties with respect to typhus abdominalis bacteria, Flexner's type c dysentery and Zonne dysentery.

The preventative properties were generally dissolved after two and three months, and sometimes after one and four months. In the majority of cases the preventative properties were absent after six months.

During the maximum increase of the preventative properties of the serum 40 to 60 percent of the passively immunized mice were protected when they were infected with 1 Dcl of the corresponding culture.

The relationship between the serological activity of the complete antigens and the strength of the preventative properties of the serum were observed in the experiments on the immunization of rabbits with typhus abdominalis. Flexner's type c dysentery and Zonne dysentery with complete antigens, differing with respect to their serological indices.

During the immunization of rabbits with Zonne series 335 dysentery antigen with high serological index (in the precipitation reaction the titer was 1:128000) their serum protected forty percent of the mice, while the serum of rabbits which were injected complete antigen of Zonne series 181 with extremely low serological indices (precipitation

reaction was 1:8000) did not prevent the death of the mice throughout the whole period of investigation.

The preventative strength of the rabbits serum, immunized with two serologically identical Flexner's type c dysentery antigens (series 82 and 388, producing the precipitation reaction in identical dilutions; 1:64000) was identical.

The same relationship was observed during the injection of two typhus abdominalis complete antigens of different serological activity into rabbits (series 293a, producing the precipitation reaction in 1:512000 dilution and series 423a -- 1:16000). However, occasionally there was a non-correspondence between these indices.

Thus, with few exceptions, it is noted that there exists a relationship between the serological indices of complete antigens and the preventative properties of the serums of rabbits which were immunized with these antigens.

Consequently, the study of whole antigens of microbes of the intestinal group, which are a part of the polyvaccine, by different laboratory methods can help to some extent in evaluating their antigenic and immunogenic properties. However, the lack of a quantitative index (a unit) for the immunogenic activity of whole antigens cannot satisfy the requirements which are placed upon the evaluation of the quality of the preparation. This necessitated further work in this area.

Conclusions

1. The comparative study of whole antigens of intestinal group microbes in three serological reactions has shown that the highest and similar indices were displayed by typhus abdominalis and Zonne dysentery complete antigens, while the lowest indices were displayed by paratyphoid A antigens. Less similar yet moderately high indices were displayed by

Flexner's type c dysentery and paratyphoid B complete antigens.

2. There is a significant coincidence between the indices of activity of complete antigens in the precipitation reaction and the complement fixation reaction, while there is a certain discrepancy between these indices and the agglutinin adsorption reaction index.

3. Typhus abdominalis complete antigens, produced by the total tryptic cooking of the broth culture, grown in vats with aeration contain Vi-antigen.

4. There is a significance between the precipitation index and the immunological activity in the antigen group with high and weak precipitation activity. These indices show some discrepancy in the group of antigens with intermediate precipitation titers.

5. The capability of complete antigens to adsorb agglutinins from the corresponding agglutinating serums coincided almost completely with their immunological activity.

6. In the case of identical subcutaneous injection of polyvaccine into rabbits, as well as of complete antigens of different serological activity, their serum contained O- and Vi-typhus abdominalis as well as Flexner's type c dysentery agglutinins, while paratyphoid A and B and Zonne dysentery agglutinins were found in extremely low titers or were not found at all.

7. No characteristic relationship was eatablished between the serological activity of complete antigens and the magnitude of the agglutinin titer in the serum of rabbits immunized with these antigens.

8. The preventative properties of the serum of rabbits, singly immunized subcutaneously with a dose of polyvaccines and complete antigens, were discovered predominantly after two and three and less frequently after one and four months. After six months the preventative properties were generally

absent.

9. With a small number of exceptions there is a relationship between the serological indices of the complete antigens and the preventative properties of the serums of the rabbits which were immunized with them.

Bibliography

[1] Troitskiy, V.L. and Kovaleva, N.I. "The Quantitative Method for the Determination of Immunogenic Properties of Vaccines for Intestinal disease," ZhMEI (Journal of Microbiology, Epidemology and Immunobiology), No.5, 60 to 67 (1947).

[2] Gekker, V.D. and Zhgutova, A.V. "Methods for the Quantitative Evaluation of Immunogenic Vaccines" ZhMEI, No.6, 27 to 33 (1947).

[3] Hekker, V.D. and Yakovleva, A.V. "On the Problem of the Standardization of Dysentery Vaccines" ZhMEI, No. 3, 32 to 36.

[4] Shvartsman, L.A. and Sidorova, N.I. "Rational Composition of the Associated Vaccines for the Prevention of Intestinal Infections" Sbornik Materialov po Obmenu Opytom Minzdrava SSSR (Collection of Data for the Exchange of Information of the Ministry of Health of the USSR), Moscow, 1955, No. 1/49, pages 1 to 9 and No. 2/50, pages 65 to 72.

[5] Zhurbina, V.I. "The Use of Adsorption of the Agglutinin Method for the Evaluation of the Quality of Whole Antigens in Polyvaccines" Nauchnyye Trudy Moskovskogo Institute Vaktsin i Syvorotok im. Mechnikov (Transactions of the Mechnikov Moscow Institute of Vaccines and Serums), Vol.7, 626 to 635 Moscow (1956).

[6] Lesnyak, S.V. "Laboratory Evaluation of the Effectiveness of Dysentery Vaccines" ZhMEI, No.3, 53 to 58 (1952).

[7] Griffits, N. "Methods for the Evaluation of the Immunogenic Properties of Vaccines," ZhMEI, No.6, 84 (1945).

[8] Luippol'd, G. "An Attempt to Evaluate Quantitatively the Immunogenic Properties of Vaccines and Their Standardization" ZhMEI, No.1, 94 (1947).

[9] Grabaret Le Minor. Ann. Pasteur, 1955, No.5, p. 88.

[10] Grasset. Atti del Secondo Congresso Internazionale di Standizzazione immunomicrobiologica. Roma, 1956, 281.

[11] Olitzki, Sharen and Godinger. International Symposium on Microbiological Standardization. Opatija, 1960.

[12] Gerichter. International Symposium on Microbiological Standardization, Opatija, 1960.

STUDY OF THE EFFECT OF MICROBE ANTIGEN ON THE PRODUCTION OF IMMUNITY AGAINST INFECTION CAUSED BY CI. PERFRINGENS

by

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In the gastric infection, caused by Cl. perfringens, is a toxic-septic illness. Its mechanism is not clear even to the present time. Therefore the role of the microbe factor in immunity against Cl. perfringens is extremely significant for the most rapid solution of the problem of active immunization. The study of the role of microbe cells in the production of active immunity against Cl. perfringens was carried out by Weinburg and co-workers (1927), Levkovich (1945), Yertova and Kozhevnikova (1950) and others.

These investigators conducted the work on the comparative study of the immunological properties of formalin treated microbe cells and Cl. perfringens antitoxin seperately and combined.

As a result of these studies all of the above authors came to the conclusion that the injection of animals with formalin treated microbe cells does not produce immunity against Cl. perfringens , in spite of the accumulation of agglutinins and precipitators in the blood. The serums of these animals did not exhibit any preventative properties.

During the testing of the effect of formalin treated microbe cells on the increase of the effectiveness of immunization with Cl. perfringens antitoxin these investigaotrs obtained discordant results.

Thus, Weinburg and co-workers, have shown that the addition of formalin treated microbe cells to the anatoxin increases the quality of the serum in comparison with antitoxic serums.

According to the data of Levkovich, Chertkova and Zel'manovich, Chertkova and Kozhevnikova the addition of two billion formalin treated microbe cells per milliliter to the anatoxin does not increase the quality of the equine serum and does not produce higher resistance to the infection in comparison with serums obtained during the immunization with anatoxin alone.

All of the above work was concerned with the study of the role of corpuscular vaccine in the production of active immunity against Cl. perfringens. Very little has been written (Chernikova and Semykina, Vygodchikov and co-workers), on the possibility of obtaining antigens from Cl. perfringens microbe cells by the methods which are used for other micro-organisms (intestinal-paratyphoid group, staphylococci, etc.)

In 1950 we used the tryptic boiling method to obtain purified and concentrated antigen, free from toxin. It was found that the obtained antigen after a double immunization with twenty day interval between the injections in mice produces 48 to 55 percent resistance with respect to 1 Dcl of broth culture of Cl. perfeingens.

Subsequently, the work conducted on the use of the tryptic boiling of the whole broth culture, containing an insignificant amount of toxin (20 to 40 Dlm/ml) and 25 billion microbe cells (g/ml) increased the immunogenic properties of the antigen. The double immunization of mice with this antigen produced 72 to 80% protection from the injection of 1 Dcl of the broth culture. This increase of the immunogenic properties of the complex antigen can apparently be explained by the presence of the toxic component, which exists in insignificant amounts (20 to 40 Dlm/ml) in the broth culture, from which the antigen was obtained. The purified concentrated antigen, prepared seperately from the filtrate of the same culture, after double immunization protected only 27 to 30 percent of the mice against the introduced 1 Dcl of

the broth culture of Cl. perfringens.

Thus, our work has shown that in order to produce active immunity against Cl, perfringens both the microbe and the toxic factors are significant.

The possibility of obtaining antigens from microbe cells of Cl. perfringens was shown by Vygodchikov and co-workers in 1957. These authors conducted tests of the immunogenic properties of three protein microbe fractions, obtained according to the method of Kholchev and dried microbe cells of Cl. perfringens, washed from the toxin. As a result of dual immunization and revaccination of rabbits, it was found that after vaccination with one microbe fraction, containing traces of toxin, all of the test animals were resistant to 1 Dcl of spore culture. Their blood serum contained small concentrations of antitoxin in the serum (0.5 AE/ml), agglutinins (titer was 1:100), and precipitant (titer was 1:1,000,000 and 1:5,000,000).

During the revaccination with a nontoxic microbe fraction (II and III) some resistance of animals to 1 Dcl of spore culture of Cl. perfringens was manifested, but it was highly incomplete: five out of nine rabbits remained alive. Their blood contained no antitoxin, but it did contain agglutinins and precipitants in the same titers as in serums obtained after immunization with the first fraction. The survival of the rabbits in these experiments cannot be explained by the presence of antitoxins, since for protection from infection the presence of antitoxin in the blood should be in excess of 0.1 AE/ml, while the blood of experimental rabbits immunized with II and III fractions contained less than 0.05 AE/ml of antitoxin.

In the immunization of rabbits with protein microbe fractions in combination with purified and concentrated antitoxin , the agglutinin and precipitin titers increased by a factor of 5 to 10, while the concentration of the antitoxin

did not exceed its level (1 to 5 AE/ml) in the blood of the control animals, immunized with antitoxin alone. In testing the intensity of immunity in this group of rabbits the authors were unable to find the difference in the resistance to the introduced 1 Dcl of spore culture of Cl. perfringens, since the antitoxin titer in the serums of all test animals was identical (1 AE/ml). To solve this problem, in our opinion, it is necessary to either increase the dose of the introduced culture of Cl. perfringens, or to conduct these experiments on animals containing smaller amounts of antitoxin in the blood (0.1 AE/ml).

The cited literature data on the role of the Cl. perfringens microbe cell antigens in the production of active immunity are insufficient. Conclusive proof was not shown in favor of the use of purified and concentrated microbe antigen in combination with anatoxin. This prompted us to carry out work on the study of the immunogenic properties of anatoxins in combination with purified and concentrated antigen, obtained from Cl. perfringens culture by tryptic boiling.

The experiments were conducted with three series of purified and concentrated anatoxins, and five series of microbe antigens, prepared from the broth culture of Cl. perfringens (Table 1).

Table 1.

Characteristics of antigens used in the experiments.

Antigen	Toxicity	Amount EC/ml	Complement fixation reaction	The resistance of single. immunized mice (in %) to the introduced 1 Dcl of the culture.
Microbe antigen	Nontoxic	-	Positive 0.004mg/ml	40 to 64
Anatoxin	Nontoxic	100	Negative	55 to 60

The antigens were found to be nontoxic when 5 ml of anatoxin and 10 mg of microbe antigen were injected into guinea pigs. The microbe antigen gave a positive complement fixation reaction with the specific serum in the amount of 0.004 mg/ml. During a single immunization 40 to 64 percent of the mice were protected against 1 Dcl of the broth culture of Cl. perfringens.

The antitoxin contained 100 EC/ml; it did not produce the complement fixation reaction with antimicrobe serums and after a single immunization it protected 55 to 60 percent of the mice against the introduced 1 Dcl of Cl. perfringens culture.

The study of antigens and the immunogenic properties of the above preparations were conducted with rabbits and mice.

In order to study the antigenic properties, one group of rabbits was injected with 50 EC of anatoxin, the second group was injected with 0.4 mg of microbe antigen, and the third group was injected with premixed anatoxin and microbe antigen in the same dose. The antigens were deposited with 8mg/ml of aluminum hydroxide and introduced both as a single injection and as two injections with a thirty day interval between them. The blood for the production of the serum was taken thirty days after the injection of the antigen.

In the obtained serums the presence of antibodies to the microbe antigen in the complement fixation reaction and the concentration of antitoxin were determined. In addition, the preventative properties of serums were tested on the experimental mice.

The experiments with the complement fixation reaction were conducted by the ordinary method at 37°C with the above rabbit serums, taken in 1:40 dilution and antigens taken in 0.08 mg/ml (Table 2).

The serum of anatoxin immunized rabbits did not react with the microbe antigen. The serum of rabbits immunized with both microbe and mixed antigens produced highly positive

complement fixation reactions at 1:40 dilution.

To study the antitoxic titer of 0.2 ml of serum in different solutions, it was mixed with a 0.3 ml test dose of standard Cl. perfringens toxin, kept at room temperature for 45 minutes and injected intravenously into mice in a 0.5 ml dose. The mice were observed in the course of forty-eight hours (Table 3).

Table 2.

The presence of antibodies to the microbe antigen in the rabbit serum, on the basis of the complement fixation reaction.

a Сыворотка крови при иммунизации	b Число опытов	c Результаты реакции связывания комплемента d Кратность иммунизации	
		1	2
e Анатоксином	4	±	±
f Микробным антигеном	4	+++	++++
g Смешанным антигеном (анатоксин и микробный антиген)	4	+++	++++

h Обозначения: ±слабая реакция; i +++положительная реакция; j ++++резкоположительная реакция. k

Key:
a- Blood serum during immunization;
b- Number of experiments;
c- Results of the complement fixation reaction;
d- number of immunization injections;
e- anatoxin;
f- microbe antigen;
g- mixed antigen (anatoxin, and microbe antigen);
h- designations;
i- ± weak reaction;
j- +++ positive reaction;
k- ++++ highly positive reaction.

Table 3.

Antitoxic titer of serums

a Сыворотка при иммунизации	f Результаты иммунизации			
	g кратность иммунизации			
	1		2	
	h число опытов	i количество AE/мл	h число опытов	i количество AE/мл
b Микробным антигеном	2	0,05	3	0,05
c Анатоксином	2	0,1	4	0,25
d Смешанным антигеном (анатоксин и микробный антиген)	2	0,25	4	0,5
e Нормальная сыворотка	2	0,05	2	<0,05

Key:
- a- Serum during immunization;
- b- microbe antigen;
- c- anatoxin;
- d- mixed antigen (anatoxin and microbe antigen);
- e- normal serum;
- f- results of immunization;
- g- number of injections;
- h- number of experiments;
- i- amount of AE/ml;

Thus, the addition of microbe antigen to anatoxin, as shown by the experiments, increases the concentration of antigen toxin in the blood of rabbits by a factor of two. Consequently, the combination of microbe antigen with anatoxin has a favorable effect on the formation of Cl. perfringens antitoxin.

The reason for the increased formation of antitoxin in the serums of animals under the influence of the microbe antigens to Cl. perfringens anatoxin requires further study.

To test the preventative properties of the serum of rabbits both whole and diluted (1:2 to 1:6) 0.5 mg doses were injected subcutaneously. Twenty-four hours after the

injection of the serum the mice were injected 1 Dcl of two hour broth culture of Cl. perfringens. The mice were observed for four days (Table 4).

Table 4.

The results of the tests of the preventative properties of serums.

a Сыворотки кроликов при иммунизации	f Результаты иммунизации									
	g кратность иммунизации									
	1				2					
	i	h		i	h	j разведение сыворотки				
	Число опытов	Неразведенная сыворотка		Число опытов	Неразведенная сыворотка	1:2	1:3	1:4	1:5	1:6
b Микробным антигеном	2	12/16		2	24/24	12/21	5/19	2/11	—	—
c Анатоксином	2	12/15		2	22/22	14/21	10/17	5/15	—	—
d Смешанным антигеном (анатоксин и микробный антиген)	2	16/16		2	22/22	25/25	19/19	20/20	15/21	14/20
e Нормальная сыворотка	2	0/15		2	0/10	—	—	—	—	—

k Примечания: числитель — число мышей, оставшееся в живых; знаменатель — число мышей в опыте.

Key:
- a- serum of rabbits during immunization;
- b- microbe antigen;
- c- anatoxin;
- d- mixed antigen (anatoxin and microbe antigen);
- e- normal serum;
- f- results of immunization;
- g- number of immunization injections;
- h- undiluted serum;
- i- number of experiments;
- j- dilution of serum;
- k- number of surviving mice; denominator number of mice in the experiment.

The preventative properties of rabbit serum, immunized with mixed antigen, both after a single as well as after a double immunization, were higher than in the serum obtained by the immunization of rabbits both with microbe antigen and with anatoxin separately.

During tests of the preventative properties of serums as a function of the antitoxic titer, it was found that at the same concentration of AE/ml the preventative properties of the serum, obtained during the immunization with complex antigen, are significantly higher than those resulting from antitoxic immunization (Table 5).

Table 5.

Preventative properties of serums as a function of antitoxic titer.

Serums used for immunization	Amount of AE/ml		
	0.25	0.12	0.06
Anatoxin	22/22	14/21	5/15
Anatoxin with microbe antigen	25/25	20/20	14/20

Note: numerator -- number of surviving mice; denominator number of mice in the experiment.

The data of these experiments on the determination of the preventative properties of the serums indicates that in the protection of animals from gas gangrene, caused by Cl. perfringens, both antitoxin as well as antibodies to the microbe factor play a significant role.

To study the effects of microbe antigen on the immunogenic properties of antitoxin, mice were immunized with anatoxin along with microbe antigen and with each of the antigens separately. The immunization of the mice was conducted by a single injection. The anatoxin was injected in 50 EC/ml, dose while microbe antigen was injected as 0.4

mg. in 0.5 ml dose. The strength of the immunity was tested thirty days after immunization by the injection of 1 Dcl of broth culture of Cl. perfringens. A total of eight experiments was conducted.* (Table 6).

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* Because during single immunization the series of microbe antigens were found to be not identical, in terms of their immunogenic properties, the results summarized in Table 6 are given separately.

Table 6.

Results of single dose immunization of mice with microbe antigen and anatoxin separately and combined.

a Число серий	b Число опытов	Микробный антиген c			Анатоксин d			Смешанный антиген (анатоксин и микробный антиген) e		
		f Количество культуры (*Dcl*)								
		1			1			1		
		g Число мышей в опыте	h Число мышей, оставшихся в живых		g Число мышей в опыте	h Число мышей, оставшихся в живых		g Число мышей в опыте	h Число мышей, оставшихся в живых	
			Абс. i	%		Абс. i	%		Абс. i	%
2	3	50	20	40	23	14	60	40	27	67
3	5	51	33	64	36	22	59	64	50	79

Key:
- a- Number of series;
- b- number of experiments;
- c- microbe antigen;
- d- anatoxin;
- e- mixed antigen (anatoxin and microbe antigen);
- f- amount of culture;
- g- number of mice in the experiment,
- h- number of mice surviving;
- i- absolute.

The obtained data indicates that mixed antigens in these experiments induce higher resistance of mice than antigens taken separately. The immunogenic properties of the preparation depended on the quality of the microbe antigen used. Thus, antigen which causes less than 50 percent protection of mice did not increase the immunogenic properties of the preparation in combination with an anatoxin. When microbe antigen possessing more pronounced immunogenic properties was used (67 percent), the effectiveness of mixed preparations increased significantly (79%).

Thus, these experiments showed that for active immunity against Cl. perfringens, it is necessary to produce preparations which include the anatoxin and microbe antibodies, displaying pronounced immunogenic properties.

In order to solve this problem, further work is necessary on improving not only the quality of the anatoxin, but also of the microbe antigen.

Conclusions

1. A single immunization of mice with anatoxin in combination with microbe antigen causes a higher pretection of mice to the introduced 1 Dcl of broth culture of Cl. perfringens (79 %), than the immunization with anatoxin (60 %) or microbe antigen separately (67 %).

2. The increase in the immunogenic properties of complex preparation depends on the quality of the microbe antigen used.

3. The serum of rabbits, immunized with anatoxin in combination with microbe antigen, obtained by the tryptic cooking method, contains a greater amount of AE/ml and better preventative properties in experiments with mice than rabbit serum, immunized with anatoxin or microbe antigen separately.

Bibliography

[1] Vygodchikov, G.V. "Effect of Antitoxic and Antibacterial Factors in the Active Immunity Against Experimental Gas Gangrene, caused by Cl. Perfringens" ZhMEI (Journal of Microbiology, Epidemology and Immunobiology), No.10, 120 to 125 (1957).

[2] Levkovich, O.I. "The Role of Antitoxic and Antibacterial Factors in the Antiperfringens Antigangrene Serums" ZhMEI, No.10 to 11, 43 to 48 (1945).

[3] Chertkova, F.I. and Zel'manovich, R.Ya. "The Effect of the Antibacterial Factor on the Protective Properties of Antigangrene Serum in the Studies with Experimental Infection" ZhMEI, No.12, 43 to 45 (1946).

[4] Chertkova, F.A. and Zel'manovich, R.Ya. "The Effect of Antibacterial Gas Gangrene caused by Cl. Perfringens and Edematiens" Collection of Data on the Exchange of Experimental Information, Moscow No. 4/47, 46 to 51 (1954).

[5] Semykina, T.G. and Chernikova, N.G. The Abstracts of Reports of Interinstitute Scientific Conference on the Problem "Anaerobes," Moscow, (1956), page 51.

[6] M.Weinburg and S.Batotte, Ann.de l'Inst. Pasteur. 1929, v. 43, pages 453 to 456.

THE ANTIRABIC MECHANISM OF DRY GAMMA GLOBULIN WHEN USED FOR THE TREATMENT OF WOUNDS INFECTED WITH A STREET VARIETY OF RABIES VIRUS

by

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The introduction of antirabic vaccination into the medical practice in 1885 by Louis Pasteur lowered the fatality of people bitten by rabid animals many fold. Even better results have been achieved in the last decade due to the achievements of the seroprophylaxis of rabies, particularly in the cases with short incubation of the disease, when a single vaccination even given in time may not necessarily prevent the disease. In such cases the introduction of antirabic serum (or its fraction) increases the incubation period of the disease, thus increasing the effectiveness of vaccination.

What happens in the immune organism? Why, after the hydrophobia prophylactic sero-vaccine series, doesn't the person become ill? What is the mechanism of immunity against rabies?

All these questions were posed by scientists a long time ago but then and now the answers to the majority of these questions are given by the investigators in the form of theories and hypotheses which have not been experimentally verified.

One of the generally accepted views is that the rabies virus which penetrates the organism may be controlled until the time that it reaches, and enters the cells of the central nervous system. In other words, as long as the virus is in the tissues where it enters with the saliva of the animal or in the peripheral nervous systems along which it moves

in the direction of the brain, preventative measures against rabies can be effective.

What helps the destruction of the virus and where is it inactivated in the case when a person or an animal has been successfully protected from rabies?

On the basis of experimental and seroprophylactic data, it is concluded that the principal factor responsible for antirabic immunity is specific antibodies.

In regard to the location where the antibodies contact the virus and inactivate it, this part of the immunity mechanism has been very little developed. Up to the present time it was only possible to show that a great role in the prevention of rabies must be played by the antibodies which penetrate from the blood into the spinal fluid, and that the penetration of the hematoencephalitic barrier plays a significant role.

We have studied the properties of powdered antirabic gamma globulin in the local treatment of wounds, infected with a street variety of rabies virus, Along with other aspects of the problem we were determined to elucidate the mechanism of the action of this preparation, namely: to determine whether gamma globulin is capable of neutralizing the virus in the wound tissues themselves.

Some indications of this possibility were obtained by Yugoslav virologists Milan Nikolitso and Zdravko Jelesitsch.

In our experiments we initially attempted to determine the effectiveness of powdered antirabic gamma gloublin in the treatment of wounds infected with a street variety of rabies virus. After it was found that this preparation is highly effective if the wounds are treated immediately after the infection, it was of interest to determine the effectiveness of gamma gloublin in local applications on different days after the infection. The preliminary results of this work were published in 1958. This article presents

more complete data on this aspect of the question.

Method

Using scissors and pincers a section of musculus masseter 1 to 1.5 cm^2 in area was opened on both sides of guinea pigs weighing 250 to 300 g. Using the sharp ends of the scissors twelve to fifteen longitudinal and transversal incisions, three mm in depth, were placed along the cleared areas. Using a syringe 0.5 ml of ten percent encephalic suspension containing street variety of rabies virus were placed on the wounded areas on each side of the guinea pig. The virus was isolated from the brain of a man who died in 1957 from hydrophobia. The diagnosis of the illness were established in our laboratory by histological (Negri bodies) and virological methods. The virus which was used for infecting the guinea pigs was passed four times through the organisms of these animals by intramuscular infection and then introduced into the brain of the rabbit.

In these experiments we used a ten percent rabbit encephalic suspension prepared with distilled water and ten percent normal equine serum. The suspension was as a twenty percent suspension at a low temperature. For each series of experiments a new series of virus was prepared.

The intracerebral titration of the virus was conducted on mice. The DL_{50} of the virus varied during different periods between $10^{-5.1}$ and $10^{-6.8}$ per 0.03 ml. The specificity of the virus suspension was determined. In the experiments we used the supernant liquid, obtained after rapid defrosting of the encephalic suspension, two fold dilution and subsequent centrifugation for 5 min. at 1500 rpm.

Antirabic gamma gloublin was prepared by the porlonged immunization of horses with the encephalic suspension from rabbits, containing the Moscow strain of the fixed rabies virus. The serum of these horses, containing a sufficiently

high concentration of specific antibodies, was subjected to the precipitation with ethanol in the cold according to the current instructions on the production of gamma gloublin. It was then dried in a vacuum apparatus by the lyophilic method. The powder obtained in the drying of one series of this preparation was subsequently used in our experiments.

A ten percent solution of gamma gloublin in 10^{-4} dilution neutralized with one hundred LD_{50} of the fixed virus was used in the neutralization reaction with mice. After the infection of the guinea pig wounds with a street variety of rabies virus, 0.2 g of the powdered antirabic gamma gloublin was applied to each affected surface.

The period of the observation of the animals was sixty days. The rabies diagnosis in dead guinea pigs was verified by histological (Negri bodies) and virological investigations.

The cited data illustrates the limited effectiveness of the treatment of wounds with antirabic gamma globulin as a function of the time of its application (Table 1). This preparation prevents the development of rabies in the majority of animals only if it is applied within fifteen to thirty minutes after infection. At a later time its application is noneffective in preventing the disease.

Nonetheless, it is known that intramuscular injection of large quantities of liquid antirabic gamma globulin into animals even forty-eight hours after the infection with a street variety of the virus prevents the majority of animals from contracting the disease. The use of this preparation in the dry form with local application on the wounds, infected with rabies virus, even one hour after the infection becomes noneffective. This fact indicates that dry gamma gloublin when applied to the wounds, has a local action with respect to the rabies virus which enters the wounded tissues.

The following experiments were also conducted in order

to investigate the validity of this assumption.

Three groups of guinea pigs were wounded on both sides in the region of the musculus masseter, but only one side was infected with rabies virus. Fifteen minutes after the infection, powdered antirabic gamma gloublin was applied to the infected side of one group of animals, while the second group received the application of gamma gloublin on the opposite side. The third group of animals was the control (Table 2).

Table 1.

Treatment of wounds with dry antirabic gamma gloublin after the infection of the wounds with a street variety of rabies virus.

e Опыт	a Обозначения	Срок обработки ран после заражения (в мин.) c					Контроль d
		3–5	15	30	80	120	
1	b Смертность	1/10	—	—	8/10	9/10	9/10
	Средний инкубационный период (в днях)	27	—	—	20	21	22
2	b Смертность	2/10	3/10	6/10	—	—	8/10
	Средний инкубационный период (в днях)	27,5	30	26	—	—	17,5
3	b Смертность	—	1/8	5/8	8/8	—	8/8
	Средний инкубационный период (в днях)	—	32	31	25	—	22
4	b Смертность	—	1/8	2/7	7/8	8/8	8/8
	Средний инкубационный период (в днях)	-	30	27,8	20	19	17

f Примечание: числитель — число павших животных, знаменатель — общее число животных.

Key: a- Designations; b- Mortality average incubation period (in days); c- elapsed time after the infection of the wound before treatment of wounds with antirabic gamma gloublin (in min.) d- control; e- experiment no. f- note: numerator -- number of dead animals denominator -- total number of animals.

Table 2.

Local and general effectiveness of dry antirabic gamma gloublin during treatment of wounds infected with street variety of rabies virus.

a Опыт	b Обозначения	Присыпание гамма-глобулином d через 15 мин.		f Контроль
		e На инфицированную поверхность	На сторону, противоположную заражению h	
1	c Смертность	2/8	5/8	6/6
	Средний инкубационный период (в днях)	24	22,5	20
2	c Смертность	3/10	8/10	8/8
	Средний инкубационный период (в днях)	22	19	17

g Примечания: числитель — число павших животных, знаменатель — общее число животных.

Key: a- experiment number; b- designations; c- Mortality average incubation period (in days); d- application of gamma gloublin after 15 minutes; e- application to the infected surface; f- control; g- note: numerator -- number of dead animals; denominator -- total number of animals; h- application to the side opposite the infected side.

The leading role of gamma gloublin in the protection of animals from contacting rabies virus disease was of the local nature. The general effectiveness of this preparation was also apparent, but it was significantly less effective.

The general action of gamma globulin is demonstrated by the low antibody titer in the blood of guinea pigs, after the application of gamma gloublin (investigations were conducted after twenty four hours, four days, ten days, fourteen days and twenty days following the application of powdered antirabic gamma gloublin to the noninfected wounded side of

fresh guinea pigs). The presence of antibodies by the tenth to the fourteenth day disappeared (see figure).

At the same time the virus which enters the wound with the saliva of the animal penetrates quite rapidly into the interior of the tissue and a higher concentration of the specific antibodies than that which is sufficient for local application of insignificant amounts of such antirabic gamma is necessary for its neutralization. Therefore, with all the due importance of the local treatment of wounds with powdered gamma gloublin and the creation of the possibility for specific antibodies to destroy the virus at the seat of the introduction of the infection the local treatment of wounds infected with a street variety of rabies virus must be used only as a measure auxiliary to the sero-vaccine prophylaxis of rabies.

Conclusions

1. The specific rabicidic antibodies can inactivate the rabies virus at the sight of its entry, i.e. in the wounded tissues.

2. The local treatment of wounds with antirabic gamma globulin alone may be insufficient for the protection against the disease of rabies, and therefore must be viewed as an auxiliary measure to the sero-vaccine prophylactic measures against rabies.

Bibliography

[1] Yeveoshenko, V.G. The Study of the Duration of Antirabic Immunity and the Effectivemess of Vaccine of the Fermi Strain of the Street Variety of Rabies Virus Isolated in Kirgiziya. Frunze, 1956 (Kirghiz Institute of Epidemology, Microbiology and Hygiene).

[2] Selimov, M.A. and co-workers. J. of Hygiene, Epidemology, Microbiology and Immunology, III, 2, (1959), Prague.

[3] Speranskiy, A.D. "Certain Conditions for the Action of Specific Antibodies in the Organism" Hygiene and Epidemology, No.3, 48 to 63 (1927).

[4] Ponomarev, A.V. and Chesnkov, A.M. "Certain Conditions for the Action of Antirabic Serum in the Organism." Archives of Biological Sciences, Vol.41, 118 to 123 (1927).

[5] De-Zhorzh, L.V. "The Significance of Hematoencephalitic Barrier in the Active Immunity Against Rabies," Herald of Microbiology, Epidemology and Paracytology, Vol. VII, No.4, 360 to 368 (1928).

[6] Solov'yev, V.D. Selimov, M.A. and Kobrinskiy, G.D. "Local Application of Powdered Antirabic Gamma Globulin for the Prevention of Rabies," Problems of Virology, No.2, 115 to 116, (1958).

[7] Baltazard, M. Bahmanyar. With the Collaboration of M. Chodssi et al. Bull Org. Geneva, Vol. 13, 5, 747 to 772 (1955).

[8] Nikolitso, M. and Jelesitsch, Zd. Arch. für Hyg. und Bakt. 7, 141 (1957).

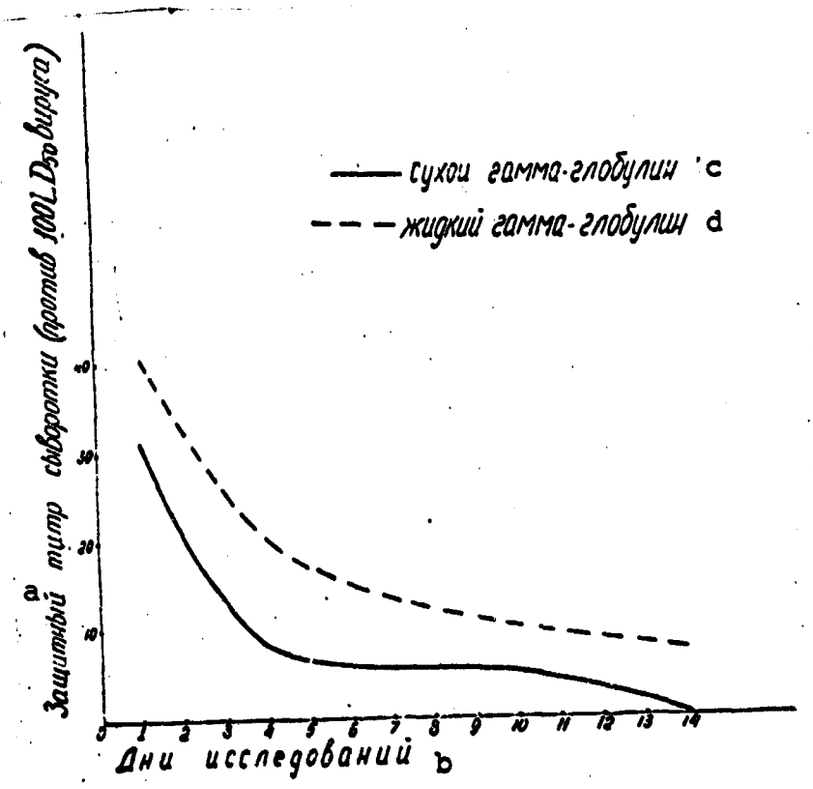

Figure. Dynamics of the level of specific antibodies in guinea pigs blood upon local application of powdered antirabic gamma globulin.

Key:
- a- Protective titer of the serum (against 100 LD_{50} of the virus);
- b- days of investigation;
- c- dry gamma globulin;
- d- liquid gamma globulin.

THE STUDY OF COMPLETE ANTIGENS OF TYPHUS ABDOMINALIS MICROBE BY DIFFUSION PRECIPITATION IN AGAR

by

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In recent years the diffusion precipitation reaction in agar has been widely used as one of the most sensitive methods for the determination of the composition of antigens in complex bacterial products. At present this method is successfully used for the study of the antigen composition of bacteria toxins, the determination of the toxigenic properties of bacteria, the study of the complex composition of antigens and the determination of the common serological properties of antigens.

The study of bacterial processes, i.e., filtrates of broth cultures, have shown that bacterial toxins have a complex antigen structure. Thus, in the native diphtheria toxin up to twenty-four antigens were detected (Pope, 1951),in tetanus toxin up to eighteen antigens (Rafyi, 1954) and Cl. perfringens anatoxin up to twelve antigens (Marmalevskaya and Blagoveschenskiy, 1959).

In type A boutlinus Biorklund (1954) found nine antigens, while Gendon (1958) discovered up to fifteen antigen fractions. In Streptococci toxin twelve antigens were discovered (Chistovich, 1956), while in staphylococci ten antigens were found (Chistovich, 1956) and seven by Kovaleva (1958).

A number of authors (Yelicheva, 1959; Rakhman, 1958; Khamagushkin, 1959) used the precipitation in agar method, to determine the toxigenic property of microbes of the intestinal and diphtheria group.

Use of this method is of great interest in the study of the antigen composition of the bacterial antigens. Thus, Goryshina (1959), used this method to study the composition

of Vi-antigens of the E. coli and typhus abdominalis cultures. The antigens were obtained using the Buavena method from strains of E. coli, S. Ballerup, Ty 1203 and V_{i_1}.

The direct and cross experiments with monoreceptor O- and Vi- serums and with serums obtained for all the strains of the investigated cultures have shown that all antigens contained serologically identical Vi-components. The antigens differed only with respect to the presence of the O- component, specific for each form of antigen. O- and Vi- antigens of the strains of E. coli, S.ballerup and Ty 1203 formed two characteristic precipitation zones. Consequently, they are two independent antigens rather than one antigen with two determinant groups.

Thus, the precipitation reaction in agar is a specific reaction, the essence of which lies in the formation of precipitation in the agar gel at the place of the reaction of the antigen with the corresponding antibody. In view of the different rate of diffusion and the different concentration of various components in the system, the zones of equivalent ratios between antigen and antibody are located at different levels of the agar gel. Each precipitation zone corresponds to only one antigen-antibody pair. In the presence of several antigens in the system they behave independently of one another and produce a corresponding number of precipation zones in the presence of the corresponding antibodies.

There are several immunobiological methods for the determination of the quality of the whole antigen (precipitation reaction, complement fixation reaction, etc.), but not one of them indicates the complex structure of whole antigen. In the earlier work (1959) on the study of whole antigen of Flexner's type c dysentery microbes, which comprise part of the NIISI polyvaccine we used the agar precipitation method as an additional investigation method in conjunction with other methods. The results of that

investigation verify the possibility of utilizing this method for the study of whole antigens.

The obtained data have shown that whole antigen of Flexner's type c dysentery microbe have a complex antigen structure. We discovered six antigens. It was also shown that from the number of precipitation zones in agar whole antigens are not standard in composition, in spite of the fact that they were obtained from the same strain and by the same method.

It is of interest to study whole antigens of typhus abdominalis microbes in this reaction, since its antigen composition has been most thoroughly investigated and serums are available which contain antibodies to individual antigens of this microbe.

The purpose of this work is to study the whole antigens of the typhus abdominalis microbe by the precipitation method in the agar in the tall medium columns, proposed by Oudin (1946, 1948). The experiments were conducted according to the method which was slightly modified by Gispen (1955). Gispen proposes the use of 0.8 and 1.6% agar, prepared with distilled water with the addition of a small amount of alkali (up to pH = 7.2). In the preparation 0.3 ml of the serum and 0.15 ml of the agar heated to 60°C are mixed together. The experiments were conducted in test tubes of a certain diameter at room temperature and the results were studied in the course of twenty-one days.

To ascertain the reliability of the obtained results, the reaction with each of the antigens was conducted simultaneously in three different test tubes. The measurements were taken after every two days. The height of the detectable zone (from the separation boundary of the first and the second layers in the agar) was measured in millimeters. At the same time it was marked on the test tube in order to

observe the dynamics of the development of the obtained zones.

The study of whole antigen was conducted with rabbit serums, immunized with live culture, formalin treated vaccine, whole antigen of typhus abdominalis microbe Ty_2 4446, obtained by the industrial production method, with monoreceptor serum O, Vi and H, and also with whole antigen, obtained by the Buawen method from O_{901} culture and Batnagar Vi culture.

For control the serum of healthy rabbits and hydrolyzed casein broth, in which the culture was grown for the preparation of whole antigens were used. The serums of healthy rabbits, checked preliminarily in the ordinary precipitation reaction in 1:2 dilution, produced negative results. In the agglutination reaction these serums indicated the presence of agglutinins to typhus abdominalis microbes, in 1:10 to 1:20 titer. We were unable to obtain serums totally free from agglutinins.

In the prelinimary experiments, using the agglutination reaction we investigated: reference culture of typhus abdominalis microbe Ty_2 4446 (from which whole antigens and serums were produced industrially) with which all of the investigations of antigens were conducted (Table 1).

Table 1.

Characteristics of the Ty_2 4446 culture on the basis of the agglutination reaction.

Culture	serum (immunization)				
	Live culture	O_{901}	Vi_1	H_{901}	Healthy rabbits
Ty_2 4446	1:20480	1:1600	1:200	1:80	1:10

The data of these experiments have shown that the live Ty_2 446 culture contained O-, Vi- and H- antigens and was full strength with respect to the whole antigen composition.

The serums, obtained in the immunization of rabbits with this live culture, was also found in full strength. It contained O-, Vi- and H- antibodies. In the agglutination reaction with live cultures O_{901} serum was agglutinated in a titer equal to 1:5120 with Vi_1 -- in a titer equal to 1:80 and with H_{901} -- in a titer equal to 1:10 240.

The monoreceptor serums O_{901}, Vi and H_{901} displayed strict specificity and agglutinated only the corresponding cultures.

In all we investigated eleven whole antigens of the typhus abdominalis microbe by two methods: 1) the generally accepted calcium precipitation for the determination of the activity and 2) by the diffusion precipitation in agar for the determination of the composition of the antigen.

The investigation of whole antigens was conducted with rabbit serums, immunized with live cultures, heated O_{901} culture, and Vi_1 and H_{901} cultures. The H_{901} serum, absorbed by the heated O_{901} culture, according to the agglutination reaction titer did not contain any O- or Vi antibodies (Table 2).[sic].

The data obtained in the calcium precipitation, indicates that whole antigens possess sufficiently high titers and contain O- and Vi- antigens.

The concentration of H-antigen was not detected.

All eleven whole antigens were studied in the precipation reaction in agar with the same assortment of serums (Table 3) [sic].

These data indicate that whole antigens are not uniform in terms of antigen composition. The experiments with O, Vi and H serums have shown that all eleven antigens reacted with O and Vi serums, while eight out of eleven reacted with H serum. With these three serums, all antigens

formed not more than three precipitation zones. The majority of antigens produced only one precipitation zone with O and Vi serum.

The control experiments with whole antigens made up of O_{901} culture and Vi_1 Batnagar culture have shown that monoreceptor serums are typically specific, since in the precipitation reaction in agar they form only one zone and only with the corresponding antigen. O_{901} antigen forms three zones with the serum of rabbits immunized with live culture, while Vi_1 antigen forms two precipitation zones.

The experiments with serums of healthy rabbits have shown that six antigens out of eleven form not more than two precipitation zones. These data indicate that the serum of healthy rabbits contains the so-called normal antibodies to the whole antigen of typhus abdominalis microbes.

The data of the precipitation reaction in agar indicate that whole antigens of typhus abdominalis microbes have complex antigen composition and contain O-, Vi- and H-antigens. It should be noted that O- and Vi- antigens in themselves are complex, which is indicated by the formation of several precipitation zones with the serum of rabbits immunized with live culture. The precipitation reaction in agar is specific since only by means of this method was it possible to detect the concentration of H-antigens in whole antigens, and also to determine the presence of antibodies to these antigens in serums of healthy rabbits.

Consequently, an attempt was made to identify the development precipitation zones according to their distance of ascension. The analysis of the obtained data indicate that zones associated with Vi antigen are located at the 8 to 10 mm level, while the zones corresponding to O-antigen are located higher at approximately the 12 to 15 millimeter level.

The data of the experiments with casein broth have shown that the zone found at 2 millimeter height is non-specific. We did not interpret the zones which were obtained

at other levels.

In addition to the conducted investigations with the serum of rabbits immunized with live culture, eleven antigens were studied in the calcium precipitation reaction and the precipitation reaction in agar with serums of rabbits immunized with formalin vaccine and whole antigen of typhus abdominalis microbes.

In order to answer the question as to how different the serums prepared by different methods are, the results of the investigations were compared to the data obtained in the same reactions with rabbit serum immunized with live culture. For visual comparison of the results conducted by these two methods the antigens were grouped according to the arbitrarily assigned indices for each of the reactions.

According to the calcium precipitation reaction the first group contained antigens with titers equal to 1:256,000 to 1:128,000 the second group contained titers equal to 1:64,000 to 1:32,000. According to the number of detected precipitation zones the antigens were subdivided in the following groups: 1) 5 and more zones, 2) 3 to 4 zones and 3) 1 to 2 zones (Table 4).

Table 4.

Characteristics of serums according to the data of ordinary precipitation and precipitation in agar.

Сыворотка (иммунизация) a	Число сывороток e	f Титр антигена		g Число зон		
		1:256000—1:128000	1:64000—1:32000	5 и выше h	4—3	2—1
Живая культура b	11	9	2	2	5	4
Полный антиген c	11	8	3	4	4	3
Формалиновая вакцина d	11	3	8	1	5	5

Key: a- serum (immunization); b- live culture; c- whole antigen; d- formalin vaccine; e- number of serums; f- antigen titer; g- number of zones; h- 5 and more.

In the calcium precipitation reaction whole antigens reacted almost identically with the serum of rabbits immunized with both, live cultures and with whole antigen.

The data of the precipitation reaction in agar have shown that with the serum of rabbits immunized with whole antigen a larger number of antigen fractions were detected in the whole antigens than with the serum of rabbits immunized with formalin vaccine.

On the basis of these data one may presume that formalin vapor changes the antigen structure of the microbe cells and the number of detectable precipitation zones increases. There is no correspondence between the calcium precipitation reaction data and agar precipitation data. These reactions indicate different properties of the investigated whole antigens.

Thus, the studies of eleven whole antigens of typhus abdominalis microbes by the calcium precipitation method and the precipitation in agar have shown the existence of a complex antigen composition. They contain O-, and Vi- and some of the H-antigens.

The composition of whole antigens, neither on the basis of the titer in the calcium precipitation reaction, nor on the basis of the number of zones formed in the precipitation in agar is sufficiently standard, in spite of the fact that they are obtained from the same strain of microbes and by the same method. In all probability this difference is not qualitative, but it is associated with the quantitative content of certain antigens in the individual series of whole antigens, determined by the agar precipitation reaction. These amounts may be insufficient to form precipitation zones.

The study of whole antigens with the use of the serum of rabbits immunized with live culture, whole antigens and formalin treated vaccines has shown that these serums are not identical with respect to their properties.

The serum obtained during the immunization of rabbits with formalin treated vaccine contained a smaller number of antibodies. Since the properties of serums depend on the quality of the antigen, which induce immunization, one may conclude that formalin has apparently a destructive effect on certain microbe cell antigens.

Apparently, the diffusion precipitation in agar may be used for the detection of the effect of physical and chemical methods, used in the preparation of typhus abdominalis vaccine, on the antigen properties of microbes. This aspect of the subject should constitute a special investigation.

Conclusions

1. According to the precipitation reaction in agar and the calcium precipitation reaction, whole antigens have complex antigen structure and contain O-, Vi- and H- antigen components.

2. The serum of rabbits, immunized with live culture, is sufficiently strong. In terms of the properties it is close to the serum obtained during the immunization of rabbits with whole antigen.

3. The precipitation in agar develops the complex structure of whole antigens and is a new highly sensitive method for the study of the quality of whole antigens which are put into the composition of polyvaccine.

Bibliography

[1] Gendon, Yu.V. "Study of the Antigen Composition of Botulin Toxins and Type A Antitoxins by Means of the Diffusion Precipitation Reaction in Agar" ZhMEI, No.5, 33 to 36 (1958).

[2] Goryshina, L.V. "Experiments on the Study of the Composition of Vi-antigens of E. coli and Typhus

Cultures by the Diffusion Precipitation Method in Gel" ZhMEI, No.2, 78 to 80 (1959).

[3] Yelicheva, I.Ya. "Characteristics of Toxigenic Properties of Grigor'yev-Shiga Microbes on the Basis of the Results of the Diffusion Precipitation in Gel," Abstracts of Reports presented at the Symposium on the Problems of Bacterial Toxins and Antitoxins," Moscow 12 - 12 [sic]. (1959).

[4] Izmaylova, N.B. "Study of Whole Antigens of Flexner's Type c Dysentery Microbes by the Diffusion Precipitation Method in Agar," Problems of Bacteriology, Virology and Immunity. Transactions of the Moscow Scientific Research Institute of Vaccines and Serums im. Mechnikov, Moscow, 163 to 170 (1959).

[5] Kovaleva, V.V. "Observations of Staphylococcus Toxin in the Diffusion Precipitation Experiments" ZhMEI, No.10, 129 to 132 (1958).

[6] Marmalevskaya, L.Ya. Blagoveshchenskiy, V.A. "Isolation of Perfringens Anatoxin for the Active Immunization Against Gas Gangrene and their Immunochemical Studies" Abstracts of Symposium, Moscow, 41 - 41[sic]. (1959).

[7] Rakhman, E.Z. "The Significance Latent Precipitation Reactions in Agar for the Determination of Toxigenic Diphtheria Bacteria" ZhMEI, No.9, 51 to 54 (1958).

[8] Khamagushkin V.V. "Certain Aspects of the Method for the Determination of Toxigenic Diphtheria Bacteria" Abstracts of Symposium, Moscow, 16 - 16, (1959)[sic].

[9] Chistovich, G.H. "Experiments with Diffusion Analysis of Antigens and Antibodies (Communication No.2)" ZhMEI, No.2, 56 to 62 (1956).

[10] Gispen, R.J. Immur.. 1955, Vol.74, p. 134 to 141.

[11] Biorklund, B. and Berengo, A. Acta. path. microbiol. Scandinav, 1954, Vol.34, pp. 79 yp 86.

[12] Oudin, S.J. Ann. Inst. Pasteur, 1948, V. 75, p. 30 to 51.

[13] Oudin, S., Compt. rend. Acad. Sci. 1946, v. 222, p. 115 to 116.

[14] Pope, C. Stevens, M. Caspary A. and Fenton E. Brit. J. exper. path. 1951, v. 39, p. 246 to 258.

[15] Rafyo, A., Mir Chanssy, H. and Debsal, J.S. Rev. Immunol. 1954, v. 18, p. 391 to 398.

STUDY OF THE ANTIGEN FRACTION OF WHOOPING COUGH MICROBE AND ITS ROLE IN THE FORMATION OF ANTIWHOOPING COUGH IMMUNITY

(Communication I)

by

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Many investigations devoted to the study of the whooping cough microbe, and its structural components, responsible for the production of insensitivity to whooping cough infection, still did not produce any new antiwhooping cough vaccines.

Up to this time the prevention of whooping cough is achieved only through the use of corpuscular vaccine, produced at present from the immunogenic strains of the first phase of the whooping cough microbe.

However, this vaccine in association with purified diphtheria antitoxins produces a severe reaction. Therefore, the production of a nontoxic soluble whooping cough antigen, possessing well developed immunogenic properties, suitable for use in the associated vaccine for a number of childhood infections, is one of the presently significant problems.

The isolation of the antigen fraction of the whooping cough microbe by the method of Buavena and Raistrik-Topli was conducted by Truskina-Tumanova (1952), Malysheva, Kholchev, Shavrova (1952), Trushina-Tumanova and Mamayeva (1955), Palant, Milel'man, Fintiktikova and Oleynikova (1955), Bayeva (1958) and others. It was found that only the Reystrik-Topli method is capable of isolating whooping cough microbe antigens with a well developed immunogenic activity. However, the doses of this antigen, which are

used for the immunization of animals significantly exceed the doses of the corpuscular vaccine. On these bases Bayeva, in particular, concluded that antigen obtained by the Reystrik-Topli method does not have any advantages over the corpuscular vaccine.

Pillemer and Burrele (1947), Smolens and Flavell (1947), Robins and Pillemer (1950) obtained antitoxins of the whooping cough bacilli, by the repeated freezing and thawing, as well as by ultrasonic irradiation with subsequent purification of the extracts of whooping cough bacilli. The purified extract displays antigenic and immunogenic properties. The authors believe that two or more than two antigen components in the purified fraction are responsible for the protective properties of the purified extract. One of the fractions, located on the surface of the microbe cell, is a specific antigen which can be obtained in large quantities without the destruction of the cells (Smolens and Flavell, 1947). Pennele and Thiele (1951) treated the whooping cough microbe suspension in an oscillator for thirty-five minutes. The suspension of destroyed cells was centrifuged. The fractionation of ethyl alcohol extracts at the appropriate pH, temperature and ionic strength and dilution of the final material permits the separation of the fraction which contained all of the immunogenic activity of the extract with low concentration of nitrogen. The immunogenic fraction contained both the agglutinin and toxin. After the removal of the toxin the antigen activity is not lost.

Pillemer and co-workers (1954) perfected the method for the preparation of the complex -- stromaprotective antigen (SPA), based on the absorption of the protective antigen on the stroma of human erythrocytes.

The study of the immunological activity of different purified fractions and the discovery of a new antigen (hemagglutinin and factor sensitive to histamine) brought us closer to the production of purified effective vaccine,

and also enabled the elucidation of the antigen complexity of the whooping cough bacilli. At the same time it is still not possible to identify the principal antigen which produces whooping cough immunity. The authors stress that it is necessary to introduce new methods in order to approach the solution of this problem.

The Japanese investigators (Kawajama and co-workers, 1956) discovered a highly effective protective antigen in the supernant liquid of whooping cough culture, grown for two - three days in a liquid synthetic medium. According to the opinion of the authors this antigen is the surface antigen of the whooping cough microbe and is the product of its metabolism, which diffuses into the liquid nutrient medium.

Barger and Soar (1958) in their study of the antigen composition of whooping cough bacilli extracted the antigen with a 2.5 M solution of urea, and showed that it is a nucleoprotein complex, containing 14.8 percent of nitrogen and 3.7 percent of phosphate. It has the characteristics of the perfected antigens in mice, intranasally infected with a live culture. The serums of mice immunized with this preparation contain agglutinins, the titers of which vary within 1:200 to 1:400 limits, while the titer in the precipitation reaction reached 1:120,000.

We believe that the establishment of the possibility of the production of immunogenic active whooping cough antigen is very important.

From this standpoint the search for new methods for the isolation of the antigen fraction of whooping cough microbes, which would insure a higher immunogenic activity of the latter, is of extreme importance.

The purpose of this work was to produce a soluble whooping cough antigen by such a method in which the microbe cell components, important for the development of immunity, would be the last subjected to trauma. The second problem

of this investigation was to study the obtained soluble antigens in order to develop a method for the production of whooping cough vaccine.

The most "mild" method is the extraction of the live microbe suspension with ether. This method was used by Larson (1945), Bell and Larson (1952, 1954) and also by Belkina and Perrosyan (1955 to 1957) for the production of immunogenic fraction of the tularemia microbe.

We utilized this method for the production of soluble whooping bacilli antigen. In this work seven strains of whooping cough microbe were used (187, 222, 248, 305, 18323, 58001 and 63), which meet all of the requirements of the first phase culture.*

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* We obtained these cultures from the State Control Institute im. Tarasevich.

In order to obtain the antigen by the ether method, the whooping cough culture was grown on a casein-carbon medium for forty-eight hours. The pure culture was removed by washing with a physiological solution. The obtained microbe suspension of certain standards was subjected to ether treatment. In some cases in order to free the culture from the cultural liquid the whooping cough bacilli culture, washed off with physiological solution (from the casein-carbon medium) was centrifuged. The obtained centrifugate was dialyzed in a flowing water system, filtered through a Berkefeld candle and freeze dried in a vacuum. The precipitate of the micromass was treated with a water-ether mixture (in 1:2.5 ratio) and the obtained antigen extract was also dialyzed, filtered and subjected to lyophylic drying.

Thus, three methods for the extraction of antigen were used in this work: the first method -- extraction of

antigen from the washed microbe culture treated with ether; the second -- ether extraction from the washed microbe mass and the third method -- from the supernant liquid of the culture wash without the ether treatment. All of the antigens were studied with respect to the chemical composition, toxicity and also serological antigenic and immunogenic properties. Using the above method twenty-nine series of antigens were obtained which were found to be nontoxic and specific. Depending on the method of extraction of the antigen, the chemical composition changes significantly. While the microbe cell antigens contain more nitrogen (seven to ten percent) and less reducing substances (5.16 to 8.28%), the antigens from the liquid phase of the culture wash contained less nitrogen (1.21 to 4.06%), but at the same time, significantly more of the reducing substances (22 to 48 %). Apparently the low reducing activity of the antigen from the microbe cells results not from carbohydrates but from the presence of other reducing substances, since the chromatographic analysis of the hydrolyzate of this antigen did not reveal any carbohydrates. The antigens extracted from the culture wash contained 11.1 to 12.1 % of nitrogen and 5.7 to 29.2 % of the reducing substances.

The study of the serological activity of antigens revealed wide variations of titers in the precipitation and the complement fixation reactions (Table 1).

In the majority of cases low titers were obtained for antigens from the supernant liquid (in five series out of six). The toxicity of the obtained preparation was checked by the subcutaneous injection into rabbits and the intraperitoneal injection into mice. The minimum toxic dose of antigen in the case of intraperitoneal injection was in excess of 5 mg. All of the investigated preparations were found to be nontoxic.

The antigen activity of the preparation was studied by the immunization of rabbits. After the completion of

Table 1.

Chemical composition and specific properties of whooping cough bacilli antigen obtained by different methods.

a Антиген	Выход антигена с 1 л (в г) f	Химический состав (в %) g				Реакция преципитации l	Реакция связывания комплемента m
		зола h	фосфор i	азот j	редуцирующие вещества k		
b Из микробной массы сер. 17	0,295	3,25	1,78	9,45	5,16	1:128000	1:40000
c Из надосадочной жидкости сер. 17	0,506	14,01	1,13	3,44	25,01	1:128000	1:40000
b Из микробной массы сер. 19	0,673	20,38	1,65	9,86	8,28	1:26000	1:8000
c Из надосадочной жидкости сер. 19	1,275	13,38	0,45	1,74	48,64	1:20000	1:8000
b Из микробной массы сер. 20	0,55	28,07	1,5	7,83	5,8	1:64000	1:8000
c Из надосадочной жидкости сер. 20	1,360	18,01	1,38	1,21	38,74	1:8000	1:10000
b Из микробной массы сер. 23	0,415	25,62	3,12	9,95	5,55	1:8000	1:8000
c Из надосадочной жидкости сер. 23	0,894	20,75	2,21	4,08	27,16	1:6000	1:20000
b Из микробной массы сер. 24	0,366	23,23	2,24	9,57	6,65	1:32000	1:16000
c Из надосадочной жидкости сер. 24	1,309	15,72	1,65	3,74	22,66	1:8000	1:10000
b Из микробной массы сер. 25	0,235	23,64	—	7,06	5,24	1:64000	1:8000
c Из надосадочной жидкости сер. 25	0,958	14,98	—	2,41	25,39	1:8000	0
d Из культуры штамма 63 3-й обработки эфиром сер. 12	—	--	—	12,1	5,7	1:128000	1:64000
e Из культуры штамма 222, обработанной эфиром, сер. 14	—	—	--	11,1	29,2	--	—

Key: a- antigen; b- from microbe mass series 17; c- from supernant liquid series 17; d- from the culture of strain number 63 of the third ether treatment, series 12; e- the culture of the strain 222, treated with ether, series 14; f- yield of antigen per liter (in g); g- chemical composition (in percent); h- ash; i- phosphorous; j- nitrogen; k- reducing substances; l- precipitation reaction; m- complement fixation reaction.

immunization, the rabbit serum was investigated for the concentration of agglutinins, complement fixing antibodies, and also for their preventative and neutralizing properties in the experiments with mice (one Dcl of live culture was used in the experiments).

The immunization schedule for rabbits was different depending on the nature of the injected antigen. The immunization cycle of rabbits involved 5 to 5.5 ml of the native antigen. The control group of rabbits was immunized with formaldehyde vaccine. One immunization cycle of rabbits entailed the injection of 4 to 9.5 billion microbes (on the basis of the intestinal standard). Six to seven days after the completion of the immunization the rabbits were exsanguinated. The serums of all of the immunized rabbits were investigated for the concentration of agglutinins and complement fixing antibodies.

The agglutination and complement fixation reactions were conducted with formaldehyde vaccine, prepared by us from the microbe suspension of the first phase virulent whooping cough culture (Table 2).

The high concentration of agglutinins was noted both in rabbit serums, immunized with formaldehyde vaccine, as well as in rabbits immunized with antigens obtained by the ether extraction method. The agglutinin titer prior to the dilution of the serums reached 1:25600, while the complement fixation titer of the antibodies was as high as 1:640. From the rabbits immunized with ether extracted antigen three had agglutinin titer, equal to 1:25600, two had agglutinin titer equal to 1:6400 and one had a titer equal to 1;12800.

The preventative properties of serums were studied on mice. Four serums were taken for the experiment (two serums obtained from rabbits immunized with formalin vaccine, and two serums from rabbits immunized with antigens extracted with ether).

Table 2.

The results of the study of the serums in the immunization reaction.

a Антиген	Серия сыворотки f	g Титры реакций: агглютинации h	связывания комплемента i
b Формоловая вакцина	10	1:25600	1:640
	11	1:25600	1:640
	12	1:25600	1:640
c Антиген, извлеченный эфиром	9	1:25600	1:640
	13	1:25600	1:640
	15	1:25600	1:320
	14	1:6400	1:320
d Антиген из надосадочной жидкости сер. 18	27	1:6400	Не ставилась j
e Антиген из микробной массы сер. 18	28	1:12800	Не ставилась j

Key:
- a- Antigen;
- b- formaldehyde vaccine;
- c- antigen, extracted with ether;
- d- antigen from the supernant liquid series 18;
- e- antigen from the microbe mass series 18;
- f- series of serum;
- g- reaction titers;
- h- agglutination;
- i- complement fixation;
- j- experiment was not conducted.

Experimental method

Twenty-four hours prior to the intranasal injection of one Dcl of the virulent culture of strain 222, the animals were injected intraperitoneally with the immune serum (0.25 ml dose) and in twenty-four hours the mice were infected

with a virulent whooping cough culture. The control mice were injected at the same time as the experimental mice a normal rabbit serum and the infective dose of the culture (Table 3).

Table 3.

Preventative properties of rabbit serums immunized with different whooping cough antigens.

Antigen	Series of serum	Form of serum	
		Immune	Normal
		Dose of culture 1 Dcl.	
Formalin vaccine	10	4/6	0/6
	12	4/6	0/6
Ether vaccine	8	2/6	0/6
	9	3/6	0/7

Note: numerator indicates the number of animals which survived the injection; the denominator indicates the total number of animals in the experiment.

While all of the control animals which were injected with the normal serum died upon the injection of one Dcl. of the virulent liva culture, the survival of the experimental mice was high when the serum obtained during the immunization of the animals with formalin vaccine was injected preliminarily. The survival is also high for the mice which

were injected, the serum obtained in the immunization with antigens obtained by the ether extraction method.

Some of the investigated serums were tested for the neutralizing properties in experiments with white mice, by the intranasal injection of the mixture of serum and the infective dose of whooping cough culture (strain 222).

Experimental method

A dose of immune serum (0.08 ml) was mixed with one Dcl. of the virulent culture contained in 0.02 ml. The mixture was kept in a thermostat for one hour and then a 0.05 ml dose of this mixture was intranasally introduced into mice. The control mice obtained the same doses of the normal serum with the infectious dose of the culture. The experimental and control animals were observed for three weeks (Table 4).

Table 4.

Antigen	Series of Serum	Type of serum	
		Immune	Normal
		Results of Neutralization	1 Dlm of the culture
Formalin vaccine	12	5/16	1/6
Ether vaccine	8	3/6	
	9	4/6	

Note: the numerator designates the number of surviving animals; the denominator indicates the total number of animals in the experiment.

The highest neutralizing activity was displayed by the

serum obtained from the rabbits immunized with formalin vaccine. The serums obtained from rabbits immunized with the ether extracted antigens, displayed a lower neutralizing ability. The control group which received the normal dose of the serum and the whooping cough culture had only one case of survival.

Having established the high antigen activity of our preparations we proceeded to the study of their immunogenic properties in experiments with mice. In the first stages of this work we evaluated the immunity of the animals which received different amounts of the culture intranasally.

The results of the serological, toxic and immunogenic properties of antigens, obtained by the ether extraction method are represented in Table 5.

Initially during the immunization of animals we titrated the minimum immunization dose of antigen from 0.01 to 0.25 mg. It was found that the minimum immunization dose is 0.01 mg. of antigen. In subsequent experiments the immunization of mice was conducted with 0.01 mg of antigen by the intraperitoneal two stage injection of this dose (with one week interval between the injections). Two weeks following the completion of the immunization the immunity of the animals was checked by the intranasal infection with virulent whooping culture. Only the series 20, antigen extracted from the microbe mass, with 0.01 mg. minimum immunization dose protected less than fifty percent of the mice against two Dcl of the culture. It, however, displayed good protecting properties when the dose was increased to 0.1 mg (66.6%).

Considering the fact that in recent years the intracranial infection with virulent culture (strain 18323) with subsequent calculation of LD_{50}, became a widely accepted method for evaluation of the immunity of test animals, in subsequent experiments we utilized this method. However, in order to establish the correspondence of the earlier obtained data with the intranasal infection method with the data obtained by the intracranial method, we re-evaluated two

series of earlier tested antigen (series 19 and series 20) by the new method. In this case series 20 antigen from the microbe mass and from the supernant liquid was mixed and injected in the same dose (0.01 mg.).

Table 5.

Serological, toxic and immunogenic properties of antigens of whooping cough bacilli extracted by the Larson method.

a Антиген	e Серологические свойства		i Токсические свойства	j Иммуногенные свойства (в %)				
	f тип			l	m проверка иммуногенности			
	реакции преципитации g	реакция связывания комплемента h	k в мг	иммунизирующая доза (в мг)	1 Del	2 Del	5 Del	164 ZD_{50}
b Из штамма 63, обработанный эфиром сер. 12	1:128000	1:64000	5	0,17	—	—	75	
c Из надосадочной жидкости сер. 17	1:128000	1:1000	5	0,01 0,1 0,2 0,25	58,3 70 63,6 72,2	— — — —	84,6 44,4 54,5 91,6	
d Из микробной массы сер. 17	1:128000	1:40000	5	0,01 0,1 0,2 0,25	84,6 58,3 91,3 75	— — — —	50 33,3 66,6 78,5	
c Из надосадочной жидкости сер. 20	1:8000	1:10000	—	0,01 0,1	60 40	— —	— —	
d Из микробной массы сер. 20	1:64000	1:8000	—	0,01 0,1	— —	44 66,6	— —	
c Из надосадочной жидкости и из микробной массы сер. 20	—	—	—	0,01	—	—	—	63
d Из микробной массы сер. 19	1:8000	1:8000	—	0,01	—	—	—	85,7

Key: a- Antigen;
b- from strain number 63, treated with ether series 12;
c- from the supernant liquid, series 17;
d- from the microbe mass, series 17;
e- serological properties;
f- titer;
g- precipitation reaction;
h- complement fixation reaction;
i- toxic properties; j- immunogenic properties;
k- in mg.; l- immunization dose (in mg.);
m- evaluation of immunogenic properties.

In series 19, only the microbe mass antigen was taken for the experiments. The tested series (20 and 19) protected 63 to 85 % of the animals, respectively, against 164 LD_{50} in this method of the verification of immunity.

Subsequently, it was of interest to verify the results of the work of the Japanese authors, Kawajama and others, who showed the possibility of obtaining highly active antigens from the supernant culture liquid after growing whooping cough microbes in the course of 2 to 3 days in a synthetic liquid medium.

On this basis, along with the ether method for the extraction of whooping cough antigen, we attempted to obtain antigens from the filtrate of the broth cultures of the first phase of whooping cough bacilli grown in a synthetic liquid medium for 48 hours.

The filtrates of the broth cultures were studied exactly as the above antigens with respect to toxicity, antigenic and immunogenic properties.

The antigenic activity of the filtrates was studied by the immunization of rabbits. The animals were immunized intravenously with 4 to 5 day intervals. The filtrates were injected in gradually increasing doses. In the course of the immunization cycle the rabbits received 25.2 ml of the native filtrate according to the scheme proposed by the above authors. Six to seven days after the last immunization the rabbits recovered.

The immunogenic activity of filtrates was studied by the intraperitoneal immunization of mice with subsequent intranasal infection with 1 Dcl of the virulent culture of whooping cough bacilli (Table 6).

The filtrate of the broth culture caused the formation of agglutinins (titer 1:100 to 1:160) and complement fixing antibodies (titer 1:150 to 1:320) in lower titers than the serum titers obtained after the immunization with other preparations (see Table 2).

Table 6.

Properties of filtrates of broth cultures.

a Сыворотка	b Свойства фильтрата							
	c антигенные		g превентивные		k нейтрализующие		l иммуногенные	
	d титр		h животные					
	e реакция агглютинации	f реакция связывания комплемента	опыт i	контроль j	опыт i	контроль j	опыт i	контроль j
4	1:600	1:320	3/6	0/6	3/6			
5	1:100	1:160			3/6	1/6	28/38	1/11

m Примечания: числитель — число выживших мышей, знаменатель — число животных в опыте.

Key: a- Serum; b- properties of the filtrate; c- antigenic; d- titer; e- agglutination reaction; f- complement fixation reaction; g- preventative; h- animals; i- experimental; j- control; k- neutralizing; l- immunogenic; m- Note: the numerator indicates the number of surviving mice, and the denominator represents the total number of animals in the experiment.

The preventative and neutralizing properties were expressed to the same extent as during the immunization with ether extracted antigens (see Tables 3 and 4).

In regard to the immunogenic activity of broth culture, it was somewhat less pronounced than in the case of antigens obtained by the ether method (see Table 5).

However, the possibility is not excluded that the production of antigen from the filtrate of the broth culture with higher immunogenic properties is possible. It may require a longer period of time for growing the culture in a synthetic liquid medium.

Conclusions

1. The use of the extraction method of the live whooping cough culture with ether enables the production of an antigen preparation which is nontoxic (Dlm is in excess of 5 mg).

2. The chemical composition of antigens, obtained by different methods, shows significant differences. The antigen from the microbe mass contained a higher amount of nitrogen (seven to ten percent) and less of the reducing substances not in the carbohydrate form. The antigens obtained from the supernant liquid, contain less nitrogen and significantly more reducing substances after the acid hydrolysis.

3. The study of the antigen activity of different whooping cough bacilli fractions has shown that their introduction into rabbits stimulates the formation of agglutinins and complement fixing antibodies. In addition, the serum of these animals possessed preventative and neutralizing properties with respect to the whooping cough microbe.

4. The antigens obtained by different methods, independently of the chemical compositi possess very pronounced immunogenic properties. Dur..g the intranasal injection of a 0.01 mg dose they protected 50 to 84.6 % of the mice from 1 Dcl infection with virulent culture. In the case of intracranial infection (strain 18323) with 164 LD_{50} of the virulent culture, 63 to 35.7 % of the mice were protected.

Bibliography.

[1] Bayeva, Ye. A. "Study of antigens obtained from Whooping Cough Microbes by the Buavena and Topli-Raystrik Method" ZhMEI, No.9, 64 to 69 (1958).

[2] Malysheva, L. A., Kholchev, N. V. and Shavrova, M. M. "Extractions of Whooping Cough Bacilli According to Buavena Method" Transactions of the Moscow State

Scientific Research Institute of Epidemology and Bacteriology," Moscow, No.5, 314 (1952).

[3] Palant, B. L., Fintiktikova, R. P. and Mitel'man, P. M. "The Effect of the Treatment Method and Structure of the Strain in the Nature of the Toxicity of Substances Obtained from Whooping Cough Bacilli" ZhMEI, No.9, 34 to 37 (1955).

[4] Palant, B. L. Fintiktikova, R. N. and Dobraya, T. Ye. "Immunization and Antigen Specificity of Toxic Substances of Different Strains of Whooping Cough Bacilli (Communication No. 15), Transactions of the Ukrainina Institute of Epidemology and Microbiology im. Mechnikov, Kharkov, Vol. 19, 36 to 41 (1952).

[5] Trushina-Tumanova, Ye. F. and Manayeva, Ye. A. "Study of the Whooping Cough Microbe Toxin" Communication No. 2, ZhMEI, No. 9, 38 to 40 (1955).

[6] Barger et Soar. Arch. Boum de pathol. exper, et microb. 17, No. 1, 119 to 123 (1958).

[7] Kawajama, Masui Asano and Matsui J. Immun. v. 76, No. 3, 175, (1956).

[8] Larson, G. L. Pub. Health Rep. v. 60, 839 (1954).

[9] Larson, G. L., Bell, Y. T. and Owen, G. R. J. Immun. v. 73, No. 4, 222 to 225 (1954).

[10] Pillemer, L. Burrele, Y. J. and Ross, O. A. Science, 106, 2741, 36 to 37 (1947).

[11] Pillemer, L. Lancet, 1954, I No. 25.

[12] Pennele, R. B. & Thiele, E. H. J. Immun. V. 66, No.6, 627 (1951).

[13] Robins, K. E. & Pillemer, L. Proc. Soc. Exp. Boil. Med. 74, I, 75 to 78 (1950).

[14] Smolens, I. and Flavell, E. H. Journ. Immunol. v. 57, No. 2, 173 (1947).

THE PRODUCTION OF SORBED WHOOPING COUGH AND WHOOPING COUGH DIPHTHERIA VACCINE AND THE STUDY OF THEIR PRINCIPAL PROPERTIES IN THE ANIMAL EXPERIMENTS AND IN A LIMITED EPIDEMOLCGICAL EXPERIMENT

(Report Number 2)

by

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In the work conducted in 1959, we utilized Larson's method for the isolation of antigen from the live whooping cough culture by extraction with an aqueous-ether mixture. Using this method we obtained thirty-one series of antigens.

The study of the principal properties of the majority of the obtained antigens in 1959 have shown that these antigens possess intensely manifested serological properties in the precipitation reactions and complement fixation reactions. The introduction of these antigens into rabbits stimulates the formation of agglutinins and antibodies (responsive to complement fixation) in high titers. The obtained serums have high preventative neutralizing properties and protect fifty percent of the mice against infection with one Dcl of the whooping cough culture. The minimum toxic dose is > 5 mg. It was established that the minimum immunization dose for the majority of the series of antigens was 0.01 mg, which protected 58.3 to 100 % of the mice from infection with one Dcl of the whooping cough culture.

Thus, in the 1959 work we established that the Larson's method may be used for the production of high quality antigens from whooping cough cultures, both in the antigen and in the immunogenic respect, and having no toxic properties.

The principal problem in this year's work was the production and study both, of monovaccine as well as of di-

and trivaccine in the association with diphtheria and tetanus antitoxins.

For this purpose we studied eight series of whooping cough antigens, obtained by the Larson's method. In this work we utilized seven strains of whooping cough bacilli (187, 222, 248, 305, 58001, 18323, and 63), which met all of the requirements for the first phase culture.*

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* These strains we obtained from the State Control Institute im. Tarasevich.

The study of the antigen composition of the obtained cultures with the monoreceptor serums L, S and O in the agglutination reaction showed different relationships in the antigen components in the investigated whooping cough cultures.

The conducted analysis of whooping cough cultures indicated the predominance of the labile antigen, significantly lower concentration of L-antigens and very low concentration of O-antigen. It should be noted that the concentration of O-antigen in the serums, obtained during the immunization of rabbits with autoclaved culture, is small. The maximum O-antibody titer reaches 1:800 to 1:1600 serum dilution.

All of the antigens were studied with respect to the chemical composition, serological properties, toxicity, antigen and immunogenic activity.

In the study of the immunogenic properties of antigens in the first stages of this work (1959) we checked the immunity of the animals by intranasal infection with different amounts of the live culture (in Dcl).

Considering the fact that recently intracranial infection with virulent culture has acquired greater acceptance, for the evaluation of the immunity of animals, strain 18323 was tested by this method with subsequent calculation of LD_{50}.

In subsequent experiments we used only this method.

In order to establish the relationship between the earlier obtained data in the intranasal infection method with the data obtained subsequently by the intracranial infection we conducted two series of experiments with earlier tested antigens (series 19 and series 20) by the new method. In this case, antigen series 20 was tested without the separation. The antigens obtained from the microbe mass and the supernant liquid were mixed, and injected into mice in the same dose (0.01 mg). In this experiment series 19 antigen was obtained only from the microbe mass.

Experiments with series 19 and 20 antigens, indicate that utilizing this method of infection 85.7 and 63 % of the animals, respectively, were protected against 164 LD_{50} of the virulent bacilli.

Table 1 shows the immunochemical characteristics of antigens, extracted from the whooping cough bacilli, by the Larson's method. Antigens of series 22, 23, 24, 25, 26, 27, 28, 29, 30 and 31, extracted from the culture as a whole, following acid hydrolysis contained a large amount of the reducing substance, reaching 33.6 %. The concentration of nitrogen in this series varied over a significant range (3.5 to 9.2 %). The exception was series 19, which was extracted from one of the microbe masses without the culture liquids. After acid hydrolysis it total nitrogen content was 9.9 %, and the reducing substances comprised of only 8.3 %.

In the precipitation reaction and complement fixation reaction, the antigen composition of ether preparations was tested by means of L-, S- and O- monoreceptor serums. The study has shown that our preparations contain all of the microbe cell antigens in different ratios. In evaluation of the toxicity of these preparations, it was found that the toxic dose of certain antigens was in excess of 10 mg. while for the majority it was in excess of 5 mg.

Immunochemical characteristics of antigens extracted from the whooping cough bacilli by the Larson's method.

a Серия	b Выход (в %)	c Химические показатели (в %) d зола	e азот	f редуцирующ. вещ.	g Серологические реакции с антисыворотками L h преципитации	L i связывания комплемента	S h преципитации	S i связывания комплемента	O h преципитации	O i связывания комплемента	LSO h преципитации	LSO i связывания комплемента	j Токсические свойства (в мг)	k Иммуногенные свойства l иммунизирующая доза (в мг)	m LD_{50} зараженных мышей	n выживаемость мышей (в %)
19		20,4	9,9	8,3	1:8000	1:61000	1:8000	--	1:16000	—	1:8000	1:8000	5	0,01	164	85,7
19		—	—	—	—	—	—	--	—	--	—	--	—	0,04	250	40,9
20		28,07	7,80	6,8							1:64000	1:8000		0,01 (микробная масса)	164	63
22		26,5	7,69	17,1	1:4000	1:4000	1:4000	1:64000	—	1:128000	1:128000	1:61000	5	0,04	250	61,9
27		19,1	4,2	30,6	1:64000	1:16000	1:4000	1:8000	—	1:64000	1:128000	1:64000	10	0,04	250	62,5
28		24,5	4,6	25,3	1:32000	1:2000	1:8000	1:8000		1:16000			5	0,04	250	62,5
29		15,3	3,1	21,6	1:61000	1:4000		1:61000		1:32000		1:64000	5	0,01	250	50
30		21,7	4,7	33,6	1:128000	—	1:2000	—	—	—	1:128000		10	0,01 0,02	238 137	37,5 75*
31		16,1	9,2	26,3	1:128000 1:61000	—	1:512000	—	—	—	1:128000		5	0,01	238 137	10 77*

Key: a- series; b- yield (percent); c- chemical indices (in percent) d- ash; e- nitrogen; f- reducing substances; g- serological h- precipitation; i- complement fixation; j- toxic properties; k- immunogenic properties; l- immunization dose (in mg); m- LD_{50} of the infective dose; n- survival of mice (in percent); o- (microbe mass).

Note: Immunity was tested at a later date (after 27 days).

The mice were immunized with a single injection of a 0.04 mg dose of antigen. The immunity was tested after twenty-one days using 238 to 250 LD_{50} of whooping cough culture. In four out of seven series, the immunity to 250 LD_{50} dose injection of the live bacilli, was developed by 50 to 62.4 % of the animals.

Antigen series 19 protected only 41.9 % of the animals. Antigen series 30 and 31 protected 10 to 37.5 % of the animals against 238 LD_{50} injection. This dose of antigen, however, protects 75 to 77 % of the mice against 137 LD_{50} dose of live bacteria, when the immunity was checked after 27 days, i.e., at a much later testing date. Apparently, later tests of the immunity revealed the immunogenic characteristics of the antigen better. The results of these experiments have shown that 0.01 to 0.04 mg doses of ether antigens, produce stable immunity in 50 to 85.7 % of the animals against infection with 137 to 250 LD_{50} of the whooping cough culture.

Having established the immunogenic quality of the ether antigen, we passed on to the development of associated preparations on their basis. We conducted the preliminary studies of the effects of sorption and association with diphtheria and tetanus antitoxins on the immunogenic properties of whooping vaccine (Table 2).

In three out of five cases the sorption of whooping cough antigen on the gel decreased the percentage of the survival of the animals, while in two cases, the immunogenic activity of the antigen increased. The lowering of the survival may be explained by the retarted desorption of the antigen. In the immunity tests conducted on the same dates as the tests with nonsorbed antigen, lowering of the antigen stimulation resulted. In those cases when sorption increased the percentage of survival of the animals, one may presume that the dose taken for immunization was sufficiently high; therefore, incomplete liberation of the antigen during the given period of time has a favorbale

effect on the manifestation of the immunogenic activity. In this case the addition of diphtheria and toxin does not have any unfavorable effects on the experimental results.

Table 2.

The effect of sorption and association on the immunogenic properties of whooping cough vaccine.

a Серия	b Состав вакцины				Иммуногенные i свойства	
	доза коклюшного антигена (в мг) c	доза дифтерийного анатоксина d (Lf)	доза столбнячного анатоксина e (EC)	доза геля f (в мг)	заражающая доза g (в LD_{50})	выживаемость мышей (в %) h
19	0,04	—	—	—	250	40,9
19	0,04	—	—	—	250	60
19	0,04	30	—	1,1	250	30
22	0,04	—	—	—	250	61,9
22	0,04	—	—	1,1	250	38,1
27	0,04	—	—	—	250	62,5
27	0,04	—	—	1,1	250	45,4
27	0,04	30	—	1,1	250	42,8
27	0,1	—	—	1,1	140	71,4
27	0,25	30	—	1,1	250	66,6
30	0,04	—	—	—	238	37,5
30	0,04	—	—	1,1	238	27,2
30	0,04	10	—	1,1	238	37,5
30	0.25	35	100	2,2	137	45,5
	0,04	—	—	—	238	10
31	0,04	—	—	1,1	238	37,5
31	0,04	10	—	1,1	238	42,8
31	0.25	35	100	2,2	137	33

Key: a- Series; b- the composition of vaccine; c- doses of whooping cough antigen (in mg); d- dose of diphtheria anatoxin (L_f); e- dose of tetanus anatoxin (EC); f- dose of gel (in mg); g- infection dose (in LD_{50}); h- survival of mice (in %); i- immunogenic properties.

Increase of the antigen dose to 0.25 mg in association with diphtheria anatoxin in the sorbed preparation insured the protection of mice against 250 LD_{50} to the extent of 66.6 % (series 27).

The whooping cough vaccine series 30 and 31 in 0.25 mg doses in the association with diphtheria and tetanus antitoxins in the sorbed preparation did not protect the animals from 137 LD_{50} (46.6 to 33 %).

The presented data indicate the necessity for thorough titration of the whooping cough component to the required amounts of the diphtheria and tetanus anatoxins in the associated sorption of the preparation.

Finally, we attempted to explain the effect of a number of injections and the location of the introduction of the vaccine on the immunogenic properties. This question was studied with one series of whooping cough vaccine (series 27) in the association with diphtheria anatoxin in the sorbed preparation.

Five variations of the immunization of mice were investigated. In two cases double immunization was used with one week interval between the injections of the sorbed whooping cough vaccine, and subsequent injection of the sorbed whooping cough diphtheria vaccine. In two injections the mice received 0.08 mg of the whooping cough vaccine and 30 Lf of the diphtheria anatoxin. Fifty percent of the mice survived the injection of 250 LD_{50}. In the second variation the dose of diphtheria anatoxin was lowered to 10 Lf. This lowering of the diphtheria component did not have any effect on the survival of the mice during the injection of 250 LD_{50} of the whooping cough culture, and again fifty percent of the mice survived the tests. In the third and fourth variations the sorbed whooping cough vaccine, and the sorbed diphtheria anatoxins were injected into mice simultaneously in different parts of the body. In the third variation 0.04 mg. of the sorbed whooping cough

vaccine was injected and 30 Lf of the diphtheria anatoxin was used. In this experiment the highest immunological effect was obtained. 71 % of the mice infected with 250 LD_{50} survived. In the first variation the dose of the diphtheria toxin was lowered to 10 Lf. Lowering of the diphtheria anatoxin dose , lowers significantly the percent of the survival of the animals (42.9 %). This fact indicates the disruption of the equilibrium of the antigen stimulation. In the last experiments,, a single application of the sorbed whooping cough diphtheria vaccine was tested. The dose of the whooping cough component was 0.25 mg while the dose of the diphtheria anatoxin in the preparation was 30 Lf.

In these tests 66.6 % of the mice survived the infection with 250 LD_{50} of the whooping cough bacilli (Table 3).

Thus, it was established that production of the associated sorbed whooping cough diphtheria and whooping cough diphtheria tetanus vaccine must be thoroughly titrated for the dose of the whooping cough component in the combination with diphtheria and tetanus anatoxins.

Consequently, for the final solution of the problem regarding the association of sorbed whooping cough vaccine it is necessary to titrate the dose of the whooping cough component in combination with diphtheria and tetanus anatoxins and to establish in the comparative study the effectiveness of a single and double vaccination as well as the effect of the duration of the immunity tests on the effectiveness of the vaccine.

In the course of the study of these aspects of the problem the most rational dose of the whooping cough component will be established in the sorbed mono-, di- and tri-vaccine for use in a single and in a double immunization proceedure.

The analysis of the experimental data indicate that the use of the water-ether extracts of the whooping cough culture according to the Larson's method will enable the production of antigen in which the microbe cell components

important for the development of the immunity are preserved. Antigens extracted from the culture as a whole contain large amounts of the reducing substances reaching up to 33.6 %. The concentration of nitrogen in these series varied over a broad range (3.5 to 9.2 %).

The obtained preparations were nontoxic -- the Dlm of antigen was in excess of 5 mg. Certain antigens had a Dlm in excess of 10 mg.

The study of the antigen composition of the preparation with monoreceptor serums L, S and O have shown that the antigen contains all of the components of the microbe cell in different ratios.

The minimum immunization dose of antigen is 0.01 mg. Upon the injection of 0.01 to 0.04 mg of antigen the immunized mice survived to the extent of 50 to 85.7 % when infected with 137 to 250 LD_{50} of the whooping cough bacilli.

To avoid lowering of the immunogenic activity of small doses of the sorbed whooping cough vaccine it is necessary to conduct tests of the immunity during a later date after immunization, than in tests of nonsorbed preparations (i.e. not earlier than a month following the immunization).

The doses of the whooping cough component must be thoroughly titrated when it is used in combination with other components in the associated sorbed preparations.

Conclusions

1. The Larson's method can be successfully used for the preparation of whooping cough antigen. The preparation is nontoxic and it possesses sharply manifested immunogenic properties.

2. The study of the antigen component of the preparation with monoreceptor serums L, S and O in the precipitation complement fixation reaction has shown the presence of all three components in different ratios.

3. For the development of the associated whooping cough-diphtheria-tetanus vaccine it is necessary to titrate the whooping component dose in combination with the diphtheria and tetanus anatoxins, to compare the immunogenic effectiveness during single and double immunizations with the associated sorbed vaccine and to establish the optimum dates for testing the immunity.

Table 3.

Effect of the number of injections and the location of injections on the immunogenic properties of the vaccine (experiments were conducted with white mice).

a Состав вакцины				b Защита (в %)
c серия	d доза в (мг)	дифтерийный анатоксин (в Lf) e	гель (в %) f	внутричерепное заражение 250 LD_{50} g
27	0,04 h через неделю 0,04	— 30	0,22 0,22	50
27	0,04 h через неделю 0,04	— 10	0,22 0,22	50
27	0,04 —	— 30	0,22 0,22	i Одновременно в разные места — 71
27	0,04 —	— 10	0,22 0,22	i Одновременно в разные места — 42,9
27	0,25	30	0,22	66,6

Key: a- Composition of vaccine; b- protection (in %); c- series; d- dose in mg.; e- diphtheria and anatoxin (in Lf); f- gel (in %); g- intracranial infection with 250 LD_{50}; h- after a week; i- simultaneously in different places.

PRODUCTION AND STUDY OF THE ACTIVE FRACTIONS OF THE DIPHTHERIA ANATOXIN

by

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In recent years highly successful results were obtained in the production of high quality diphtheria toxins and anatoxins.

For a long time the diphtheria anatoxins with 2000 Lf/mg of the total weight of nitrogen wer considered to be the highest (Iton, 1936; Pillemer, 1947, 1948, and others).

In recent years reports occurred in literature on the preparation of anatoxins with purification indices reaching up to 3200 Lf/mg of the total nitrogen (Holt, 1951; Pope, 1951; Bowen, 1952; Pope, 1953; Khabas, 1957 and others.)

As it is well known the production of a highly effective preparation entails the use of a complex single stage purification method.

It should be noted that significant losses of the specific antigen are encountered in the process even when the most "merciful" purification method and standard nutrient media are used.

When different purification methods are used the percentage yield of the active units varies even within a single starting series of the diphtheria anatoxin. This is apparently explained by the fact that different precipitants cause disaggregation or cause other structural changes in the protein which has an effect on the yield of active substances (Table 1).

Acccrding to the presented arguments one must assume that the starting anatoxin contains several specific antigens, which differ in their chemical composition and physico-

chemical properties, or there exists one specific antigen which forms strong compounds with different proteins.

Table 1.

Characteristics of purified anatoxins, obtained by different methods.

a Метод осаждения	Характеристика нативного анатоксина (i)				o Выход (%)	h Характеристика очищенного анатоксина			
	j Серия	l Lf/мл	m продол. флокуляции (в мин.)	n общий азот (мг %)		m продол. флокуляции (в мин.)	n общий азот (мг %)	l Lf/мл	p К-во Lf/мг общего азота
b По Холту	120	52	5	154	18	428	20	24,3	1750
b По Холту	120	52	5	154	26	208	30	27,6	730
46-процентной фосфатной смесью	Смесь k 2	52	5	154	12	216	45	27,8	770
c 50-процентной фосфатной смесью	Смесь k 2	52	5	154	19	363	40	29,1	1200
c 62-процентной фосфатной смесью	Смесь 2	52	5	154	19	400	45	27	1480
d Насыщенным раствором серно-кислого аммония	44	42	40	176	16,6	474	15	25,2	1480
e Сухой солью серно-кислого аммония	44	42	40	176	24	372	25	30,6	1226
f Соляной кислотой	5a	46	35	154	47	72	20	7,7	935
g Трихлоруксусной кислотой	Смесь 3	51	20	134	68	285	20	14,2	1995

Key: a- Precipitation method; b- according to Holt; c- 46 % phosphate mixture; d- saturated solution of ammonium sulfate; e- with dry ammonium sulfate; f- sulfuric acid; g- trichloroacetic acid; h- characteristics of purified anatoxin; i- characteristics of native anatoxin; j- series; k- mixture; l- Lf/ml; m- duration of flocculation (in min); n- total nitrogen (mg %); o- yield (%0; p- amount of Lf/mg of the total nitrogen.

The purified anatoxin, concentrated by a factor of five or more, has 730 to 2000 Lf/mg indices for the total nitrogen with the variation of the yield of active units, in the proximity of 12 to 68 %. The Lf/mg indices for the total nitrogen above 2000 were not obtained using a variety of methods for their isolation.

The structure of the obtained purified concentrated diphtheria anatoxins were investigated by paper electrophoresis and the diffusion precipitation method in agar according to the Chistovich modification of the Yuden method. The zone electrophoresis method recently acquired importance not only for analytical, but also for preparative purposes.

The work presents the results obtained in the study of highly active diphtheria anatoxins and active fractions which were isolated from them.

The problem of the fractionation of diphtheria anatoxin has not been sufficiently described in literature. In our country the first investigators who were concerned with these problems were Krestovnikova and Ryakhina (1937), Khalyapina (1937, 1954), Chistovich (1955) and Khabas (1957). Abroad the investigators delving into this area are Pope (1951), Bowen (1952), Pulik (1952, 1953, 1958) and others.

The electrophoretic studies of diphtheria anatoxins were conducted in our laboratory. It was established that in order to achieve the successful separation of protein fractions the anatoxin must be preconcentrated in cellophane sacks by means of ventilation or by means of lyophilic drying. For electrophoretic separation a chamber with horizontal placement of strips was used. The strips were preliminarily saturated with a buffer solution by siphoning or simply by dipping the paper strips into the buffer and drying them slightly between large sheets of filter paper. The tested material was placed by means of a Pasteur pipet on the cathode end. In the whole series of preliminary

experiments the suitability of various buffer solutions for the analysis of anatoxins was investigated. In choosing a buffer solution, as it is well known, it is desirable to find such a solution in which more complete separation of the protein mixture and its preservation in an unaltered state takes place (without altering the proteins themselves.)

We tested the following buffers: veronal-medinal buffer, pH = 8.5 = 8.6 and ionic strength 0.1; Michaelis buffer, pH = 8.5 = 8.6 and ionic strength 0.05 - 0.1; phosphate-citrate buffer of pH = 5.2 - 5.4 and ionic strength 0.1 - 0.2; acetate buffer of pH = 5.2 - 5.8 and ionic strength 0.1 - 0.2.

Satisfactory results were obtained with acetate buffer of pH = 5.2 - 5.4 and ionic strength of 0.1 - 0.2. The electrophoresis were conducted for four to five hours.

The following factors play an important role in the separation of the components of the protein mixture: electric field strength, current intensity, temperature and duration of the electrophoresis.

In practice, however, the duration of electrophoretic separation of the proteins on paper, with all other things being equal, depends primarily on the current intensity. In our separation experiments we applied 410 to 530 volt potential.

Paper strips were stained with various dyes: acid blue-black, acid chrome blue, aniline black, brom-phenol blue.

It should be noted that the best staining of the anatoxin fraction was obtained with 0.2 % blue-black dye in ten percent acetic acid (Figures 1, 2, 3).

The electrophoretic separation of diphtheria anatoxins and toxins is described in literature only in isolated cases, primarily in relation to the problem of fractionation of only highly purified diphtheria anatoxins. The work of

Bowen (1952) has shown that anatoxin, purified by the Holt method, consists of proteins with the mobility which corresponds to the mobility of γ = and β_2 globulins. Pulik (1952, 1953) having access to purified anatoxin with Lf/mg of the total nitrogen equal to 2,400 obtained on the filter paper two to three bands in Michaelis buffer and veronal buffer at pH = 8.55. However, the nature of the dispersion of anatoxin proteins was similar to the dispersion of proteins which we obtained. Kabas (1957), in the separation of anatoxin with 2200 to 2800 Lf/mg activity of the total nitrogen in a starch block obtained two fractions.

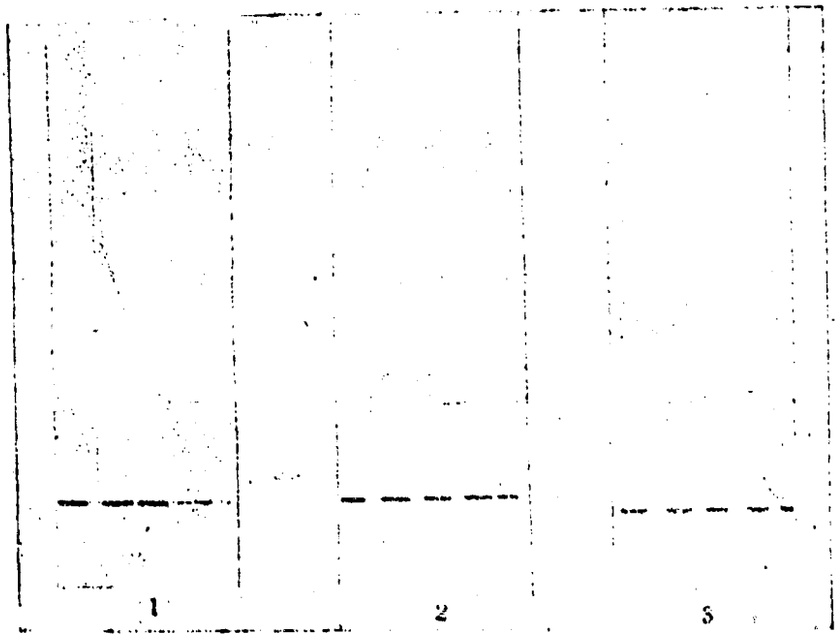
From this one can conclude that diphtheria anatoxins, obtained in different laboratories, have different total nitrogen indices, but the fractionation structure of the anatoxin is practically the same. The purified anatoxin consisted of two to three fractions. In conjunction with the phoretic separation we also conducted a study of the antigen structure of diphtheria anatoxins and obtained from it the fractions by the diffusion precipitation method in agar according to the Chistovich modification of the Yuden method. Several experiments were conducted in order to obtain a general understanding of the dynamics of the occurrence of bands and their nature. Bands were obtained at room temperature. The first bands were always wider, less dense and more pronounced than the bands formed later.

Subsequently, experiments were conducted in order to determine the concentration threshold of the formation of the band (Figure 4). The concentrated purified anatoxin was placed in layers on the agar in decreasing amounts (100, 60, 30, 15, 5, 3, 1 Lf/ml). The diphtheria flocculating serum was taken in all cases in the amount equal to 100 AE/ml. The start of the formation of precipitation rings was noted by the 28th to 30th hour, while after 7 to 12 days, when the formation [original text pp. 134 to 135 not furnished].

3. Electrophoretic separation of anatoxins of filter paper indicates molecular nonuniformity of the purified diphtheria antigens. The purified anatoxins have two to three fractions, while the native anatoxins contain one fraction.

4. The study of the native and the purified anatoxins, and also of the individual fractions, isolated from the anatoxin by the precipitation diffusion in agar method, has shown the nonuniformity of their composition.

5. The purified diphtheria anatoxin in the Phywe type ultracentrifuge were found to be nonuniform in composition (we observed two peaks).



Figures 1 to 3.

STUDY OF THE PROPERTIES OF COMPLETE ANTIGENS, OBTAINED FROM THERMOLABILE ANTIGEN CONTAINING FLEXNER'S TYPE c AND f DYSENTERY MICROBES

(Communication II)

by

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In our 1959 investigation of the thermolabile antigens of Flexner's type c and f dysentery microbes we have shown that certain cultures contain another antigen which is relatively thermolabile in addition to somatic thermostable antigens. The cultures, containing the above antigen, display strong immunogenic properties in the experiments with mice, in comparison with cultures without thermolabile antigens, which proved the significance of this antigen in the development of immunity.

The purpose of this work was to study the stability of thermolabile antigens in the course of the production of complete antigens from the selected dysentery cultures, and also to study their properties. First of all, we found it necessary to study the stability of the thermolabile antigens in microbes grown in a reactor with aeration and during boiling. For this work we selected five dysentery cultures: three Flexner's type f cultures, and two Flecner's type c cultures. One of the latter two cultures was the production strain 144 which was included in the experiment as a control.

Characteristics of the Culture

All cultures were biochemically typical and in the S-form, displayed strong typifying properties. They all contained 1:1600 titers of the studied, relatively thermolabile antigens

with the exception of Flexner's type f culture (strain number 37). The titer of the thermolabile antigen in this culture was equal to 1:3200. In the study of the toxicity of cultures, mice were given intravenous injections of the vaccine heated in the course of one hour at 60°C. Each culture was checked in three doses: one, two, and four billion microbes. Five mice were injected with each of the above doses.

In these experiments it was found that the studied cultures were of low toxicity. During the study of virulence each culture was titrated in three doses: 50, 100 and 200 billion microbes - five mice for each dose. The indicated amounts of microbes in 0.2 ml volumes with the addition of 0.8 ml of 0.4% of the imported agar were injected intraperitoneally into the mice. Thus, each mouse received one billion microbes of the agar suspended culture intraperitoneally.

The results of the experiments have shown that Flexner's type f strain 66 culture was the most virulent. One Dcl of this culture under our experimental conditions was equal to 50 million microbes, while 1 Dcl of Flexner's type f strain number 37 culture and Flexner's type c strain 580 culture was equal to 200 million microbes. Flexner's type f strain 490 culture was the least virulent in comparison with other cultures. The antigen properties of the cultures were checked on rabbits, which were immunized first with formaldehyde treated vaccine, and subsequently with live culture. The agglutination reaction titers with live cultures were equal to 1:6400 to 1:12800.

In four cases the antibody titer to the relatively thermolabile antigen, was equal to 1:1600 and with one Flexner's type f culture strain number 37 the titer was more than 1:3200, for the serums obtained by adsorption of the autoclaved cultures, i.e., the cultures containing only the somatic antigens.

Subsequently, we studied the immunogenic properties of

the cultures on mice. To preserve the thermolabile antigen, mice were immunized with formaldehyde treated vaccine. Thirty mice were used for each culture.

Each mouse was injected subcutaneously with two doses of 250 million microbes of the formaldehyde vaccine in 0.5 ml volume with a seven day interval between the two injections. On the tenth day the resolving dose of the culture was introduced intraperitoneally. The mice were infected with one and two Dcl (fifteen or ten mice per dose).

The results of the experiments indicate high immunogenic properties of the formaldehyde treated vaccine (Table 1).

Table 1.

Immunogenic Properties of Flexner's Type c and f Dysentery Culture.

Культура Флекснера (a)	d Разрешающая доза (в *Dcl*)	
	1	2
	e выживаемость мышей (в %)	
b Типа *c* штамм 580	95	—
b Типа *c* штамм 144	96.7	—
b Типа *c* штамм 490	80	40
c Типа *f* штамм 66	60	50
c Типа *f* штамм 37	70	20

Key: a- Flexner's culture; b- type c strain; c- type f strain; d- resolving dose (in Dcl); e- survival of mice (in %).

From the above dysentery cultures, complete antigens were produced by the hypogeal industrial culture method (in a reactor with aeration).

In the process of the preparation of complete antigen (during the growth of the culture) samples were taken from the reactor periodically in order to study the intensity

of the culture growth and the stability of the thermolabile antigen titers. The intensity of the growth of the dysentery culture was determined from the increase of the bacteria count, by taking samples every two hours beginning with the fourth hour after the beginning of the experiment, and continuing for the next sixteen hours (Table 2).

Table 2.

Intensity of Growth of Dysentery Culture in the Reactor with Aeration.

Высевки по часам роста a	b Количество культуры (в мард. микробных тел)				
	c Флекснера типа *f*			d Флекснера типа *c*	
	e Штаммы				
	49C	66	37	580	144
4	11	9	12	9	19
6	13	23	20	19	25
8	24	30	25	26	30
10	27	35	35	29	35
12	33	42	41	34	39
14	42	52	41	36	39
16	44	55	—	39	39

Key: a- Sampling according to the hours of growth;
b- Amount of culture (in billion microbes);
c- Flexner's type f; d- Flexner's type c;
e- Strain number.

The microbe count determined in samples of broth culture by hours, increased rapidly and reached 41 and 55 billion/ml for Flexner's type f culture and 39 billion/ml for Flexner's type c culture by the sixteenth hour of growth. It should be noted that certain cultures, (for example, Flexner's type

c strain number 144 and Flexner's type f strain number 37; completed their growth by the twelfth hour, since the microbe count by the sixteenth hour remained the same.

It was of great interest to observe the changes of the thermolabile antigen titer during the growth of the culture in the reactor with aeration. For this purpose the broth culture, sampled from the reactor every two hours during the growth, was used in an open agglutination reaction with the adsorbed Flexner's type c and f serums, i.e., with serums void of antibodies to somatic antigen (Table 3).

Table 3.

Titer of Thermolabile Antigen of Dysentery Cultures During the Growth in the Reactor with Aeration

a	e Культура			
	c Флекснера типа f		d Флекснера типа c	
	f Штамм			
Выемки по часам роста	490	37	580	144
	g Титр			
b Исходный материал	1:1300	1:3200	1:1600	1:1600
4	1:1600	1:1600	1:1600	1:1600
6	1:1600	1:1600	1:1600	1:800
8	1:400	1:1600	1:1600	1:800
10	1:400	1:800	1:800	1:800
12	1:200	1:800	1:400	1:800
14	1:400	1:800	1:400	1:800
16	1:400	—	1:400	1:800

Key: a- Sampling by hours of growth; b- Starting material; c- Flexner's type f; d- Flexner's type c; e- Culture; f- strain; g- titer.

In growing Flexner's type c and f cultures in the reactor with aeration, the thermolabile antigen titer after eight to ten hours of growth gradually decreased in comparison with the starting titer. By the end of the growth of the culture (i.e., in sixteen hours, as it is normally accepted in production practice) the titer of the investigated cultures decreased by a factor of four as compared with the titer of the starting culture, grown on the slanted agar. In the production culture of Flexner's type c strain, number 144, the titer decreased by a factor of two after six hours of growth and subsequently the thermolabile antigen titer remained unchanged.

The obtained results are important, as it was found that prolonged growth of dysentery culture with aeration leads to the lowering of the thermolabile antigen concentration. This is also applicable to production strain 144 containing this antigen. It is, therefore, necessary to find conditions under which the relatively thermolabile antigen would be preserved, specifically the use of a shorter culture growth time.

Subsequently, we studied the following stages in the production of complete antigens - tryptic boiling of the dysentery culture. The rate of boiling of the culture was determined by the microbe count after four and forty-eight hours of growth.

The results of the experiment have shown that the digestion of Flexner's type f cultures occurred very slowly in comparison with the digestion of Flexner's type c culture. It can also be noted that the digestion of Flexner's type f cultures in the period starting with the fourth hour, through the forty-eighth hour increased very little.

Characteristics of Complete Antigens

One of the basic questions is: does a relatively thermolabile antigen exist in the experimental series of complete antigens which we obtained, and does it exist in the production series obtained on the basis of Flexner's type c culture strain 144, also containing, according to our data the thermolabile antigen?

It was found impossible to determine the presence of thermolabile antigens in complete antigens in the direct experiment with the precipitation reaction using adsorbed serums and complete antigens, since the serum, diluted after adsorption in 1:50 ratio, did not produce a ring during stratification.

We were also unable to detect the presence of thermolabile antigens in the complete antigen in the second experiment involving the precipitation reaction with complete antigen, both native as well as autoclaved (in which thermolabile antigen was destroyed).

The precipitation reaction titer with the autoclaved complete antigens remained almost the same as with native complete antigen in nearly all of the cases.

A third experiment was conducted using the agglutination reaction with rabbit serums, immunized with complete antigen, adsorbed by the homolytic autoclaved culture, i.e., with serums which did not contain antibodies to somatic thermolabile antigen. The agglutination reaction was conducted with the live cultures. The existence of the positive agglutination reaction would indicate the presence of antibodies to thermolabile antigens in the serum and consequently, the presence of thermolabile antigen in complete antigen, used for the immunization of rabbits during the production of these serums (Table 4).

The serums of rabbits, immunized with complete antigen, adsorbed by autoclaved homolytic cultures, give a positive

agglutination reaction with live cultures at the 1:400 and 1:800 dilution of Flexner's type f serum. With similar exporimental Flexner's type c serum, the agglutination titer was significantly lower (1:200).

Table 4.

Antibody Titer to Thermolabile Antigen in the Agglutination Reaction with Adsorbed Rabbit Serums, Immunized with Complete Antigens.

a Сыворотка к полному антигену Флекснера	e Живая культура				
	f Флекснера типа f			g Флекснера типа c	
	h Штамм				
	37	66	490	580	144
	i Титр				
b Типа f штамм 37	1:800	1:400	1:400	—	—
b Типа f штамм 66	1:200	1:400	1:200	—	—
b Типа f штамм 490	1:800	1:800	1:800	—	—
c Типа c штамм 580	—	—	—	1:200	1:200
c Типа c штамм 144	—	—	—	1:200	1:200
d Производственному типа c сер. 326 штамм 144	—	—	—	1:800	1:800
Производственному типа c сер. 502 штамм 144	—	—	—	1:800	1:800
Производственному типа c сер. 633 штамм 144	—	—	—	1:200	1:200

Key: a- Serum to complete Flexner's antigen;
b- type f strain 37; c- type c strain 580;
d- production type c series 326 strain 144;
e- live culture; f- Flexner's type f;
g- Flexner's type c; h- strain; i- titer.

Of the three rabbit serums immunized with industrially produced antigens of Flexner's type c culture strain number 144, in two cases antibody titer for the thermolabile antigen was equal to 1:800 and in one case it was equal to 1:200.

The results obtained in this experiment convince us that thermolabile antigen is preserved in complete antigens, but it is there in very small amounts.

We thus observed the change of the thermolabile antigen titer throughout the whole process of the preparation of complete antigen. On the basis of the above investigation, one may conclude that the principal loss of the thermolabile antigen occurs in the course of the growth of the culture in the reactor with aeration. However, there are apparently some factors which affect the lowering of the thermolabile antigen titer in further preparation processes of complete antigens, as indicated by the low thermolabile antigen titers in the complete antigens which we obtained.

We evaluated the quality of the obtained complete antigen on the basis of complete antigen precipitation reaction titer with hyperimmune serums of rabbits immunized with live culture. For a control we conducted experiments with the industrial Flexner's type c complete antigen, obtained at the end of 1956 and at the beginning of 1957.

The positive precipitation reaction with complete antigen, prepared from the investigated Flexner's type f culture, was found in 1:128000 to 1:256000 titers. In complete antigens from Flexner's type c culture positive precipitation reaction was noted in titers equal to 1:64000 to 1:128000. The titers of five production control complete antigens, prepared from Flexner's type c culture strain 144 were analogous.

Toxicity of Complete Antigens

Each complete antigen in 0.5, 1.2 and 4 mg. amounts was introduced intraperitoneally into mice in 0.5ml volume. Five animals were used for each dose. The mice wer observed for three days. It was found that complete antigens, obtained from Flexner's type f culture, are much less toxic in comparison with complete antigens of Flexner's type c culture,

with the exception of the Flexner's type f culture strain number 66 antigen.

The toxicity of the investigated complete antigens is shown by the eighty to hundred percent death of mice upon injection of 1 mg of complete antigen. The lower dose (0.5 mg) did not cause 100% death of the mice. Thus, the toxicity of the investigated complete antigen differs little from the toxicity of the control antigens produced industrially.

In order to determine the antigen properties of complete antigen, obtained from the selected cultures, the rabbits were immunized and their serum was studied in serological agglutination and precipitation reactions.

The positive precipitation reaction was noted at complete antigen dilutions equal to 1:64000 to 1:256000.

In the agglutination reaction, conducted with the same serums and homologous cultures, the positive reaction was noted at 1:3200 and 1:6400 dilutions of the serum. Only with one serum of rabbits immunized with complete antigen of Flexner's type c culture strain number 580, the positive reaction was noted at 1:1600 dilution.

On the basis of the serological study of rabbit serums, immunized with complete antigen, obtained from the cultures containing relatively thermolabile antigen, one may conclude that the above complete antigens have antigen properties and do not differ from the industrially produced complete antigens.

The principal evaluation of the quality of the complete antigens in industry is conducted on the basis of their immunogenic indices. The immunogenic indices of the experimental complete antigen were checked on mice, also according to the instructions established for the approbation of the industrially produced series.

The immunizing dose of complete antigen, equal to 010625 mg. was singly injected into mice subcutaneously with depot in 0.5 ml volume. On the tenth day the dose of preliminarily titrated culture in 0.2 ml with 0.3 ml of 0.4% import agar was introduced intraperitoneally. Generally thirty mice

were immunized with the antigen. The infection was made in three doses, ½, one and two Dcl - ten mice for each dose (Table 5).

Table 5.

Immunogenic Properties of Complete Antigen of Flexner's Dysentery Culture

Infection dose (in Dcl)	Type of culture				
	f			c	
	Strain				
	490	66	37	580	144
	Survival of mice (in %)				
1	90	60	40	73.3	76.6
2	30	40	10	-	-

During the immunization of mice with complete antigen of Flexner's type f strain number 66 and 490 cultures sixty and ninety percent survival of mice was observed, while for complete antigen strain number 37 only forty percent survival was observed when the infection dose was equal to one Dcl.

During the immunization of mice with complete antigen of Flexner's type c culture and the infectious dose of one Dcl the survival of mice was equal to 73.3 and 76.6%. On the basis of these data one may conclude that complete antigens, obtained from the selected cultures, containing relatively thermolabile antigen, contain sufficiently high immunogenic properties.

We have shown that in obtaining complete antigens from the selected cultures, the relatively thermolabile antigen which they contained is lost to a significant extent in the course of culture growth in the reactor with aeration. The thermolabile antigen in combination with somatic antigen, in combination with somatic antigen plays a significant role in the formation of immunity.

We were unable to determine what factor affects the lowering of the thermolabile antigen titer during the growth of culture by the reactor method. It was found however, that with increase of the microbe mass the thermolabile antigen titer slowly decreases. This lowering for different cultures is noted primarily after an eight to twelve hour growth. It was noted at the same time that with two Flexner's type c cultures strain number 144, and Flexner's type f culture strain number 37, the microbe count ceased to increase after a twelve hour culture growth. With other cultures, the microbe count increases very insignificantly after twelve and fourteen hours.

On the basis of this,one instantly thinks of the possibility of shortening the growth period for dysentery cultures in the reactor in order to minimize the decrease of the thermolabile antigen titer. It is apparently also necessary to find conditions for increasing the rate of digestion of the microbe mass of Flexner's type f culture and to terminate the digestion at the time when the antigen yield is the highest.

The results, obtained in the determination of the toxicity antigenic properties and immunogenic properties of four complete antigens show that three antigens (Flexner's type f culture strain number 490, and 66 and Flexner's type c culture strain number 580) differ very little from complete antigens. of the industrially produced antigen from Flexner's type c culture strain number 144. This verifies the possibility of passing these cultures into production for the final

evaluation of their properties under industrial conditions.

Conclusions

1. In selecting Flexner's type c and f dysentery cultures, it was shown that cultures containing the thermolabile antigen, display high immunogenic properties.

2. In the preparation of complete antigens from Flexner's type c and f dysentery cultures, containing thermolabile antigen significant lowering of its titer (by a factor of four) was noted after sixteen hours in the course of the growth of the culture with aeration. The complete antigen, produced from dysentery culture, containing thermolabile antigen, contained it in low titers.

3. Complete antigens, produced from select cultures, still contain sufficiently high antigen and immunogenic properties, not differing from the properties of complete antigens obtained under the industrial production conditions from Flexner's type f and c cultures.

4. In the process of the production of these antigens from Flexner's type f and c dysentery cultures it is necessary to find conditions which would insure the preservation of the thermolabile antigen, which in conjunction with the somatic antigen plays an important role in immunogenesis.

Bibliography

[1] Ignatova, M. I. "Study of the Thermolabile Antigen and Its Immunogenic Properties in Flexner's Type c and f Dysentery Microbe" ZhMEI, No. 9, 85 to 89 (1959).

PROPERTIES OF COMPLETE ANTIGENS, OBTAINED BY DIFFERENT METHODS FOR INCREASING THE QUALITY OF THE FLEXNER'S B DYSENTERY COMPONENT IN POLYVACCINES

by

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In recent years more and more frequently one confronts an opinion of the investigators regarding the low effectiveness of the preparation for dysentery inoculation, particularly polyvaccine. Thus, for example, Devoyno in epidemological observations (1959) noted that polyvaccines produced by the Institute im. Gamaley and by the Moscow Scientific Research Institute of Vaccines and Serums im. Mechnikov were ineffective with respect to dysentery. The inoculation also did not have any significant effect on the infectious process in the inoculated patient and did not shorten it in comparison with those who were not inoculated.

The problem of improving the quality of the dysentery component in the vaccine should be viewed from all aspects. It is widely known that in order to obtain an effective typhus abdominalis preparation selection of strains, containing Vi-antigens and possessing better immunogenic qualities is required.

In addition to the choice of cultures the method of the growth of the culture may have a significant effect on the production of complete dysentery antigens which comprise one of the components of polyvaccine.

The nature of immunity during this infection may be very significant for the effective control of dysentery. It should be pointed out that all of these questions have not been answered yet. There is no unified opinion, particularly

with respect to the effect of typical properties of Flexner's dysentery causal organisms in the production of immunity. Certain investigators believed only in the immunity pertaining to species (Kashayeva and co-workers). Others, for example Yakhniaa (1949), Zak (1951, 1952), in the study of passive immunity came to the conclusion regarding the presence of a sharply defined type related immunity.

There are also investigators who believe that in the case of Flexner's dysentery there exists not only a type related, but also an intertypical immunity. Thus, Yakhnina (1949) notes that antimicrobe immunity in the homologous type of Flexner's bacteria is significantly more pronounced and reaches 90 to 100% protection for mice, while for heterologic types only 24 to 45% protection is reached. Zelekina and Khabas (1944) who studied the immunity caused by the corpuscular vaccine in complete antigen for Flexner's bacteria, arrived at the analogous conclusions. According to the data of these authors vaccines and complete antigens are almost equally immunogenic and cause protection to the homologous cultures to the extent of 35 to 90%, while with respect to the heterologic cultures, the protection is only to the extent of 4.7 to 50%. The authors conclude that in order to obtain immunity with respect to five serological types of Flexner's type microbe it is sufficient to immunize animals with only three of the types, i.e., types c, f and e. Naryshkina (1959) came to the conclusion that in the presence of a large number of serological types (fourteen), it is extremely significant to select the appropriate strains which would contain several of the leading types of antigens and would possess sufficiently pronounced immunogenic properties. This author was able to select only twelve out of 1260 strains which contained three or four typical antigens. However, in evaluating the immunogenic properties it was found that polyantigens of dysentery strains cannot replace

the vaccines with type specific strains. Finally, Potapchik (1959) in the immunization of mice with a mixture of monovaccines obtained immunity which was equal in strength to the average of the typical and the specie related immunity.

In our work, concerned with the improvement of the quality of the dysentery component in polyvaccine, we were faced with the following problems:

1. To select the most immunogenic cultures from the cultures of Flexner's types c, f and e bacteria, and to attempt to explain the presence of thermolabile antigen, analogous to Vi-antigen of typhus abdominalis culture, as well as to determine its significance in the production of immunity.

2. To compare the immunogenic effectiveness of complete antigens obtained in growing the microbe mass on agar and in reactors with aeration.

3. To evaluate the immunogenic properties of complete antigen, obtained from the experimental mixture of five types of Flexner's cultures.

In our work in 1957 and 1958 the presence of labile antigen in the majority of Flexner's types c, f and e dysentery cultures was shown and its great significance in the formation of immunity. However, we noted that in growing the microbe mass in the reactor with aeration lowering of the labile antigen titer occurs. The low indices of the labile antigen were also observed in complete antigens, obtained by the above method of growing of the microbe mass. This necessitated the study of the immunogenic properties of compelte antigens obtained from the microbe mass, grown in the reactor and in agar in a comparative experiment.

To resolve these questions, three series of complete antigen from the production culture of Flexner's type c strain 516 bacteria were obtained. Two of them were obtained from the microbe mass grown on agar, and one from the microbe

mass grown in the reactor with aeration by the industrial production method. Flexner's type e strain 516 was agglutinated with hyperimmune rabbit serum of the homologous type at 1:2800 dilution. The serum absorbed by the autoclaved culture (i.e., free of antibodies to somatic antigen), produced positive agglutination reaction at 1:1600 dilution, which we call labile antigen culture titer. It was found that after an eighteen hour growth in the thermostat, microbes from the agar wash had a labile antigen titer equal to 1:800, while microbes obtained from the reactor after a sixteen hour growth had a titer equal to 1:400.

Thus, we noted that labile antigen titer decreased by a factor of two, when the culture was mass grown on the agar matrix, while in the reactor it decreased by a factor of four in comparison with the starting labile antigen titer of the culture which was taken for seeding.

Subsequently, a method used for obtaining whole antigens from the microbe mass, grown under different conditions, was the same (tryptic boiling). The quality of the obtained complete antigens was determined both in serological reactions as well as in experiments on verifying the development of immunity in mice.

In the precipitation reaction, complete antigens, obtained by the agar method, had indices four times higher in comparison with complete antigens obtained by the production method. It should also be noted that complete antigen of series one, obtained from the microbe mass in agar, had an ash content equal to 17.7%, and in series two - 11.8%, while the complete antigen obtained from the microbe mass grown in the reactor with aeration had a significantly higher ash value - 49.8%.

The advantage of different methods for growing the microbe mass for the production of complete antigens from Flexner's dysentery culture should also be based on the

indices of their immunogenic properties. The immunogenic properties of the three above complete antigens were investigated. In our experiments we took twenty mice for each testdd dose (Table 1).

Table 1.

Immunogenic Properties of Complete Antigens, Obtained by Different Methods of Growing the Microbe Mass of Flexner's Type c Strain 5.6 Culture.

Complete antigen for immunization	Flexner's culture	Survival of mice (%)			
		Amount of culture injected (in Dcl)			
		1	2	4	8
Microbe mass from agar series one	type c	100	85	55	30
Microbe mass from agar series two	type c	90	70	50	10
Deep well method with aeration series 172	type c	75	45	15	0
Control culture	type c	0	0	0	0

Two experimental complete antigen series, obtained from the microbe mass of Flexner's type c culture, grown on agar had almost identical indices for the survival of the mice: upon injection of one Dcl 90 to 100% of the mice survived, tow Dcl - 70 to 85%, four Dcl - 45 to 50%, and eight Dcl only 10 to 30%. The complete antigen obtained in growing the same Flexner's type c culture in the reactor with aeration had

lower protection indices for mice: upon injection of one Dcl, 75% of the mice survived, two Dcl - 45 %, four Dcl - only 15%. All of the control mice died upon the injection of any of the above doses.

Thus, complete antigens, obtained from the microbe mass of Flexner's type c culture, grown on agar, are more effective in experiments on mice from the standpoint of their immunogenic properties in comparison with complete antigens obtained from the microbe mass grown in the reactor with aeration.

Subsequently, considering the literature data and the acceptance of the significance of typical immunity by the majority of the investigators, one could think that the low effectiveness of the polyvaccine in controlling Flexner's dysentery may be explained primarily by the fact that the polyvaccine includes only one type c. This cannot insure protection against all of the other frequently encountered types of dysentery. However, the impossibility of burdening the polyvaccine by the inclusion of complete antigens of all five types of Flexner's bacteria in the same ratios as type c lead us to the search for a new method which would furnish the polyvaccine with antigens of other types of Flexner's bacteria. For this purpose, on the basis of the proposal by Professor Krestovnikova, a study was undertaken of the properties and the immunogenic effectiveness of the complete antigen, obtained experimentally from a mixture of five typical Flexner's cultures - c, f, e, a and d (one billion microbes after an eighteen hour growth of each type of the culture). After a thorough mixing the mixture of cultures was seeded into a semifluid agar, from which in order to obtain antigens bacteria were seeded without passages through the organism of a carrier. The mixture of cultures, seeded into the semiliquid agar produced a positive agglutination reaction on the glass with all five single type agglutinating serums. The same mixture of cultures produced positive agglutination reaction with the

hyperimmune rabbit serum, immunized with a single type culture, included into the mixture, at the same dilutions of the serum as a homologous culture, or sometimes one dilution lower (1:3200 to 1:1600).

When we were convinced that a mixture of cultures seeded into a semiliquid agar contained all five types of Flexner's culture, we simultaneously obtained from it complete antigen of the microbe mass, grown in agar and the formaldehyde treated vaccine.

The rabbits were immunized with the complete antigen and formaldehyde treated vaccine for the study of the antigen properties of a mixture of cultures. The results of serological investigations have shown that the mixed culture possesses sufficiently well developed antigen properties with respect to each of the five types of cultures, since hyperimmune rabbit serums contain antibodies both to the homologous mixture of the culture and to the typical cultures, included into the mixture in 1:1600 to 1:3200 titers.

The principal evaluation of the quality of the mixture of cultures with respect to each type of the culture is the evaluation of the immunogenic indices.

The evaluation of the immunogenic properties of a mixed culture was carried out on mice, immunized with complete antigen, and for control immunized with formaldehyde treated vaccine, obtained from the above mixture of cultures. The immunizing doses, used in these experiments did not exceed those established by the instructions of the State Control Institute im. Tarasevich for each of the issued complete dysentery antigens and formaldehyde treated vaccines. The mice were immunized with a single subcutaneous injection of complete antigen with the depot dose equal to 0.0625 mg per mouse. The mice were immunized with formaldehyde treated vaccine as usual in two vaccinations subcutaneously with a dose equal to 250 million microbes. Thus, during the immuni-

zation both with complete antigen as well as with formaldehyde treated vaccine, obtained from a mixture of cultures, the content of each type of antigen in the immunizing does was decreased by a factor of five, in comparison with the doses used for the immunization of single type antigens. (Table 2).

Table 2.

Immunogenic properties of complete antigen of formaldehyde treated vaccine, obtained from a mixture of cultures No. 1.

a Тип культуры Флекснера для заражения мышей	c Антиген для иммунизации мышей				
	Полный антиген d		e Формалиновая вакцина		
	f выживаемость мышей (в %)				
	g Доза для разрешения (в *Dcl*)				
	1	2	1	2	4
e	80	77,7	100	100	100
c	100	80	90	90	40
f	90	0	50	70	0
d	70	0	70	40	20
a	45	15	25	25	0
bКонтроль *d*	10	0	10	0	0
a	0	5	0	5	0
c, e, f	0	0	0	0	0

Key: a- Flexner's type culture for the infection of mice; b- Control; c- Antigen for immunization of mice; d- Complete antigen; e- Formaldehyde treated vaccine; f- Survival of mice (in %); g- Resulting dose (in Dcl).

The absence of immunity of Flexner's type a culture is apparently explained by insufficient immunogenic activity of this type of culture. As a control we simultaneously checked

the immunogenic properties of the formaldehyde treated vaccine, also obtained from the same mixture of cultures. The highest immunity indices were displayed with respect to type e culture (i.e., 100% survival of the mice upon injection of doses equal to one, two and four Dcl, and 70% survival upon the injection of eight Dcl). The lowest indices were displayed with respect to type a culture.

It was of interest to compare the above results of the survival of mice to each type of culture during the immunization with complete antigen, obtained from a mixture of cultures with the survival of mice immunized by the same dose of monotypic complete antigen (Table 3).

Table 3.

Immunogenic properties of the monotypic complete antigens of Flexner's bacteria.

Type of antigen for immunization of mice	Type of culture for testing	Survival of mice (in %)		
		Antigen dose (in Dcl)		
		1	2	4
e	e	100	101	95
c	c	100	85	-
f	f	90	30	-

The survival of mice upon immunization with the monotypic complete antigens type c and f was analogous to the survival upon the immunization with complete antigens from a mixture of cultures. The survival of the mice was somewhat higher for those immunized with a monotypic type e complete antigen.

Thus, it should be noted that the complete antigen obtained from a mixture of five Flexner's type cultures,

possesses high immunogenic properties of four types of Flexner's cultures (c, f, e and d) without the increase of the immunizing dose of the complete antigen, generally used for the immunization of mice with the monotypic complete antigens.

Thus, taking the results obtained in these investigations, into consideration one may conclude that at present there are no bases for accepting the impossibility of ensuring specific prophylaxis in the control of dysentery. There are however, reasons to believe that at present, not all the factors have been taken into account in preparing the prophylactic preparations.

The principal attention in this respect must be directed to the study of the antigen structure of the Flexner's dysentery cultures, particularly in selecting the cultures containing the labile antigens and the preservation of this antigen in complete antigens.

Attention must also be given to the problem of ensuring the inclusion of antigens of the most popular Flexner's type cultures into the preparation designed for porphylactic purposes. In view of this, we believe that it is worthwhile to collect immunogenic data on the epidemiological experiments obtained with complete antigens from the microbe mass of mixed culture grown on agar. One must keep in mind the fact that during immunization with this antigen the dose of the introduced antigen is not increased, and at the same time the immunity to four types of Flexner's bacteria is being developed.

It is of great interest to study the properties of mixed cultures, seeded into semifluid agar, upon storage and their variability during passages. This will be the subject of our future investigations.

Conclusions

1. Complete antigens, obtained from a microbe mass of Flexner's type c dusentery culture, grown on agar, have better serological and immunological indices than complete antigens obtained from the microbe mass grown in the reactor with aeration.

2. The experimental culture mixture of five types of Flexner's bacteria were seeded into semifluid agar and not passed through various media. It possesses sufficiently well expressed antigen properties with respect to each of the types of bacteria which were included into the mixture.

3. Complete antigen, obtained from a mixture of five types of Flexner's cultures during the growth of the microbe mass on agar, in experiments with mice produces high immunity indices to four types of Flexner's cultures (c, e, f and d) without the increase of the established immunization dose for the injected antigen. The survival indices for mice meet the requirements for the preparation of complete antigen for preventative purposes.

4. The epidemological study of complete antigens obtained from a mixture of five Flexner's type cultures, grown in agar, must be conducted.

Bibliography

[1] Devoyno, L. V. "The Specificity of Dysentery Prophylaxis with Complete Antigen Vaccines," ZhMEI, No. 4, 23-27 (1959).

[2] Yakhnina, N. A. "Species Specificity of the Immunity in Dysentery, Communication No. 1" ZhMEI, No. 7, 58-58 [sic] (1949).

[3] Yakhnina, N. A. "Type Specificity in the Immunity to Flexner's Group Dusentery. Communication 2." ZhMEI, No. 7, 63, (1949).

[4] Yakhnina, N. A. "Type Specificity of the Passive Immunity in Dysentery Under Experimental Conditions. Communication 3." ZhMEI, No. 4, 55-61, (1950).

[5] Zak, S. F. "A Characteristic of the Immunogenic Properties of Flexner's Dysentery Vaccines as a Function of Their Tpes Composition" ZhMEI, No. 4, 51-56, (1951).

[6] Zak, S. F. "Type Specificity of the Immunity Against Flexner's Type Dysentery in the Passive Immunity Experiments with White Mice," ZhMEI, No. 3, 48-52, (1952).

[7] Zelikina, M. I. and Khalas, I. M. "Comparative Date on the Study of the Serological Properties of the Flexner's Hiss Group Dysentery Microbes and of Complete Antigens Obtained from Them by the Bauer Method," Transactions of the Leningrad Institute of Vaccines and Serums, No. 1, 43-57, (1944), Leningrad.

[8] Naryshkina, Z. P. "A Comparative Evaluation of the Immunogenic Properties of Mannitol Fermenting Strains of Dysentery with Polyantigen and Type Specific Structure," ZhMEI, No. 4, 85-86 (1950).

[9] Potapchik, Yu. L. "Experimental Study of the Type-Specific and Intertypical Immunity, Caused by Flexner's Dysentery Microbe," ZhMEI, No. 4, 34-42, (1953).

STUDY OF THE PRINCIPAL PROPERTIES OF DIPHTHERIA DIAGNOSTIC PREPARATIONS AS A FUNCTION OF THE DURATION OF THEIR STORAGE

(Communication I)

by

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The most popular practical serological method of investigation is the agglutination reaction, which is used for the diagnosis of many infectious diseases. This reaction was not used in the study of diphtheria infection until very recently. Only the serological type of diphtheria microbe was determined by means of this reaction (Bell, 1922; Smith, 1923; Hammerschmidt, 1924; Orr, and Eusing, 1933; Robinson and Peeney, 1936; Murray, 1936; Hewitt, 1947; Huang, 1949; Ivaneko, 1940; Yablokova, 1952; Tarasyuk, 1954; Kochurovskiy, 1956; Davydova and co-workers, 1956 and others).

Certain authors recommended this reaction for differentiation of the diphtherial microbe from other representatives of the Corynebacterium genus (Lyubovskiy, 1900; Schwonner, 1909; Langer, 1916; Tarasyuk, 1955; Nemchenko, 1955; Sadykova, 1958).

The possibility of detecting specific agglutinins in the serum of the diphtheria patient by means of the agglutination reaction was proven by many Soviet investigators (Delyagina, 1950; Fisher and co-workers, 1956; Finkel'shteyn, 1956; Markova, 1956; Vvedenskaya and others, 1956).

The possibility of using this reaction for the retrospective differentiation of a typical form of diphtheria from nonspecific angina in diphtheria bacteria carriers, was studied by Delyagina (1956), Sukhareva (1957), Aliyeva (1956), Tsimbalist and Ivanov (1958) who recommend it for use in everyday practice.

On the other hand, Kroger, Thofern and Schulz (1952), Grinshteyn (1957), Shiryayeva, Filosofova, Shekhter (1958) consider that the so-called diphtheria "Vidal" [a diagnostic preparation] must be viewed very critically. Fisher and co-workers (1956), Finkel'shteyn (1956), Markova (1957) and Khrushcheva (1959) recommend that the agglutination reaction be conducted with live diphtheria cultures. Under the practical laboratory conditions, this is associated with a great number of difficulties (storage of the pathogenic cultures, preparation of microbe samples, etc.). Other investigators (Delyagina, 1950; Sviridenko and Aliyeva, 1950) used formaldehyde treated dead diphtheria cultures for this purpose.

The instructions for the production, control and use of the diphtheria diagnostic preparation was developed by Delyagina, a staff member of the Moscow Scientific Research Institute of Vaccines and Serums im. I. I. Mechnikov, and ratified on February 28, 1957.

In order to verify the agglutination reaction value to obtain extensive practical experience it was necessary to produce diphtheria diagnostic preparation under industrial conditions and to study the principal properties of this preparation as a function of the storage time. This work was conducted in the course of 1957 to 1958.

During the preparation of diagnostic reagents, the existing instructions were strictly followed. The diphtheria cultures were selected and studied according to the instructions.

For the production of diagnostic preparations, strains of different serological type of diphtheria cultures were seeded into two to three test tubes with coagulated normal equine serum and placed into a thermostat for twenty-four hours at 37°C. The culture grown was washed with 10 ml of 3% sodium chloride solution, the pH of which was equal to 7.6, established by the addition of a 5% solution of Na_2HPO_4. Subsequently, 10 ml of the culture were seeded on mats with

20% serum agar. After an 18 to 20 hour incubation of the seeded mats in the thermostat and after evaluation of the purity (microscope smear) the culture from each mat was washed with 30 to 50 ml of 3% saline solution of pH 7.6 and collected into sterile bottles. After determining the concentration of the culture (according to the optical standard method of the State Control Institute im. Tarasevich) the obtained suspension was diluted with three percent NaCl solution to a concentration of one billion microbes per ml. A 0.1% solution of commercial formalin, with the established neutral reaction was added to the microbe suspension.

The obtained mixture was left for three days at room temperature. After three days the sterility of the solution was checked by seeding it on the following media: two test tubes with coagulated serum; two test tubes with tapered agar gel; two test tubes with beef-peptin broth and 0.5% glucose and two test tubes with Kitta-Tarocci medium. The seeded test tubes were kept in a thermostat at 37°C for five days. In addition, Saburo and beef-peptin broth (two test tubes each) were also seeded, and kept at temperatures ranging from 20 to 22°C for five days.

When the seeded control contained diphtheria bacilli growth the diagnostic preparation was kept under the same conditions for an additional three days, after which the repeat seeding was conducted to determine sterility. When the microscopic smears of macroscopic examination of the seeded cultures indicated the presence of the vulgar microflora, the diagnostic preparation was destroyed. Prior to distribution into ampules, the diagnostic preparation was checked for its agglutinating ability with homologous agglutinating serums. Subsequently, the specificity was checked with three serums of healthy people and three serums of patients with fever.

The whole batch of diagnostic material, obtained in one day from one suspension, collected together comprised of one

series. Prior to each experiment diagnostic preparations should be well shaken and filtered through cotton, placed on the tip of a Pasteur pipet. The results of the reactions should be judged by the naked eye or through a lens.

The duration of the usefulness of the diagnostic preparation from the day of its synthesis according to the instructions was eight months.

The diphtheria culture preparations which we used were stored in the laboratory in dry form for several years (four cultures were stored for two to 2.5 years, and one culture was stored for four years). The drying was conducted after the study of all of the properties of the culture at the time of its transmittal to the museum.

The cultures were checked for all of their properties, generally one to one and a half months prior to the synthesis of diagnostic preparations and were found to satisfy all of the requirements specified by the instructions.

In the course of all of this work one and the same dry agglutinating rabbit serum with titers equal to 1:1600, 1;3200 was used.*

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* Prior to use all glassware was checked for the alkalinity of the glass.

The following properties of the diagnostic preparation were checked: concentration of microbes, agglutinating ability (homologous and heterologous) and also the pH of the solution. At the beginning of the work our laboratory had some diagnostic preparations which were stored for different periods of time (one to five months). Nineteen series of the preparations were studied. Twleve series of the preparations were stored after the synthesis, in a refrigerator at 4 to 6°C and seven series were stored at room temperature (ranging from

20 to 25°C).

By the time the preparations were issued, five of the seven series stored at room temperatures did not meet the requirements as specified in the instructions with respect to one or several of the indices (e.g., group reactions were higher than the permissible titers of preparations produced nonspecific reactions). By the time of issuance, twelve series of the preparation stored in the refrigerator, met all the requirements as specified by the instructions. (Table 1).

Table 1.

Changes of the principal indices of the preparation as a function of the duration of storage in a refrigerator.

Число a	Показатель b	g Срок хранения (в месяцах)									Число серий с измененными показателями h
		1	2	3	4	5	6	7	8	9	
12	*pH*	5/5	4/4	2/2	—	—	—	1/1	—	—	12
	Концентрация c микробов	5/0	5/1	4/0	3/2	5/5	—	1/0	—	—	8
	Степень агглютинабильности с гомологичной d сывороткой	5/0	9/0	10/1	7/0	6/1	—	4/0	—	2/1	3
	Групповая агглютинабильность e выше допустимого титра	5/0	9/4	7/1	4/1	6/1	—	2/0	—	—	7
	Специфичность f	5/3	2/1	3/2	4/3	3/0	—	1/0	—	—	9

Note: numerator - number of the investigated series; denominator - number of series with changed indices.

Key: a- Number; b- Index; c- Concentration of microbes; d- Degree of agglutination with homologous serum; e- Group agglutination above the permissible titer; f- Specificity; g- Duration of storage, (in months); h- Number of series with changed indices.

For different series of preparations the pH was measured one to eight times. The lowering of the pH was observed in all series, moreover, significant changes were observed from pH 7.6 to 6.0 to 6.21 in eight of the series. In six series the pH having decreased somewhat remained at the same level, in some series for as long as five to nine months. In six series the pH continued to decrease from month to month. The lowering of the pH occurred soon after the synthesis of the diagnostic preparation, thus from five, four and two series, studied after one, two and three months respectively, lowering of the pH was detected in all series at the moment of measurement.

The concentration of microbes was checked for different series one to five times, four to five times more frequently. After one month, five series were checked. All of them contained the starting concentration of (2 billion/ml) microbes. The same level was retained in four series after two months. In one out of five series the concentration after two months decreased to 500 million, i.e., to one quarter of its initial level. In two out of three series checked after four months the concentration decreased by a factor of two, After five months in three series the concentration decreased from one billion microbes/ml to 500 million microbes/ml and in two others it decreased from two billion microbes/ml to 1.5 billion microbes/ml. In four series the concentration remained without change in the course of five to seven months. Thus, in eight series lowering of the concentration of microbes was noted. In individual series it decreased after two months, while in the majority it decreased after four to five months.

The initial agglutinating properties were retained completely in nine series of the diagnostic preparation in the course of the whole series of observations (seven to nine months). Only in three series lowering of the titer by a

factor of two to four was noted after three, five and nine months of observation respectively.

The existence of group agglutinating ability was verified in each series two to seven times, (four to five time in the majority of cases). By the time of the issuance of the diagnostic preparations, seven out of twelve series of preparations did not produce any group agglutination reaction, while in four series this reaction was observed at 1/16 and in one series at 1/32 of the level of the principal titer. In two series the existence of group agglutination ability was not noticed until the end of the experiment.

In three series group agglutination reactions took place, but within the limits of the titers permissible by the specifications. In seven series monthly increase of the group agglutination reaction titer was noted and after two to four months it became higher than specifications permit. The existence and the increase of the group agglutination reaction titer was noticed in the first few months with serums of different serological types.

The evaluation of the specificity of the diagnostic preparation was conducted with the serums of feverish patients and with the serums of healthy people. The existence of nonspecific reactions was noted in nine series of diagnostic preparations during the evaluation in the course of one to four months storage; in three out of five, in one out of two, in two out of three and in three out of four series respectively. The nonspecific reactions amounting to 1:50 titer were observed in four out of nine series (five out of seventeen, four out of ten, and one out of six respectively), and in 1:100 and higher titers in five series. The number of serums with which diagnostic preparations produced nonspecific agglutination reactions, are sometimes very great, thus for example: three to four months after the synthesis of the diagnostic preparations 27, 41 and 17 serums out of

127, 120 and 21 evaluated serums respectively, gave positive results 13, 17, and 17 serums in each of the above series had titer equal to 1:100.

We were unable to establish any relationship between the individual properties of the diagnostic preparations. It was established that four series which decreased in pH insignificantly to 7.4 were different with respect to all other properties. In the diagnostic preparation of one series, all the other indices did not change, while in three other series lowering of the standard by a factor of two and the occurrence of nonspecific reaction was noted, while in another series lowering of the group reaction was observed.

In four series the pH decreased to 7.2 to 7.0. In one series, the formation of a flaky sediment in the diagnostic preparation was observed. The characteristics of the other series were principally the same as those of the above described group.

In four series the pH decreased to 6.88 to 6.0. The diagnostic preparation of one series did not change in terms of other indices. In four other series the development of weak nonspecific reaction was observed (in four out of fourteen, one out of six and one out of ten) in 1:50 titer, while in another series the titer of the group agglutination reaction became equal to the principal titer and sediment formation in the diagnostic preparation was also observed.

Only three series of the diagnostic serum retained the standard titer level and produced group reactions within the permissible titers: nonspecific agglutination ability was either absent or very weak at different pH values (7.0; 7.4 and 6.8).

In four series the lowering of the standard titer coincided with a large number of group reactions and nonspecific reactions at pH equal to 7.4; 7.29: 6.4 and 6.6. The lowering of the

standard titer and a large number of nonspecific reactions also coincided in four series at pH equal to 7.4, 7.4, 7.2 and 7.28. Only in one series of the diagnostic serum did the lowering of the pH and the lowering of the titer with the homologous serum coincide with the factor of two.

Thus, in the course of two to three month storage of diphtheria diagnostic serums, lowering of the pH was noted in twelve series, change in the concentration of microbes in eight series, the occurrence and increase of group reactions in seven series, and the occurrence of nonspecific reactions in nine series.

In seven series of preparations, stored at room temperature in the course of the first month of storage the pH decreased from 7.8 to 7.4 to 7.5, while in six series, lowering of the concentration by a factor of two was observed (Table 2).

The initial agglutinating properties were preserved in five series of the diagnostic preparation in the course of four months of observation. The change of these properties was noted in three series of the preparation after three to six months.

The group reactions above the permissible titer occurred in two out of four series of the diagnostic preparation, after one month of storage, and in one out of two series after three months of storage.

The loss of specificity was observed in three out of five series after one month and in one out of two series, after two months, i.e., in all seven series of diagnostic preparations changes of the principal properties were observed: pH, concentration; specificity and agglutinating ability.

Thus, in the study of nineteen series of diphtheria diagnostic preparations made from the same culture, according to the existing instructions, five series were found to be

unacceptable for work at the time of issue, and fourteen series were unaccepatble after one to three months of storage in refrigerators and at room temperature.

Table 2.

Change of the principal indices of the preparation as a function of storage time at room temperature.

Число серий a	Показатель b	Состояние показателей в момент выпуска c	Срок хранения (в месяцах) d						Число серий с измененными показателями e
			1	2	3	4	5	6	
	pH	7/0	7/7	—	—	—	—	—	7
	f Концентрация	7/0	7/6	—	—	—	—	—	6
7	g Степень агглютинабильности с гомологичной сывороткой	7/0	7/0	7/0	7/1	6/1	—	5/1	3
	Групповая агглютинабильность выше допустимого титра h	7/3	4/2	2/0	2/1	—	—	—	6
	Специфичность i	7/2	5/3	2/1	1/0	—	—	—	6

Remarks: the numerator indicates the number of series; the denominator designates the number of series with changed indices.

Key: a- Number of series; b- index; c- State of indices upon completion of the preparation; d- Duration of storage (in months); e- Number of series with changed indices; f- Concentration; g- Agglutination ability with homologous serums; h- Group agglutinating ability above the permissible titer; i- Specificity.

Conclusions

1. The method, recommended by Delyagina for the production of diphtheria diagnostic preparation does not insure the suitable quality of the preparation over the eight month storage period as indicated in the instructions.

2. The principal properties of the diagnostic preparation (pH, specificity, agglutinating ability, concentration of microbes) are unstable even in the course of the first month of storage.

3. At present synthesis of this preparation on the industrial scale does not appear to be possible.

4. It is possible that part of the difficulty is associated with the additional industrial environment changes, since the experimental conditions and the industrial preduction conditions are not equivalent. Each of the preparations must pass the experimental-production phase, which has not been anticipated by the instructions.

Bibliography

[1] Aliyeva, R. O. "Agglutination Reactions in Dephtheria Microbe Carriers," Pediatriya (Pediatrics), No. 4, 31, (1956).

[2] Vvedenskaya, Ye. F., D'yakonova, T. V. and Gratsionova, A. I. "Agglutination Reaction in the Diphtheria Diagnostics," Trudy Moskovskogo Nauchno-Issled. In-ta Vaktsin i Syvorotok im. I. I. Mechnikova (Transactions of the I. I. Mechnikov Moscow Scientific Research Institute of Vaccines and Serums), Moscow, No. 6, 23, (1956).

[3] Davydova, I. S., Bazhanova, A. G. and Banikova, V. F. "Study of the Biochemical, Serological and Virulent Properties of C. Diphtheriae, Isolated from Patients and Carriers in the City of L'vov" Tezisy Dokladov Konferentsii po Voprosu Immuniteta i Epidemiologii Stolbnyaka i difterii (Abstracts of the Reports Presented at the Conference on the Problem of Immunity and Epidemology of Tetanus and Diphtheria), L'vov, 30-31 (1956).

[4] Delyagina, L. P., Serologicheskoye tipirovaniye vozbuditelya i serodiagnostika difterii, (Serological Typing of the Causal Organisms and the Serodiagnostics

of Diphtheria) Candidate's Dissertation, Moscow, 1950.

[5] Delyagina, L. P. and Kravchenko, N. A. "Agglutination Reactions in Diphtheria in the Practice of Regional Laboratories," Trudy Moskovskogo Nauchno-Issled. In-ta. Vaktsin i syvorotok im. I. I. Mechnikova, Moscow, 6, 19, (1956).

[6] Delyagina, L. P., Kravchenko, N. A., Ter-Avaryan, N. I. and Miroshkina, S. K. "The Significance of Agglutination Reactions as a Method for the Differentiation of Diphtheria from Anginal Etiology in Diphtheria Bacteria Hosts," Pediatriya, No. 4, 28 (1956).

[7] Delyagina, L. P. and Shapiro, S. L. "Serological Characteristics of Diphtheria Cultures, Isolated in Leningrad and Moscow," Trudy Moskovskogo Nauchno-Issled. In-ta. Vaktsin i Syverotok im. I. I. Mechnikova, 6, 9 (1956).

[8] Ivnaenko, P. D. "Serological Classification of the Diphtheria Bacilli by Means of the Agglutination Reaction" Trudy Ukrainskogo Instituta im. I. I. Mechnikova (Transactions of the Ukrainian Institute im. I. I. Mechnikov), Kharkov, No. 5, 51 (1940).

[9] Ivanov, Yu. A. "Data on the Laboratory Diagnostics of Diphtheria," Okrana Materinstava i Mladenshestava (Protection of Pregnant Mothers and Infants), No. 3, 9 (1959).

[10] Kil'disheva, A. Ye., and Potapchik, Yu. A. "Data Related to the Biology and Serology of Diphtheria Bacilli," ZhMEI, No. XVIII, Issue 6, 811 (1937).

[11] Kocherovskiy, Yu. E. "Kharakteristika Difteriynykh bakteriy v g. Tashktnte (Characteristics of Diphtheria Bacteria in Tashkent), Candidate's Dissertation, Tashkent, 1956.

[12] Mikayelyan, V. G. and Amyan, D. A. "Serological Characteristics of Diphtheria Cultures in Yerevan,"

Tezisy Dokladov XII Vsesoyuznogo S"yezva gigivenistov, epidemilogov, mikrobiologov i infektsionistov (Abstracts of Reports Presented at the XII All-Union Congress of Hygienists, Epidemologists, Microbiologists and Infectious Disease Specialists), Moscow, 395 (1956).

[13] Lenkina, M. S. "Serological Properties of Diphtheria Causal Organisms Isolated in Khabarovsk in 1956 - 1957" Chapter in the Collection: Nauchnyye raboty i avtorefераty Khabarovskogo Institut Epidemiologii i Mikrobiologii (Scientific Work and Author's Abstracts of the Khabarov Institute of Epidemology and Microbiology), No. 4, 67, (1958).

[14] Nemchenko, A. G. "Experience with the Identification of the Diphtheria Microbes by the Serological Method" in the collection: Trudov Mezhinstitutskoy nauchnoy konferentsii 1955 g. (Transactions of the 1955 Interinstitute Scientific Conference) Infectious Child Disease, Moscow, 180 (1957).

[15] Rozanov, S. N. "Evaluation of the Method for the Early Diagnosis of Diphtheria. Problems of Clinical Practice, Pathogenesis and Treatment of the Acute Child Disease" Sbornik Rabot Gosudarstvennogo nauchno-issledovatel'skogo pediatricheskogo Instituta (Collection of Works of the State Scientific Research Institute of Pediatrics), Moscow, 206, (1957).

[16] Sviridenko, Ye. T. and Aliyeva, R. O. "Serological Diagnosis of Diphtheria" ZhMEI, No. 4, 29, (1958).

[17] Sukhareva, M. Ye., Blyumental' K. V. and Smirnova. V. V. "Diagnosis of Larynx Diphtheria on the Basis of the 1948-1949 Data, S. P. Botkin Hospital" Pediatriya, No. 5, 36, (1955).

[18] Sukhareva, M. Ye, and Flekser, S. Ya. "Characteristics of Diphtheria in Recent Years" Voprosy Okhrany Materinstva i mladenchestva, No. 3, 3, (1959).

[20] Finkel'shteyn, A. F. "The Significance of Serodiagnostics as an Auxiliary Method in the Diagnos of Diphtheria," Chapter in the book: Novyye Metody Laboratornoy diagnostiki vazhneyshikh infektsionnykh Bolezney (New Methods of the Laboratory Diagnostics of the Important Infectious Disease), Leningrad, 102 (1956).

[21] Fisher, M. N. and Vayzer, A. M., "Experiments with Serodifferentiation of the Diphtheria Cultures and Serodiagnostics of Diphtheria" in the book: Biologicheskiye Antiseptiki (Biological Antiseptics), Leningrad, 266, (1950).

[22] Fisher, M. N., Mekler, S. S. and Shmidt, M. M. "Experiments with the Serodiagnostics of Diphtheria According to the 1954 - 1955 Data of Botkin Hospital" in the book: Novyye Metody laboratornoy diagnostiki vazheyshikh infektsionnykh Bolezhney, Leningrad, 102 (1956).

[23] Khrushcheva, V. A. "Microbiological and Serological Characteristics of Patients, Entering the Hospital with Diagnosed Diphtheria and'Diphtheria'" Okhrana materinstva i Mladenshestva, No. 3, 12 (1953).

[24] Khorsova, N. I. "Serological Types of Diphtheria Microbes Isolated from Patients in Alma-Ata" XXVI Itogovaya Nauchnava Konferentsiya (XXVI Scientific Summary Conference), Alma-Ata, 104, (1957).

[25] Tsimbalist, D. F. and Ivanov, Yu. A. "Innovations in the Laboratory Diagnostics of Diphtheria" ZhMEI, No. 11, 148, (1957).

[26] Shiryayeva, A. G., Filosofova, T. T. and Shekhter, A. B. "Agglutination Reaction in Diagnostics of Diphtheria," in the collection: Voprosy epidemiologii detskikh infeketsiy (Problems of The Epidemology of Infectious Children's Disease), Kiev, 283, (1958).

[27] Shumakova, G. V. "The Serological Classification of Diphtheria Bacilli on the Basis of the Data Obtained

in Moscow. Problems of Infectious Pathology and Immunology" Trudy Akad. Med. Nauk SSSR (Transactions of the Academy of Medical Sciences of the USSR), Moscow, 164, (1949).

[28] Yablokova, T. B. Sravnitel'naya otsenka metodov tipirovaniya difteriynkh bakteriy po kul'tural'no-biokhimicheskim i serologicheskim priznakam (Comparative Evaluation of Methods for the Type Classification of Diphtheria Bacteria on the Basis of Biochemical Cultures and Serological Indices). Candidate's dissertation, Moscow (1952).

29. Bruno, H. Über Diphtheriaagglutination und Serodiagnostik. Berlin. Klin. Wschr. 1898, 35, 1127.

30. Cummins, C. S. Some observation on the nature of the antigens in the cell wall of Corinebacterium diphtheriae. Brit. J. Exp. Path. 1954, 35, 166—180.

31. Freeman, V. J., Minzel, G. H. Serological studies of C. diphtheriae. II Antigenic relationship between small colony types of C. diphtheriae and C. diphtheriae— eike bacilli as determined by the method of slide agglutination. Am. J. Hyg. 1950, 51, 3, 305.

32. Ferris, A. A. Serological Types of C diphtheriae. in Australia. J. Path. Bact. 1950, 62, 165.

33. Hammerschmidt, J. Uber Gruppenbildung bei den Diphtheriebazillen. Zbl. f. Bakt. I. Abt. Orig. 1924, 93, 443.

34. Hewitt, L. F. Serological typing of C. diphtheriae. Brit. J. Exp. Path. 1947, 28, 338.

35. Huang, C. Further studies on the serological classification of C. diphtheriae. Am. J. Hyg. 1949, 35, 325.

36. Kroger, E., Thofern, E. und Schulz, K. Serologische Untersuchungen bei Diphtheriebakterien Ztschr. Hyg. Infekt. Krankh. 1952, B 5, 3, 254—274.

37. Langer, A. Die Agglutination der Diphtheriebazillen Zbl. f. Bakt Orig. 1916, 78, 117.

38. Lubowski, K. Über einen atoxischen u. avirulenten Diphtheriestamm und über die Agglutination der Diphtheriebazillen. Ztschr. f. Hyg. u. Infect. 1900, XXXV, 87.

39. Lautrop, H. Studies of antigen structure of Corynebacterium diphtheriae. Acta Path. micr. Scand. 1950, 27, 3, 443—447.

40 Minzel, J., a Freeman, V. Serological studies of C. diphtheriae, I. The use of surface active agent in the preparation of uniform suspension of C. diphtheriae for serological typing. Am. J. Hyg. 1950, 51, № 3.

41. Murray. The serological relationships of 250: strains of C. diphtheriae, J. Path. a. Bact. 1935, 41.

42. Orr-Ewing, J. The Serological Grouping of the Starch Fermenting strains of C. diphtheriae J. Path. Bact. 1933, 37, 345.

43. Robinson, D. T. a. Peeney, L. A. The serological types among gravis strains of C. diphtheriae and their distribution. J. Path. a. Bact. 1936, 43, 403.

44. Smith, A. A study of diphtheriae bacilli with special reference to their serological classification. J. Hyg. 1923, 22, 1.

45. Sia a Huang. Serological classification of C. diphtheriae. Proc Soc. Exp. Biol. a. Med. 1939, 41, № 2, 348.

46. Schwonner. Wien. Klin. Wschr., Biol, 15, 1902

THE DEVELOPMENT OF METHODS FOR IMPROVING DIPHTHERIA DIAGNOSTIC PREPARATIONS

(Communication II)

by

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Up to the present time the diagnostic preparations for diphtheria were prepared in our laboratory in small series. In working with such series Delyagina established that the initial properties of the diagnostic preparation are stable in the course of eight months. However, during the production of the first series of the diagnostic preparation under industrial conditions and during the study of its properties at different storage times we found that its principal properties (pH, concentration, agglutinating ability, specificity) do not remain stable even in the course of the first few months of storage.

The principal difficulties during the production of diphtheria diagnostic preparation lie in that it is not possible to obtain an absolutely homogeneous suspension of the diphtheria microbes. The varying degrees of inhomogeneity (amorphity according to Robinson and Peeney) are always apparent and visible in the agglutinoscope, and in the case of unfiltered preparation it is visible through a magnifying glass.

In the attempt to prepare stable homogeneous suspensions of the diphtheria microbes the use of high density liquids (Lyubovskiy, 1900), mechanical effects (Eving, 1933; Hammerschmidt, 1924), selection of different concentrations of hypertonic NaCl solution from 0.85% (Kil'disheva and Potap-

chik, 1937) to five percent (Ferris, 1950), different chemicals such as polyhydroxymethylene, sorbinate, monoaliate, Twin-80 (Minzel and Freeman, 1950), suspension of diphtheria microbes in specific and nonspecific serums (Kröger, Thofern and Schulz, 1952) did not produce any desirable results. Delyagina's method also did not produce the desired results. She used two to three percent hypertonic solution of NaCl with pH equal to 7.6 which permitted the production of only a relatively homogeneous suspension, which could be stored for thirty days at the most, It was found that during storage, the pH decreased from 7.6 to 5.6 to 5.4.

Subsequently, in accordance with the proposal of Shanina, the pH of the hypertonic solution was stabilized by the addition of a five percent solution of Na_2HPO_4. The use of this solution, according to the data of Delyagina, aided the preservation of the initial titer and the pH of the diphtheria diagnostic preparation at the 7.6 pH level in the course of eight months. Nevertheless, prior to each experiment the diagnostic preparation required vigorous shaking and filtering through cotton, wadded on the end of the Pasteur pipet, and the results of the reaction was determined by the naked eye or with the aid of a lens. This indicated that these diagnostic preparations can be considered only relatively homogeneous.

The purpose of this work was to determine the cause of the instability of the principal properties of the diagnostic preparations and the development of the method for their removal.

It is possible that it is necessary to conduct more rigorous selection of the cultures, used in the production of the diagnostic preparations (than was indicated in the existing instructions), and to choose only the select gravis and mitis type cultures, tested in a sample by boiling.

It is also possible that the concentration of salt and the pH indicated in the instructions is not the optimum, and

also the culture medium may not be the most suitable for the production of a homogeneous suspension of the diphtheria microbes.

It is possible that the attempt to dry the diagnostic serum per se or in conjunction with fillers directly after its preparation, when it satisfies the requirements of the instructions, will aid the final solution of the problem.

The methods for the selection of Salmonella, Snigella and many other microbe strains have been developed in the greatest detail. In regard to the selection of the strains of the diphtheria microbes, the work has been conducted only with the industrial strains PW8 Toronto and others, used for the preparation of the diphtheria toxins, anatoxins, therapeutic serums and are concerned strictly with the toxicogenic properties of the microbes.

The problem of the selection of the diphtheria strain for use in the production of the diagnostic preparations, has been left completely uninvestigated even though this is an extremely significant problem.

In storing strains under laboratory conditions on a culture medium disassociation processes can take place, as a result of which not only the morphological and biological but also the serological properties undergo changes. It is also possible that the loss of type specificity occurs and agglutination reactions may develop and increase with agglutinating serum of other serological types of diphtheria. At the same time all of the properties of the industrially used strains must be stable.

The selection of strains may be conducted in two ways:

1) selection of subcultures in the experiments in vitro;
2) passage through the organism of the receptive animals.

For the selection of diphtheria microbe strains, we utilized the former method.

The diphtheria cultures used in the preparation of diagnostic serums were stored dry in the laboratory in the

course of several years (two to four years). Therefore, we conducted simultaneously selection of the museum cultures, as well as selection and study of the freshly isolated cultures. Altogether, ninety-two freshly isolated cultures and eighteen museum cultures were studied.

In conducting the first preliminary agglutination reaction on the glass with diphtheria agglutinating rabbit serums, sixty-four freshly isolated cultures were immediately excluded from the investigation since ten of them produced positive agglutination reaction with several serological types of serums, and fifty-four were spontaneously agglutinated in three percent saline solution.

Among the freshly isolated types the serological types I and VI cultures were not found. In recent years, cultures of these serological types have almost totally disappeared. Therefore, we studied only the museum serological type cultures I and IV.

Serological type II, III, and IV, cultures were studied both as freshly isolated and museum varieties (Table 1).

In the investigated material serological type Gravis III and Gravis IV were the predominant toxigenic cultures.

The boiling time used by Bernhof for the detection of the initial stage of the disassociation of the culture, has been widely used in selecting cultures of typhoid-paratyphoid, and dysentery groups for the production purposes.

We also utilized this test, but the microbes were suspended not in the physiological solution, but in three percent saline solution, the pH of which was equal to 7.6, as maintained by the addition of five percent solution of Na_2HPO_4. The degree of homogeneity of the culture was determined by the naked eye, and also with the use of a lens and the agglutinoscope. A suspension of one billion microbes were boiled for one hour on a steam bath and placed for an additional eighteen hours in the 37° thermostat, after which

it was re-examined. (Table 2).

Table 1.

Characteristics of the investigated diphtheria culture.

a Культура	e Число культур	f Источник выделения: g Больной	f Источник выделения: h Бактерионоситель	i Биохимический тип: Gravis	i Биохимический тип: Mitis j	k Серологический тип: I	k: II	k: III	k: IV	k: VI	l Токсигенность: +	l Токсигенность: —
b Свежевыделенная	28	18	10	25	3	0	5	8	15	0	23	5
c Музейная	18	12	6	13	5	1	3	3	9	2	11	7
d ВСЕГО	46	30	16	38	8	1	8	11	24	2	34	12

Note: Designations: + toxigenic culture; - nontoxigenic culture.

Key: a- Culture; b- Freshly isolated; c- Museum; d- Total; e- Number of cultures; f- Source; g- Diphtheria patient; h- Bacteria carrier; i- Biological type; j- Mitis; k- Serological type; l- Toxigenicity;

The flaky deposit formation phenomenon was observed to almost the same extent both in the freshly isolated as well as in the museum cultures and equally for gravis and mitis types. Fifty percent of the cultures (twenty-three out of forty-six) displayed it at 100°C and it was observed in thirteen cultures during heating at 100°C for one hour. Only the suspensions of 10 cultures withstood the boiling test, however, after incubation in a thermostat for 18 hours at 37°C they were also found to be nonhomogeneous when examined through the lens and through the agglutinoscope.

In spite of the fact that none of the investigated cultures passed the boiling test, this test permits the

detection of the difference in the stability of suspensions of diphtheria cultures with respect to heating.

Table 2.

Boiling tests for Diphtheria cultures.

Гомогенность культур при температуре a	Число культур e	f В том числе			
		Негомогенные g	Гомогенные h	Свежевыделенные i	Музейные j
				gravis mitis	gravis mitis
a 50—90°	46	16	30	18 / 4 / 22	7 / 1 / 8
b до 100°	30	7	23	13 / 2 / 15	7 / 1 / 8
c 100° в течение 30 мин.	23	6	17	8 / 2 / 10	6 / 1 / 7
d 100° в течение 60 мин.	17	7	10	5 / 2 / 7	2 / 1 / 3

Key: a- Homogeneity of cultures at the indicated temperatures; b- up to 100° C1; c- 100°C for 30 minutes; d- 100°C for 60 minutes; e- Number of cultures; f- Including; g- Nonhomogeneous; h- Homogeneous; i- Freshly isolated; j- Museum.

The most stable culture with respect to this test (ten cultures), and also four museum cultures (mitis type VI - one culture; gravis type III - two cultures; mitis type I - one culture) of those serological types which were not found through boiling tests were seeded on an agar-blood culture medium. After a twenty-four hour incubation period, ten to twenty colonies of each culture were seeded into three test tubes containing Py medium. After the incubation in a thermostat for eighteen hours, one test tube with culture was placed in a refrigerator, the second was used for the agglutination reaction and the third was used for conducting the test with boiling. This test was conducted only after the study of 10

to twenty subcultures of each strain in the agglutination reaction on glass. Only those subcultures were selected which produced an agglutination reaction with homologous serological type serum.

It can be seen that serological types I, II, and VI were represented only by the mitis cultures and the serological type IV was represented only by the gravis culture, while the serological type III was represented both, by gravis and by mitis cultures (Table 3).

Table 3.

The serological and biological characteristics of cultures taken fro further selection

Diphtheria culture	Serological type									
	I		II		III		IV		VI	
	gravis	mitis	gravis	mitis	gravis	mitis	gravis	mitis	gravis	matis
Freshly isolated	-	-	-	1	-	1	4	-	-	-
Museum	-	1	-	2	2	1	1	-	-	1
TOTAL	-	1	-	3	2	2	5	-	-	1

The agglutination reaction on the glass 210 subcultures of different serological types were investigated. Seventy-three subcultures were agglutinated simultaneously with several serological type serums, fifty-four spontaneously in hypertonic NaCl solution, therefore, only eighty-three subcultures were subjected to the boiling test.

The number of the selected subcultures were different for each strain and varied between two and ten.

The boiling test was passed by 28 subcultures, obtained from nine cultures: four - mitis type (I, II, III, VI) and five - gravis type (IV), In these cultures two were of the mitis type,(I and VI) and were museum specimens (Table 4).

Table 4.

The boiling test of diphtheria subcultures selected by the serological method

a Гомогенность культур при температуре	e Число субкультур	f В том числе: g Негомогенные	h Гомогенные	i Субкультуры: j Свежевыделенные — l Серологический тип: II mitis	III mitis	IV gravis	k Музейные — l Серологический тип: I mitis	II mitis	III mitis	III gravis	VI mitis
50—90°	83	22	61	5	10	19	8	11	3	3	2
					34				27		
b до 100°	61	17	44	3	10	16	8	—	3	3	2
					29				16		
c 100° в течение 30 мин.	44	10	34	2	9	12	7	—	1	1	2
					23				11		
d 90° в течение 60 мин.	34	6	28	2	8	7	7	—	1	1	2
					17				11		

Key: a- Homogeneity of the culture at the following temperature; b- up to 100°C; c- 100°C for 30 mins. d- 100°C for 60 mins. e- Number of subcultures; f- including; g- Nonhomogeneous; h- homogeneous; i- Subcultures; j- freshly prepared; k- Museum; l- serological type.

After these cultures were kept for an additional eighteen hours in a thermostat at 37°C an insignificant inhomogeneity of the microbe suspension was detected in eighteen subcultures during the examination in the agglutinoscope.

Thus, the suspensions of the diphtheria cultures had a different degree of stability to heating. This stability coincided in a number of cases with the greater homogeneity of the microbe suspension.

The investigations, conducted in this laboratory (Delyagina and Sadykova), and also at the State Control Institute im. Tarasevich (Suslova), have shown that the best method for preserving diphtheria cultures is storage in the dry state. The specimens were freeze dried. This method permits prolonged storage (three to four years of observation) without charges of the initial properties of the culture. During the lyophilization of the diphtheria culture either normal equine serum or a saccharose-gelatin medium are used as fillers. We used the latter. The preliminary index of the satisfactory lyophilization was the external appearance of the culture (compact precipitate, not adhering to the walls of the ampule, solubility in the course of two to three minutes and low residual moisture content - 1.35%).

The freeze-drying of the culture was conducted immediately after the selection. The duration of the storage of the cultures in the refrigerator did not exceed seven to ten days.

Only four of the selected subcultures, three of which passed the boiling test, preserved their initial serological properties prior and after lyophilization. The remaining cultures, partially prior to and partially after lyophilization lost their properties and agglutinated spontaneously in hypertonic solution with sodium chloride, or produced positive agglutination reactions with the heterological type diphtheria serums. (Table 5).

Since diphtheria cultures are highly labile, and have a tendency to agglutinate spontaneously, their selection process is extremely time consuming, complex and laborious.

However, the study of cultures, the selection of subcultures on the basis of the agglutination reaction and the boiling test aid in the selection of the most suitable strains.

Table 5.

Serological properties of the selected diphtheria culture prior to and after lyophilization

a Гомогенность культур при температуре	f До сушки: j Число культур	h В том числе негомогенные			i Реакция агглютинации			g После сушки: j Число культур	i Реакция агглютинации		
		k глазом	l через лупу	m в агглютиноскоп	n только с сыворотками своего серологического типа	o с сыворотками других серологических типов	p спонтанная		n только с сыворотками своего серологического типа	o с сыворотками других серологических типов	p спонтанная
50—90°	4	4	—	—	3	1	—	3	1	1	1
b до 100°	1	1	—	—	1	—	—	1	—	—	1
c 100° в течение 60 мин.	2	2	—	—	—	—	2	—	—	—	—
d После часового кипячения в термостате в течение 18 час.	7	3	4	—	5	2	—	5	3	—	2
e ВСЕГО	14	10	4	—	9/9	3 / 5	2	9	4/4	1 / 5	4

Key: a- Homogeneity of the culture at the following temperature; b- up to 100°C; c- 100°C for 60 minutes; d- After one hour boiling in a thermostat for eighteen hours; e- Total; f- Prior to drying; g- After drying; h- Including non-homogeneous; i- Agglutination reaction; j- Number of cultures; k- Naked eye; l- With the use of a lens; m- In the agglutinoscope; n- Only with homologous serological type serums; o- With serums of other serological types; p- Spontaneous.

The subsequent lyophilization permits the storage of these strains for long periods of time.

Simultaneously with the selection of cultures different culture media were investigated. For the production of

homogeneous diphtheria microbe suspensions the optimum concentration of NaCl and the pH were selected.

Five different culture media were investigated: 1) three percent beef-peptone agar containing 20% normal equine serum (the medium recommended by the instructions); 2) Py medium (coagulated equine serum); 3) 2.5% Martin's agar with doubled concentration of beef water, and twenty percent normal equine serum; 4) 2.5% Martin's agar with doubled concentration of beef water and two percent of manifester (instead of serum); 5) 2.5% Martin's agar with doubled concentration of meat water, two percent manifester and twenty percent normal equine serum.

We selected five different serological type diphtheria cultures for investigation. Each culture was seeded in test tubes containing the above media.

After the cultures were held in the thermostat in the course of a day at 37°C the culture was washed from the medium with three percent saline solution, a standard dilution was made to the concentration of one billion microbes/ml and the microbe suspensions were examined in the agglutinoscope. Different cultures, grown on the same media developed different degrees of inhomogeneity.

The best medium for the production of homogeneous suspensions was found to be the Py medium. Even the culture 163, producing a completely inhomogeneous suspension on all other media, gave an almost homogeneous suspension on this medium. The worst medium was 2.5% Martins-agar with doubled concentration of meat water with the addition of two percent of manifester (Table 6).

To determine the effects of the concentration of NaCl on the homogeneity of the microbe suspensions, we prepared solutions containing different concentrations of sodium chloride (three to twelve percent).

The investigated one day diphtheria cultures were washed

with the above salt solutions and subjected to the boiling test. The best results were obtained with three percent saline solution. Flaky sediments were formed only after one hour boiling of the microbe suspension. With increase of the concentration of sodium chloride, flaking of the suspensions occurred earlier and at lower temperatures. Thus, for example, when the concentration of NaCl was four to five percent, the flaky sediment formation occurred at 100°C while when the concentration of sodium chloride was increased to six to twelve percent, sediments were formed at 90°C.

We also investigated the effect of pH on the homogeneity of the microbe suspension, since the buffer mixture, recommended by the preparation instructions, was insufficiently effective, apparently dur to the presence of only neutral salts, (NaCl) and basic salts (Na_2HPO_4) in it, and the absence of the acid salt.

For our work we selected a phosphate-buffer mixture, consisting of two salts; basic - Na_2HPO_4 and acid - KH_2PO_4. Buffer solutions of different pH's were prepared: 7.6, 8.04, 8.4, and 8.64.

We prepared one billion formaldehyde treated microbe suspensions with the phosphate buffer solution of different pH's. The homogeneity of the suspensions at the time of the preparation was determined by the naked eye, through the lens and through the agglutinoscope. A slight inhomogeneity was detected only in the examination of the suspension in the agglutinoscope.

After thirty day storage of the suspensions in the refrigerator, their homogeneity and pH's were tested again. These tests revealed no change in the degree of homogeneity. The pH of the microbe suspension and of the control buffer salt solutions was changing.

The change in the pH did not occur due to the presence of the microbe cells in the solution, since the pH of the control

saline solution was also changing simultaneously. The smallest change of the solution was observed at pH equal to 7.6.

Table 6.

Study of the homogeneity of diphtheria microbe suspensions grown on different culture media.

a Культура	b Серологический тип	c Биохимический тип	d Гомогенность взвеси, полученной с питательных сред (учет в агглютиноскопе)				
			e 3-процентный мясо-пептонный агар и 20% нормальной лошадиной сыворотки	i 2,5-процентный мартеновский агар и 20% нормальной лошадиной сыворотки	j 2,5-процентный мартеновский агар с 2% манфестатора	k 2,5-процентный мартеновский агар с 20% нормальной лошадиной сыворотки и 2% манфестатора	l Среда Ру
42	I	Mitis	f Негомогенность	f Негомогенность	h Значительная негомогенность	g Незначительная негомогенность	g Незначительная негомогенность
692	II	Mitis	.	.	.	f Негомогенность	.
151	III	Gravis	g Незначительная негомогенность	.	.	.	.
163	IV	Gravis	h Значительная негомогенность	h Значительная негомогенность	.	h Значительная негомогенность	.
702	VI	Mitis	g Незначительная негомогенность	g Незначительная негомогенность	.	g Незначительная негомогенность	.

Key: a- Culture; b- Serological type; c- Biochemical type; d- Homogeneity of the suspension obtained from various culture media (on the basis of agglutinoscope examination); e- Three percent meat-peptone agar and twenty percent normal equine serum; f- Nonhomogeneity; g- Insignificant nonhomogeneity; h- Sigificant nonhomogeneity; i- 2.5% Martin's agar and twenty percent normal equine serum; j- 2.5% Martin's agar with 2% manifester; k- 2.5% Martin's agar with 20% normal equine serum and 2% manifester; l- Py medium.

Table 7.

Change of pH of the microbe suspension upon storage in the refrigerator.

pH of the Microbe Suspension		pH of the Control Suspension	
At the time of preparation	After 30 days	At the time of preparation	After 30 days
7.6	7.44	7.6	7.44
8.04	7.36	8.04	7.43
8.4	6.61	8.4	7.58
8.64	7.59	8.64	7.59

Thus it was established that the most homogeneous suspensions of the serum microbes were obtained from cultures grown on a Py medium with the use of three percent NaCl solution, the pH of which was adjusted to 7.6 with a phosphate buffer mixture.

However, the diagnostic preparation, produced from the selected cultures, grown on the best culture medium with the use of optimum concentration of NaCl and pH did not display any stable properties in the course of the first few months of storage.

We made an attempt to dry the diagnostic preparation per se or with the use of filler. The dry diagnostic preparations would help us to solve a number of difficulties, particularly those associated with the stabilization of the principal properties of the preparation. This would also solve the problem of prolonged storage of the preparation. In the role of fillers we used saccharose-agar-gelatin medium (distilled water - 100 ml, saccharose 10 g, gelatin 1 g.,

and agar 0.1 g) and saccharose-gelatin medium (distilled water 100 ml, saccharose 10 g, gelatin 1 g). A one day diphtheria culture grown on a Py medium was washed with one of the above media as well as with three percent NaCl solution.

The obtained suspensions of the diphtheria microbes were examined with the naked eye, through a lens and in the agglutinoscope. Only the suspensions, prepared on a three percent saline solution were homogeneous, but the small granular consistency was detected even in these when viewed in the agglutinoscope. The nonhomogeneity of the microbe suspensions with fillers was detected during the examination with the aid of a lens. The subsequent lyophilization of diagnostic preparations, made on the basis of these microbe suspensions, increased their nonhomogeneity even more, and the preparation was found to be totally unsuitable for work. Lyophilization also ruined the diagnostic preparations dried per se.

Thus, the instability of the principal properties of the diagnostic preparations, are determined by the fact that diphtheria microbe cells contain predominantly hydrophobic protein compounds, which in fact determine the nonhomogeneity of the microbe suspension. The slight inhomogeneity is always present in the preparation even at the moment of its synthesis. Storage of the diagnostic preparation produces even greater inhomogeneity, which manifests in the formation of larger and larger conglomerates in the solution. These particles, precipitate and are then removed during the filtration of the diagnostic preparation through the cotton filter. This in turn produces the change in the concentration of the microbe suspension.

At the same time lysis of the microbe cells occurs, as a result of which acid products are liberated (for example ribonucleic acid), which in turn decreases the pH of the solution.

The agglutinogenic properties of the preparation change. The acidified solutions facilitate even greater precipitation of the particles from the solution which in turn leads to the sedimentation of flakes.

Conclusions

1. Since diphtheria cultures are extremely labile and have a tendency to undergo spontaneous agglutination, the process of selection of the diphtheria cultures is extremely complex and laborious.

2. The study of the strains, the selection of subcultures on the basis of the agglutination reaction and the boiling test aid in the detection and selection of the most suitable strains from the standpoint of their antigenic properties.

3. The subsequent lyophilization enables one to stabilize the properties of these strains for long periods of time (time of observations three to four years).

4. The most homogeneous suspensions of diphtheria microbe were obtained when the cultures were grown on Py culture medium with the use of three percent sodium chloride solution, the pH of which was adjusted to 7.6 by means of phosphate buffer mixture.

5. However, the use of the selected cultures, grown on the optimum culture medium and under the optimum concentrations of NaCl and pH did not respond by the stabilization of the principal properties of the diagnostic preparation even for the first few months of storage.

6. The lyophilization of the diagnostic serum per se and in combination with fillers increases the degree of the nonhomogeneity of the preparation even more, and it becomes totally unusable.

7. The instability of the principal properties of the diphtheria diagnostic preparation results from the fact that

at present it is not possible to obtain totally homogeneous suspensions of the diphtheria microbe. Therefore, subsequent work in the area of the improvement of diphtheria diagnostic preparations by this approach, i.e., as a bacterial suspension prepared on the industrial scale offers little promise.

Bibliography

[1] Delyagina, L. P. Serologicheskoye tipirovaniye vozbuditelya i serodiagnostika Difterii (Serological Typing of the Causal Organisms and Serological Diagnosis of Diphtheria). Candidate's dissertation, Moscow, 1950 (Ministry of Health of the USSR. Bureau of Institutes of Vaccines and Serums. Moscow Scientific Research Institute of Vaccines and Serums im. I. I. Mechnikov.

2. Ewing, J. The serological grouping of the starch—fermenting of diphtheriae. J. Path. a. Bact. 1933, 37, 3, 345.
3. Freeman, V. J. a. Minzal, G. H. Serological studies of Corynebacterium diphtheriae. Am. J. Hyg. 1952, 55, 74—82.
4. Ferris, A. A. Type specific agglutinins in Corynebacterium diphtheriae infection. J. Path. Bact. 1950, LXII (2), 157.
5. Hammerschmidt, J. Ueber Gruppenbildung bei den Diphtheriaebazillen. Zbl. f. Bakt. I. Abt. Orig. 1924, 93, 443.
6. Kröger, E., Thofern, E. und Schulz, K. Serologische Untersuchungen bei Diphtheriaebakterien. Ztschr. Hyg. Infekt. Krankh. 1952, B. 5, s. 254.
7. Minzel, G. H. a Freeman, J. Serologic studies of C. diphtheriae. Am. J. Hyg. 1950, 51, 3.

THE PRODUCTION OF ADSORBED AGGLUTINATING SALMONELLA O-SERUMS

by

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The adsorbed agglutinating o-serums of the salmonella group are used for the classification of the microbes into one of the serological groups (A, B, D, C, E, etc.) according to the Kaufman-White classification.

One of the principal stages in the production process of adsorbed agglutinating serum is the preparation of antigen for the immunization of animals. The methods, used for the preparation of antigens, are based on the action of different antigens on the antigen composition of the microbe cell. As it is well known, the microbe cell of salmonella consists of several antigens, differing both in their chemical composition and their location in the cell, as well as in their reactions to physico-chemical interaction. Thus, the flagellum type H-antigen is a protein type substance. It is thermally labile and it is totally destroyed during 2½ hour boiling of the bacteria suspension. Its partial or total inactivation is also observed upon the addition of alcohol or phenol to the culture. Chemically, O-antigen is a lipoid-polysaccharide-protein complex and it is bound to the body of the microbe cell. O-antigen is stable for long periods of time when exposed to elevated temperatures, and also to a number of chemical substances such as alcohol, ether, acetone, formaldehyde and phenol (in low dilutions). The stability of O-antigen of the microbe cells to certain physico-chemical interactions was used as the basis for the development of the production method for antigen, used in the immunization of animals,

which in turn produce immune O-serum. For the preparation of O-antigens, Kaufman (1954) recommends broth or agar cultures, sterilized by 2½ hour boiling at 100°C on a steam bath or autoclaving. Thus, according to Kaufman, a total destruction of the flagellum apparatus of the microbe is achieved. The alcohol treatment of cultures for the preparation of O-serum is not recommended by Kaufman. He considers that during the action of alcohol on the microbes only their H-agglutinating properties are eliminated, while their agglutinogenic ability is totally preserved. In the opinion of Edwards (1951), during the treatment of the culture with alcohol, more or less complete inactivation of the H-antigen is achieved, therefore, serums obtained during the immunization of animals with alcoholic antigen have relatively low H-antibody titer. Edwards also notes that when boiled antigens are used the obtained serums generally have low O-antibody titer. In order to obtain highly active O- and H-serums, he recommends the treatment of cultures, designated for immunization according to the method proposed by Roschka (1950).

The Roschka Method

A twenty hour agar culture is washed with physiological solution and the obtained suspension is heated in a stream of steam in the course of 2½ hours. The heated suspension is centrifuged, dissolved in 96 proof alcohol and held in a thermostat at 37°C, following which it is again centrifuged and washed twice with acetone. The newly obtained precipitate is dissolved in several milliliters of acetone and poured into a Petri dish, and placed into a thermostat for drying for a period of 14 to 18 hours. The powder prepared by this method is ground with a mortar and pestle, dissolved in physiological solution and used as antigen for the immunization of animals.

Weil (1918) also recommends the alcohol treatment of the culture for the preparation of O-antigens. He indicates that even from the culture, existing in H-form, during the treatment with alcohol it is possible to obtain pure O-antigen, The alcohol treated antigens have the advantage that they are less toxic than the boiled antigens. Using the alcohol treated antigens for the immunization of animals Chernokvostov and Letavet obtained high quality serums, which could not be obtained by boiling or formaldehyde treatment of antigens from the Shig strain of dysentery due to the low survival of tha animals.

Since 1954 our laboratory also uses alcohol treated antigens for the production of the salmonella O-serum, since the use of boiled antigens for this purpose constantly produces O-serums with low titers. The alcohol treated antigens are prepared by the method analogous to the one which was proposed by Chernokhvostov and Letavet (1941) for the production of Shiga strain dysentery antiserum.

The Chernokhvostov and Letavet Method

An eighteen to twenty-hour agar-grown culture is washed with physiological solution, placed in the preweighed test tubes and centrifuged. Subsequently, the liquid is decanted and physiological solution is added to the weighed suspension (based on the calculation one ml/g of the precipitate) and ten times the volume of alcohol. The antigen is placed into a thermostat at 37°C, for two days, then centrifuged and to the precipitate 0.5% carbolic acid treated physiological solution is added in the amount sufficient to produce ten billion microbes per ml. After the preparation is checked for sterility it is ready for the immunization or animals.

The use of alcohol treated antigens in the course of a number of years enabled us to obtain serums with sufficiently high O-antigen titers (1:12,800 to 1:25,600). Having found

in literature indications that the highest O-antibody titers were obtained during the immunization of animals with boiled alcohol-acetone antigen (according to Kaufman - boiling) we decided to conduct parallel immunization of rabbits with all three forms of O-antigen and to analyze the obtained results. The rabbits were immunized with antigen prepared from the following cultures S. paratyphi A. S. paratyphi B, S typhi murium, S heidelberg, S. cholerae suis, S. newport and S. london, in order to obtain serums for the microbes most widely spread in the Soviet Union (i.e. salmonella groups A, B, C, D, and E according to Kaufman-White classification scheme).

Since only smooth colonies can be used for the preparation of O-antigens the strains which we collected were seeded on the agar plate for the selection of isolated colonies in the S-form. This enabled us to exclude the morphologically coarse shapes and to use only the shiny convex colonies, stable in physiological solutions and not producing spontaneous agglutination during boiling. In terms of their biochemical and serological properties the strains were typical. They produced very high agglutination titers (1:12800 to 1:25600) with homologous O-serum and did not produce positive reactions with heterological serums.

For the production of antigens from each salmonella group, cultures were grown on a dense carbolic acid treated Martin's agar in a thermostat at 37°C. The microbes were washed with physiological solutions and the whole collected suspension was subdivided into three equal parts, from which boiled, alcoholic and boiling-alcohol-acetone treated antigens were prepared, according to the above-described method. After the four day testing of the sterility of the obtained antigens, immunization of the rabbits was attempted. The antigens were introduced intraveneously with a four day interval, at the following doses: for the first injection - 500 million, for the second

injection - one billion, for the third injection - 1.5 billion and for the fourth injection - 2 billion microbes. Seven days after the last injection blood was taken from each rabbit for the determination of O- and H- agglutinin titer. The titer is defined by the instruction on the production of monoreceptor serums as the maximum dilution of serum at which agglutination, characterized by three phases (+++) is still observed. The O-agglutinin titers in serum's, were determined in the linear agglutination reaction by means of the same cultures as were used for immunization. Prior to the agglutination experiment these cultures were heated for thrity minutes, in order to destroy their H-agglutinating properties.

In order to determine the H-agglutinin titers the serum was checked with specially selected cultures, belonging to other serological groups and consequently having the possibility of reaction with the experimental serums only due to their H-antigens. Thus, for example, serums E (O), obtained by immunization of rabbits with S. london (antigen formula - 3.10; 1, v; 1.6) were checked as follows: the magnitude of the O-antibody titer - with boiled S. london culture; the magnitude of H-antibody titer - with live S brandenburg culture (antigen formula - 4, 12, 1, v; e, n, z_{15}) and with S. inverness (antigen formula - 3, 8; [illegible]: 1, 6). The last two cultures were used for the determination of antibodies to the first and the second H-antigen of S. london in the serums, by means of which these serums were obtained.

The examination of the results of the immunization of rabbits with all forms of antigens showed that the highest O-antibody titers (1:12800; 1:25600) were displayed by serums, obtained with alcohol treated antigens. (see figure).

From the obtained native serums, having O-antibody titer not lower than 1:6400, the corresponding adsorbed agglutinating serums were prepared. In spite of the fact that the serums obtained with the use of alcohol antigens had higher H-antibody

titers than analogous serums obtained by means of boiling and boiling-alcohol-acetone treated antigens, adsorbed serums were prepared form these with equally high specific O-titers.

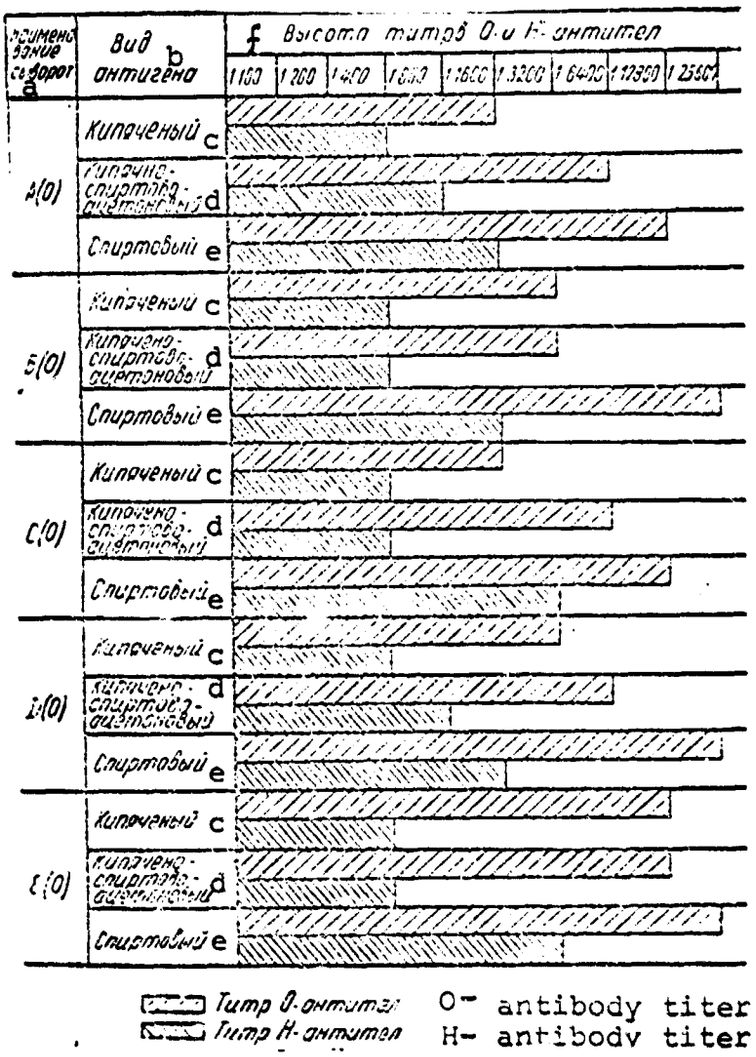

Figure 1. Summary table for O- and H- antibody titers in serums obtained by the immunization of rabbits with different O-antigens.

Key: a- Type of serum;
b- Type of antigen;
c- Boiling;
d- Boiling-alcohol-acetone;
e- Alcohol;
f- Magnitude of O- and H- antibody titers.

Apparently, this is explained by the fact that due to high O-titers alcohol serums can be diluted more with physiological solutions prior to adsorption, which lowers the concentration of the H-antibodies in them and more favorable conditions are created for the adsorption process.

Conclusions.

1. In the preparation of the salmonella O-serums, the highest O-antibody titers were obtained by immunization of animals with alcohol treated antigens. In this case the serums had O-agglutinin titers equal to 1:12800 to 1:25600. Lower O-antibody titers (on the average 1:3200 to 1:6400) were observed when boiling alcohol acetone-treated vaccines were used (Raschka method). Boiled antigens were found to be even less effective. The O-serum titers obtained with the use of boiled antigens, in the majority of cases did not exceed 1:3200.

2. In spite of the fact that the immunization of animals with alcohol treated antigens caused the formation of higher H-antibody titers than other experimental O-antigens, adsorbed agglutinating O-serums of high specificity were prepared from these serums.

Bibliography

[1] Kaufman, F. Semeystvo kishechnykh bakteriy (Intestinal Bacteria Family), Moscow, State Medical Publishing House, 1959.

[2] Chernokhvostov, V. A. and Letavet, K. S. "Preparation of Antidysenterial Vaccines" ZhMEI, No. 5 - 6, 105-112, (1959).

[3] Roschka, R. Eine neue Methods zur Herstellung hochwertiger O-Immunseren der Salmonelly teuppe. [A new method for producing highly effective O-serums of the Salmonella type.] Klinische Medizin [Clinical Medicine] 1950, H. 2, 88-93

A METHOD OF ULTRATHIN SLICES[1]

by

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Starting in 1940, several foreign companies began the production of electron microscopes, and from that time on electron microscopy began to be widely used in laboratories as a new research method.

In analyzing the work on the electron microscopy of biological objects (the number of these works exceed one thousand), one instantly notes a difinite systematicity both in the choice of the investigated objects, as well as in the methods used for the investigations.

Thus, initially the objects of investigation were only finely dispersed objects such as bacteria, viruses, and colloidal particles. As a result of this the cytology of bacteria, the morphology of viruses, and the processes of the interaction of bacteriophage with microbe cells were investigated.

Subsequently, in addition to this method was proposed for the artificial dispersion of fiberous structures and this permitted the study of such objects as muscles, collagen, etc.

The attempts to study the surfaces of certain more or less complex bodies (bone, hair, teeth, seeds, leaves, etc,)

lead to the development of another method - the so-called replica method, The essence of this method lies in that an imprint of the surface of the dense object is prepared from a thin transparent film, which is subsequently studied under the electron microscope.

Nonetheless, all these methods have one general drawback, they do not permit the investigator to penetrate into the details of the inner structure of the investigated object due to the almost total impermeability of the investigated objects to the electron beam.

Due to relatively low density, i.e. light atoms, which comprise of organic structures, the thickness of the object, which can be studied in the electron microscope, must be of the order to tenths or even hundredths of a micron. This situation was a stimulus for the research directed towards the production of ultrathin slices, sufficiently penetrable by the electrons.

The histroy of the preparation of ultrathin slices began in 1939, when Ardenne first obtained ultrathin slices and investigated them on the electron microscope. The author was displacing a block with tissue of an angle to the ordinary histological microtome, thus obtaining slices in the form of wedges, the base of which had a thickness of one micron, while the apex had a thinner edge and was suitable for study on the electron microscope.

However, this method of "wedge microtoming" was not widely accepted, and later Richards, Anderson and Hance (1943), using an ordinary histological microtome with a special transmission, were able to decrease the thickness of the slices to 150 mμ. This method of "slow microtoming" was later improved and the thickness of the slices was decreased further to 100 mμ.

In order to avoid the deformation of the slices during the cutting operation, O'Brien and McKinley (1943) used high

speed microtoming, the method by which the object was fed to the knife, rotating at 40,000 rpm. Such a method produced slices thinner than 100 mμ.

A new method was proposed by Newmen, Borisko and Swerdlow (1950). The thick object was enclosed into butylmethacrylate monomer. After polymerization freezing lead to the compression of the block, after which during the subsequent thermal expansion on the ordinary microtome slices were obtained, the thickness of which was equal to 100 mμ.

Finally, in recent years one more method was proposed for obtaining thin slices, the originality of the design of which had a tremendous impact on the achievements in ultramicrotoming.

In 1953, Sjöstrand constructed a microtome, in which the feeding of the rotating object to the fixed knife is realized through the thermal expansion of the rod upon which the mounted block is resting.

The proposed model of the microtome differs favorably from all of its predecessors by the absence of feeding micrometer screws, which inevitably have some play. The play in the screws and rocking in the bearings do not permit one to obtain serial slices of thickness less than 0.4 mμ. On the other hand the Sjöstrand ultramicrotome permits one to obtain a series of slices of thickness down to 0.01 mμ.

However, the basic improvement in the technology of the production of ultrathin slices in addition to the inventiveness of the instrument builders depends to a significant extent on the use of new methods for treating the investigated material and the preparation of blocks.

An important condition for obtaining satisfactory results in the preparation of slices is rapid and reliable fixing of the investigated tissues. In this area the most successful work was conducted by Palade. The author developed in great detail a method which permits fixing biological

materials without disruption of their microstructure. He determined the most important favorable conditions such as the pH of the medium and the temperature of fixing.

Coating of the investigated material with paraffin or collodion, used widely in histological practice, was found to be totally inapplicable in the preparation of slices, the thickness of which is of the order of a few hundredths of a micron. In 1950 Newmen, Borisko and Swerdlow proposed a new method for coating with monomers, butyl and methyl methacryhates. While these substances are sufficiently transparent, they also permit the production of blocks of the necessary hardness when they are mixed in different ratics.

Finally, one more improvement, instrumental in the progress of ultramicrotoming was, the proposal of Latta and Hartmann (1949) on the use of glass knives. It resulted in the exclusion of the very complex sharpening process (involved in the use of metal knives), and thus simplified the preparation procedure for the cutting of the blocks.

The technical and methodology achievements in the area of microtoming resulted in a significant expansion of electron microscopic investigations directed towards the study of the inner microstructure of cells and tissues. Much recent work encompasses a diversified assortment of the investigated objects. Without pausing on detailed considerations of the obtained results, we should only point out the most significant ones.

Thus, by means of electron microscopic investigation of ultrathin slices of many objects, particularly those which are most important and perform complex functions in the organism (nerve tissue, retina, pancreas, etc.) a surprising ordering of the microstructure was discovered, which is called "submicroscopic layering," indicating a definite space orientation of the macromolecular compounds. The discovery of such ordering of the structure of cells of different

sections of the organism is a morphological basis for the well-known rigorous sequence of physiological and biochemical processes in various tissues.

Many new facts have been accumulated by means of ultrathin slices and the study of the cytology of bacteria. Relatively recently, serial slices of virus particles were obtained by means of this method.

Without posing the problem of analyzing the factual material accumulated through the use of the ultrathin slices method in biology we would like only to stress by this brief historical survey the following.

The work of recent years has shown that the method of ultrathin slices may be used successfully for the solution of a number of physiological immunological problems, which opens wide horizons for the use of electron microscopy in the new areas of biology and medicine.

Bibliography

1. Ardenne, M. Z. Zschr. wiss. Mikr. 1939, 56, 8—23.
2. Latta, H., Hartmann, J. F. Proc. Soc. exp. Biol. a. Med. 1950, 74, 436—439.
3. Newmen, S. B. Borisko, E. Swerdlow, M. Science. 1949, 110, 66—68.
4. Newmen, S. B., Borisko, E., Swerdlow, M. J. Res. Nat. Bureau Standards. 1949, 43, 183—199.
5. O'Brien, H. C., McKinley, G. M. Science. 1943, 98, 455—456.
6. Palade, G. E. J. exp. Med. 1952, 95, 285—297.
7. Richards, G. A., Anderson, Th. F., Hance, R. T. Proc. Soc. exp. Biol. a. Med. 1942, 51, 148—152.
8. Sjöstrand, F. S. Experientia, 1953, 9, 114—115.

THE USE OF MIXERS AS CONTAINERS FOR CONDUCTING OPSONO-PHAGOCYTIC REACTIONS

by

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In recent years in our scientific literature a number of works have been published (Latysheva, 1955; Berzin' and Blyumberg, 1958; and others) the purpose of which was to develop the optimum standard conditions for the study of opsono-phagocytic reactions.

In these investigations there are two quite limiting conditions associated with the volumes of blood required by different methods. The method proposed by Latysheva requires a large volume of blood (of the order of 10 ml), while the method of Berzin' and Blyumberg permits the utilization of small volumes of blood (of the order of hundredths of a milliliter). Each of the investigators wishing to express his preference for a given method must base his conclusions on the specific conditions of his own work. In work with large animals (dogs, rabbits, etc.) the method proposed by Latysheva is totally suitable. In work with small animals (rats, mice, etc.) it is preferable to use a method which requires small volumes.

The necessity of working with small animals (rats) forced us to develop a microvolumetric method for conducting opsono-phagocytic reactions. We utilized mixers (mixers which are used in the dilution of blood), which replaced in our experiments both the micropitet and the test tubes.

The simple method which we proposed enables one to study the reaction in the volume of ~0.02 ml, i.e. 0.005 ml. of 2% solution of sodium citrate, 0.01 ml of blood and 0.005 ml containing two billion microbes in suspension. We indicate

the approximate accuracy of the absolute volumes because we did not use the accurately calibrated micropipets, but mixers for erythrocytes in which the volume proportions are accurately graduated. In the available mixers the volume of the capillary part to the marker "1" is approximately 0.01 ml.

Experimental procedure: 1) sodium citrate was taken up into the capillary part of the mixer to "0.5" marker and then sucked into the expended part of the mixer; 2) blood from the candel vein of a rat was taken to marker "1" of the same mixer and sucked into the expended part after which both liquids were carefully mixed; 3) the microbe suspension (Gertner's bacilli in our experiment) was taken up to "0.5" marker of the same mixer and sucked into the expended part and again mixed with the citrate diluted blood.

After a thorough but careful mixing of the liquid in the mixer, the mixers were placed in the horizontal position (on special stands) in a thermostat at 37°C for thirty minutes.

After the expiration of the indicated period of time a smear was prepared from the content of the mixture (fixed with methyl alcohol and stained with azurin-eosin solution).

The sodium citrate and microbe suspensions are best aliquoted not from the large vessel, but from micropipets, by making a contact between the openings of the micropipet and the mixer. This produces the highest accuracy. If during sucking of the blood from the capillary part of the mixer into the expended part of the mixer, a bubble is formed it may be removed easily by means of a thin wire introduced through the capillary of the short end of the mixer.

Using the microvolumetric method with mixers, we studied the opsono-phagocytic reaction for two groups of animals (for adrenalectomized rats, and rats with normal adrenal glands) before and after passive immunization with the rabbit serum (rabbits were immunized with formalin treated vaccines

from Gertner's bacilli culture). We determined the phagocytic activity of the neutrophil in smears (the percent of active cells out of the total number of cells counted) and the phagocytic number (i.e., the number of microbe cells per active phagocyte). The results of the investigation are shown in Table 1.

Table 1.

Results of the Investigation of opsono-phagocytic reactions

Index	Experimental conditions			
	Prior to immunization		After immunization	
	Animals			
	Adrenalectomized	With normal adrenal glands	Adrenalectomized	With normal adrenal glands
Phagocytic activity of neutrophils (in %)	26	60	23	92
Phagocytic number	3.3	3.2	2.6	6.4

The cited data indicate two conditions: first of all opsono-phagocytic reaction i adrenalectomized animals is weakened in comparison with the same reaction in animals with normal adrenal glands; secondly, passive immunization increases opsono-phagocytic reaction under the action of normal adrenal glands and does not change in adrenalectomized animals.

On the basis of the cited data one may conclude that the adrenal glands play a significant role in the determination of the functional state of microphages and participate in the

functioning of the mechanism through which the passive immunization effect is realized. It is possible that inclusion of the function of the adrenal glands into the reaction to the injected immune serum is one of the physiological mechanisms of the preventative action of such serums.

The comparison of the earlier data on the sharp lowering of the natural resistance of animals to infections under the influence of adrenalectomy with the above results may lead to another conclusion: the indicated lowering of the resistance of animals to infection may be associated with lowering of the phagocytic reaction under the influence of adrenalectomy.

Conclusions

1. The use of mixers in carrying out opsono-phagocytic reactions is convenient since it enables one to carry out the reaction in microvolumes and to insure maximum protection of the neutrophils during aliquoting of the blood.

2. The opsono-phagocytic reaction in microvolumes enables one to conduct chromological experiments on the same animal.

3. The indicated method was used for the study of the role of the adrenal gland in the activity of microphage and it was established that.

a) adrenalectomy leads to weakening of the opsono-phagocytic reaction;

b) the absence of the adrenal gland hinders the activation effect of the immune serum on the phagocytosis;

c) the adrenal glands are found to be one of the physiological links in the complex mechanism by means of which passive immunization is realized.

Bibliography

[1] Berzin', V. K. and Blyumberg, M. Ya. "On the Method for Conducting and Evaluation of the Opsono-Phagocytic

Reaction" ZhMEI, No. 1, 124 (1958).

[2] Ivanov, V. M. "Effect of Certain Hormones on the Resistance of Animals to the Toxic Effect of Vaccine of Gertner's Bacilli Vaccine" ZhMEI, No. 6, 113, (1960).

[3] Ivanov, V. M. "On the Role of the Hypophysics and Adrenal Glands in the Natural Resistance of Animals in the Immunological Reconstructions of the Organism During Vaccinations" 3-ya Vsesoyuznaya konferentsiya parofiziologov 4-9 Iyulya 1960 (Third All-Union Pathophysiology Conference July 4-9 1960) (Abstracts of reports) Sverlovsk, 1960.

[4] Latysheva, N. I. "A Method for Conducting Phagocytic Experiments," ZhMEI, No. 1, 76 (1955).

THE EFFICIENT USE OF HORSES FOR THE PRODUCTION ANTITETANUS SERUM

By

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Up to the present time, antitetanus serum has been widely used in the prevention and treatment of tetanus. This necessitates the improvement of the quality of this preparation. The success of the production of highly active antitetanus serum depends on the ability of the organism of the animal, to produce actively the antitoxin in response to the injected antigen, on the conditions of exploitation of the producing animals, on the care and feeding, on the use of nonspecific stimulants in the process of the exploitation of animals and to a large extent on the amount and the quality of antigen used for the immunization (Kats, 1946; Georgadze, 1948; Ryzhov, 1955; Mikhaylova and Temereva, 1953; Yefimova, 1957; Lukin and Kovshov, 1957; Panin and Panina, 1957 and others).

The data of our previous investigations (1953, 1954, 1956, and 1958) have shown that after the first cycle, the use of small doses of high quality tetanus anatoxin produces high titer serum in ninety to ninety-five percent of the horses, while the injection of low immunogenic tetanus anatoxin, even to highly active producers to this form of antigen, leads to low antitoxin titer in the blood. Therefore, the necessity arises to immunize horses starting with the first cycle with tetanus toxin.

The investigations reported here were devoted to the problem of the efficient dosage of tetanus anatoxin in the first immunization cycle of "fresh" horses and selection of the

optimum amount and the quality of antigens and the conditions for the use in subsequent cycles of the exploitation of producers.

In order to determine the optimum antigen dose for starting the cyclic immunization we began to immunize twelve "fresh" horses with different doses of tetanus anatoxin. We used tetanus anatoxin series 459 as antigen. It had anatoxin combining properties equal to 0.01 ml, (measured according to Becker) and immunogenic properties insuring 100% survival of guinea pigs injected with 100 Dlm of toxin.

The immunization of the horses was conducted as follows. In the two months prior to the cyclic immunization of the animals, they were conditioned with two injections (ten and twenty ml) of tetanus anatoxin with a twenty-one day interval between the two injections. On the basis of the concentration of antitoxin after the conditioning of the animals, they were subdivided into three groups: each group consisted of four horses with different concentrations of antitoxin in the blood after the conditioning. The interval between the injection was equal to four to five days (Table 1.)

Thus, after the first immunization cycle, the horses in group I had serum titers lower than the titers in groups II, and III with extended cycles. Therefore, we consider that one ml of tetanus anatoxin for the beginning of the cyclic immunization of horses, conditioned preliminarily, is not the optimum dose and consequently it is not worthwhile to begin the immunization of horses with a one ml dose of tetanus anatoxin.

At present the better dose for the beginning of the immunization of the majority of "fresh" horses is five ml, since the majority of horses immunized with this dose (three out of four) responded by the accumulation of high antitoxin titer.

In the process of production we were also interested in the quality of antigen and in the effect of chan[illegible] of the

antigen on the serum titers, particularly in the horses in which the antitoxin titer decreased in the process of prolonged immunization with ordinary tetanus anatoxin.

Table 1.

a Группа	Лошадь b	Количество АЕ перед цикловой иммунизацией c	d Цикловая иммунизация: Первая доза (в мл) e	Последующая доза (в мл) f	Длительность цикла (в днях) g	h Титр (в АЕ/мл): После цикла иммунизации i	Средний j
I	889	>1/2<1	1	2, 5, 10, 20, 40, 80, 100	48	200	500
	895	>5 <8	1	2, 5, 10, 20, 40, 80, 100	43	400	
	897	=1/2	1	2, 5, 10, 20, 40, 80, 100	41	800	
	906	>1/10<1	1	2, 5, 10, 20, 40, 80, 100	45	800	
II	898	>1 <3	5	10, 20, 40, 80, 100 etc.	35	800	700
	905	>1/2 <1	5	10, 20, 40, 80, 100 etc.	35	800	
	908	>1/10<1/2	5	10, 20, 40, 80, 100 etc.	41	1100	
	934	>1/2<1	5	10, 20, 40, 80, 100 etc.	48	100	
III	899	>1/10<1/2	20	40, 80, 100 и т. д.	35	200	650
	900	>1 <3	20	40, 80, 100 и т. д.	29	500	
	911	>1/2 <1	20	40, 80, 100 и т. д.	25	1500	
	910	>1/10<1/2	20	40, 80, 100 и т. д.	35	300	

Key:
- a- Group;
- b- Horse;
- c- Amount of AE prior to cyclic immunization;
- d- Cyclic immunization;
- e- First dose (in ml);
- f- Subsequent dose (in ml);
- g- Duration of the cycle (in days);
- h- Titer (in AE/ml);
- i- After immunization cycle;
- j- Average.

One group of producing horses was tested for the effect of the change of antigens, differing in the quality indices, particularly: ordinary anatoxin, partially detoxicated and native toxins.

We investigated the effect of the change from the immunization with anatoxin to immunization with native toxins and partially detoxicated toxin and visa versa on the magnitude of antitoxin titers in serum producing animals. The partially detoxicated toxin which was taken for immunization was native toxin, treated with 0.35% formaldehyde solution and kept in a thermostat for seven to ten days. The strength of the toxin varied within 100,000 to 4,000,000 Dlm limits. Its combining ability was equal to thirty to hundred EC.

The partially detoxicated toxin (after seven day detoxication) injected subcutaneously in ten ml dose, had a lethal effect on guinea pigs on the fourth to fifth day after the injection. The toxin obtained in ten day detoxication produced tetanus symptoms in guinea pigs on the fifth or sixth day and death on the tenth to twelfth day after the injection.

The native toxin was characterized by the following indices: Dlm was equal to 100,000 to 2,000,000, combining ability was equal to thirty to 100 EC. The first immunization cycle of horses (which passed through five to eighteen immunization cycles with anatoxin) with the toxin was conducted with the use of one of the following three doses: 50, 100, 200 ml; 100, 200, 400 ml; 150, 300, 500 ml. The interval between injections was four to five days. The subsequent immunization cycles with toxin were conducted according to the same scheme, as the previous immunization cycles with anatoxin. The titers of serums of twenty-five serum producing horses, which were changed to immunization with partially detoxicated toxins in the same doses, of the same of higher quality as anatoxins used previously, were equal to 300 - 1300 AE/ml. The average titer was equal to 650 AE/ml. After the animals were changed to the immunization with partially detoxicated toxins on a sixth,

to nineteenth cycle, the antitoxin titers were equal to 600 to 1700 AE/ml, the average titer was 980 AE/ml, i.e., the average titer increased by 330 AE/ml or by a factor of 1.5.

The comparison of subsequent data indicate the rationale of transferring those horses, in which titers during immunizatior with anatoxin decrease, to partially detoxicated toxin. It should be noted that the quality and the dose of the latter must be not lower than the titers and the dose of the previously used preparation. The situation is totally different with antitoxin titers in horses changed over to immunization with smaller doses of partially detoxicated toxins, or when the quality of the toxin is lower (Tables 2 and 3).

Table 2.

The titer of serums of producing horses, changed over to immunization with smaller doses of partially detoxicated or native toxin.

Лошадь a	Обычный анатоксин b		Недообезвреженный токсин e		Нативный токсин f	
	Доза (в мл) c	Титр d (в АЕ/мл)	Доза (в мл) c	Титр d (в АЕ/мл)	Доза (в мл) c	Титр d (в АЕ/мл)
374		500				400
393		300			50, 100, 200	300
406		250				300
400		400				400
425		400		400		
45	150, 300, 500	350		400		
129		600	100, 200, 400	600		
142		350		200		
153		400		400		

Key: a- Horse; b- Ordinary anatoxin; c- Dose (in ml); d- Titer (in AE/ml); e- Partially detoxicated toxin; f- Native toxin.

Table 3.

Titers of serums of producing horses in change over to immunization with quality lower partially detoxicated toxins.

Horse	Ordinary anatoxin			Partially detoxicated toxin		
	Dose (in ml)	Quality (in EC)	Titer (in AE/ml)	Dose (in ml)	Quality (in EC)	Titer (in AE/ml)
663		100	500		30	300
705		100	500		50	500
477	150, 300, 500	150	600	150, 300, 500	50	500
668		100	800		30	500
673		100	500		30	400

The analysis of the above presented data indicate that decrease of the dose of partially detoxicated toxin or its lower quality does not increase the antitoxin titer in the blood of the horses, and in many cases it leads to lowering of the titer.

When the producing horses are changed from partially detoxicated toxin to the native toxin no positive results were observed either. Thus, nineteen horses during the immunization with partially detoxicated toxins have an average titer equal to 520 AE/ml, while upon transfer to the immunization with the native toxin they produced average titer equal to 510 AE/ml.

Thus, the comparative evaluation of antigens, used for the immunization of antitoxin producing horses had shown that the native and partially detoxicated toxin upon the injection to the producing animal whose antitoxin titer in the blood has decreased, leads to no increase of the antitoxin

titer when the quality of the toxins and their doses are not lower than the corresponding doses of the previously used preparation.

According to our data the toxins in comparison with partially detoxicated toxin of the same quality do not have any advantages. The change of the immunization of horses from one form of tetanus antigen to other forms must be conducted only in those cases when in the animals immunized with this antigen low antitoxin titer is obtained. In the same cases, when the producing horses develop antitoxin in sufficiently high titer during the immunization with ordinary anatoxins change to immunization with other preparations is not beneficial.

The antigens must meet the following requirements: they must have high immunogenic properties and their immunizing strength must be expressed in standard immunizing units. The antigens must produce minimum reactions.

We studied the reactions and hemodynamics in producing horses in the course of the immunization process with different antigens.

The immunization with partially detpxocated toxins did not produce any more serious reactions in horses then ordinary immunization with tetanus anatoxin. The immunization of horses with native toxin produced more severe reactions: the animals were irratated, showed muscular tics, heavy breathing, did not accept food for two to three days and were in a depressed state.

Conclusions

1. The initial cyclic immunization of previously conditioned horses is best done with a five ml dose.

2. It is worthwhile to change horses which produce lower titer during immunization with ordinary anatoxin to partially detoxicated toxin (seven to ten day detoxication). The quality and doses of the detoxicated toxin must not be lower than the

dose of the previously used preparation.

3. In our experiments the toxin was not found to be any more advantageous than partially detoxicated toxins.

Bibliography

[1] Vorb'yeva, M. I., Gindin, A. I., Stankevich, I. A., Ushakova, A. V., Lyutikova, O. I. and Ryabkov, G. Ye. "The Experiments with Nonspecific Stimulation of Antitoxin Formation in Horses Which Produce Therapeutic Serums" Materially po obmenu opytom Ministerstva Zdravookhraneniya SSSR (Materials for the Exchange of Scientific Information of the Ministry of Health of the USSR), Moscow, 2/52, 171-177, (1956).

[2] Georgadza, I. A. K voprosu o poluchenii protivostolbnyachnoy syvorotki vysokogo antitoksicheskogo titra. The Problem of Obtaining Antitetanus serums with High Antitoxic Titer. Author's Abstract of the Candidate's dissertation. Tbilisi 1948.

[3] Yefimova, N. P. "Physiological Characteristics of the Immunological Reactivity" ZhMEI, No. 1, 94-99, (1957).

[4] Kats, F. M. Izucheniye usloviy zakonomernogo polucheniya sil'nukh protivostolbnychnykh syvorotok (Study of the Conditions for the Regular Producing of Strong Antitetanus Serum), Candidate's Dissertation. Khar'kov, 1946.

[5] Lykin, Yu. B. and Kovshov, A. A. "A Comparative Evaluation of Certain Schemes for the Injection of Tetanus Antigen in the Production of Antitetanus Serum" Trudy Ufimskogo IVS (Transactions of the Ufa Institute of Vaccines and Serums), Ufa, No. 4, 191-195 (1957).

[6] Mikhaylova, G. M. and Temereva, V. I. "Production of Antitetanus Serum in a Short Period of Time with Minimum Expenditure of Antigen" Byulleten'po Obmenu opytom (Bulletin for The Exchange of Scientific Information),

Moscow, Vol. 2/43, 25-30 (1953).

[7] Mikhaylova, G. M. and Temereva, V. I. "Study of the Flocculation Reaction as a Method for the Titration of Antitetanus Serum" Sbornik materialov po obmenu opytom (Collection of Data for the Exchange of Information), Moscow, No. 2/45, 36-43, (1954).

[8] Mikhaylova, G. M., Nikitina, M. T., and Temereva, V. I. "The Flocculation Reaction as a Method for Selecting Tetanus Antigens for the Immunization of Horses." Materialy po obmenu opytom, Moscow, 2/52, 193-203 (1956).

[9] Mikhaylova, G. M. Nikitina, M. T., Budylina, V. I., Nikitin, V. P., Ryzhov, A. P. and Popov, F. S. Materialy po obmenu opytom, Moscow, 1/54, 114-119 (1958).

[10] Panin, F. T. and P[illegible]ina, N[illegible] P. "The Possibility of Using Weakly Reacting Horses During the Immunization with Tetanus Antigen as Producers of Serum" Trudy Stavropol' skogo IVS (Transactions of the Strvropol' Institute of Vaccines and Serums), Stavropol', No. 4, 3-9, (1957).

[11] Ryzhov, A. P. Ratsionalizatsiya kormleniya loshadey-produtsentov lechebnykh syvorotok (Selection of Optimum Diet for Horses which are Used in the Production of Therapeutic Serums), Candidate's Dissertation, Moscow, 1955.

STUDY OF THE CONCENTRATION OF LIPIDS IN THE PLACENTAL BLOOD SERUM IN THE PROCESS OF THE PRODUCTION OF GAMMA GLOBULIN

by

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During the production of gamma globulin from the placental serum formation of sediment in the preparation occurs when the latter is stored over different periods of time, and frequently soon after filtration. The cause of this is not always understood.

Earlier (Dubova and Nechayeva, 1958) investigated the effects of the residual alcohol on the physical properties of gamma globulin. The results of the work have shown that the presence of small amounts of alcohol in the finished preparation is not the principal cause for its instability (Table 1).

Table 1.

Effect of the residual alcohol on the stability of the finished gamma globulin product.

Срок хранения (в годах) a	Содержание спирта (в %) b	Число серий c	Характер физических свойств d		Стабильность (в %) g
			Изменился e	Не изменился f	
1,5	0	12	2	10	83
1,5	1,5–3	33	3	30	90
1,5	3–4	28	4	24	85
1,5	4–4,5	11	2	9	82

Key: a- Duration of storage (in years);
b- Concentration of alcohol (in percent);
c- Number of series;
d- Nature of physical properties;
e- Changed;
f- Did not change; g- Stability (in pe.cent).

Thus according to our data, the presence of alcohol up to 4.5% in the finished gamma globulin preparation is not the sole reason for formation of sediment. According to the work of Rozenberg, Kholcheva and others, who determined the reason for the formation of turbidity in antimeasles serums upon storage, there are bases to believe that the physical properties of gamma globulin are affected more significantly by the presence of lipids.

The purpose of this investigation was to determine the effects of residual lipid components on the stability of gamma globulin. It is known that blood serum consists of protein fractions, which are closely related to lipids. Approximately, one fourth of the proteins in the serum is combined with lipids, forming lipoprotein complexes. We investigated the concentration of lipids, using the extraction method in a Soxhlet apparatus, both in the starting placental serum and at different stages of the production process.

In comparison with serum of venous blood, the concentration of lipids and cholseterol in the placental serum is significantly higher (Table 2).

Table 2.

Concentration of lipids in placental blood serum.

Serum		Density of serum	Concentration of lipids (in mg %)	Concentration of free cholesterol (in mg %)
Venous		1.026	400-1400	40-120
Placental	1	1.020	2400	123.7
	2	1.020	1800	140.8
	3	1.012	780	152.3
	4	1.022	1920	113.6
	5	-	1640	140
	6	-	1000	125

The obtained data on the concentration of lipids in the placental serums correspond to the data of Balakhovskiy and Travina, which indicate that during pregnancy the overall content of lipids in human blood serum increases. The increase of the concentration of lipids in the placental serums necessitates their removal in the course of the production of gamma globulin. According to the data of Anichkova (1956), Blix (1941), Oncley (1950) and Cohn (1945), the lipids are plimarily bound with α- and β-globulins, but in the course of the fractionation the lipid component remains in other fractions as an undesirable impurity.

In order to determine the concentration of lipids in various stages of the fractionation process three industrial series were subjected to the investigation. It was found that in individual processes the concentration of lipids varied significantly in the same protein fractions. This depends both on the concentration of lipids in the starting serum, as well as on the correct adherance to the conditions of the technological process. A large part of the lipid components is gradually removed in the production of gamma globulin. The principal mass of free lipids is noted in β-fractions after the second precipitation. In this stage of the production process the removal of the cholesterol also takes place. The determination of the cholesterol was conducted according to the method of Engel'gard and Smirnova (1926). The finished gamma globulin product contains only traces of cholesterol, while other lipid fractions are not removed in the course of fractionation. These unremoved fractions are primarily phosphorus containing lipids which are tightly bound to proteins.

The difficulty of removing certain lipid fractions lies in the fact that they are in the bound form, and therefore the total removal of lipids from the blood by simple ether extraction is not always possible. In this case it is first of all necessary to destroy the lipid-protein complex. Phospho-

lipids are invariably found in the tightly bound protein lipid fractions in gamma globulin. A more extensive study of the lipid components was conducted in the gamma globulin fraction and in the finished preparation obtained under industrial conditions. In these investigations attention was also given to the physical properties of the finished gamma globulin prior to packaging into ampules. The causes for the difficulty of filtration through the candles and the formation of flaky deposits during storage were investigated (Table 3).

Table 3.

The results of the investigation of the physical properties of gamma globulin

Series of gamma globulin	Free lipids (in mg %)	Physical properties of gamma globulin
5	No	Completely transparent
32	No	Completely transparent
36	Traces	Completely transparent
26	400	Flake formation after 2 months of storage
118 b	597	Increased viscosity
	515	Impaired filtration through candles
56 b	476	Impaired filtration through candles
34	650	Impaired filtration through candles
104	620	Impaired filtration through candles

In the absence of free lipids in gamma globulin or in the case of negligible concentrations of the latter the gamma globulin solutions display stable physical properties. The

presence of free lipids impairs the filtration of the solution of gamma globulin.

The data of our previous work have shown that vacuum filtration aids in the removal of alcohol which remains in the gamma globulin solution after fractionation with alcohol.

As a result of this investigation it was established that in the course of the filtration process through pulp or candles partial removal of lipids from the gamma globulin solution also takes place. In the course of the investigation in the first experiments the concentration of lipids in the pulp through which the preliminary filtration of gamma globulin solution was made was determined as well as its concentration in the physiological solution which was used for washing the candles after filtration.

In the second experiment the candles through which the gamma globulin was filtered were washed with a small amount of ether in which some extracted lipids were found.

The conducted work has shown that small amounts of lipids which exist in the placental serum are removed in the course of fractionation in the second stage along with the β-globulin fractions, to which they are tightly bound.

Under normal fractionation traces or insignificant amounts of lipids remain in the gamma globulin fraction. Gamma globulin contains primarily phospholipids (lecithin). In the case of difficulties arising in the fractionation process the concentration of lipids in gamma globulin may be high, which in turn may produce instability of the preparation.

Conclusions

1. The concentration of the residual alcohol up to 4.5% is not the principal cause of instability of the preparation. The concentration of lipid component in the placental serum exceeds the concentration in human venous serum.

2. In the course of the production of gamma globulin in

different stages, particularly in the second precipitation stage, gradual removal of the lipid components takes place.

3. Increase of the concentration of lipids in gamma globulin has a significant effect on its physical properties and stability.

4. The next problem is the total removal of lipids from the gamma globulin in fractionation of proteins of the human placental serum.

Bibliography

[1] Anichkov, N. N. Meditsinskiy Rabotnik (Medical Worker), No. 79, 81-82 (1956).

[2] Balakhovskiy, S. D. and Balakhoskiy, I. S. Metody khimicheskogo analiza krovi (Methods for Chemical Analysis of the Blood), State Medical Publishing House, Moscow, 1953.

[3] Belozerskiy, A. N. and Proskuryakov, N. I. Prakticheskove rukovodstvo po biokhimii rasteniy (A Practical Handbook on the Biochemistry of Plants), State Medical Publishing House, Moscow, 1951.

[4] Dubova, V. A. and Nechayeva, A. S. "The Effect of Residual Alcohol on the Physical Properties of Gamma Globulin" Turdy Moskovkogo Nauchno-Issledovatel'skogo In-ta im. Mechnikova (Transactions of the Moscow Scientific Research Institute im. Mechnikov), Moscow, Vol. 10, 85-91 (1958).

[5] Rozenberg, G. Ya. Bolotina, T., and Papush, N. O. "Denaturation of Human Plasma Protein and Blood Serum During Storage and Heating" Voprosy meditsinskoy khimii (Problems of Clinical Chemistry), Vo. 5, 175 (1953).

[6] Travina, O. V. Rukovodstvo po biokhiomicheskim issledovaniyam (Handbook for Biochemical Investigations), State Medical Publishing House, Moscow, 1955.

[7] Kholchev, N. V. and Kolesnikova, L. N. "Precipitate in Antimeasles Serums" Trudy Moskovskogo gosudarstvennogo

Instituta epidemiologii i bakteriologii (Transactions of the Moscow State Institute for Epidemology and Bacteriology), Moscowm Vol. IV, 202-120 (1942).

[8] Engel'gardt, V. A. and Smirnova, L. G. "A Simplified Method for Determination of Cholesterol in the Blood and Serum" Zhurnal eksperimental'noy biologii i meditsiny (Journal of Experimental Biology and Medicine), No. 7, 108 (1926).

[9] Blix, G., Tiselius, A. and Stevenson, H. J., Biol. chem., 137, No. 2, 485, 1941.

[10] Oncley, J. Gurd, F. J.

[11] Cohn, E., Medicine, 24, 3, 333-338, 1945.

CHANGES OF THE THERAPEUTIC ACTIVITY AND THE AVIDITY OF ANTIDIPHTHERIA SERUMS AS A RESULT OF PURIFICATION AND CONCENTRATION ACCORDING TO THE DIAFERM-3 METHOD*

by

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* The work was conducted in the Perm Scientific Research Institute of Vaccines and Serums.

In the study of the therapeutic properties of native antidiphtherial serums we became convinced that highly avid serums have certain advantages from the therapeutic standpoint in comparison with low avidity serums.

In this connection we considered it necessary to verify whether the advantage of highly avid serums is preserved also when they are purified and concentrated by the Diaferm-3 method.

In previous articles, we reported that the results of the concentration of antitoxic serums according to the above method produces a significant increase in the avidity. We also decided to test whether the increase of the acidity also corresponds to the increase of the therapeutic activity of the serum.

In the available literature we were unable to find any direct investigations in this area. We were only able to familiarize ourselves with the investigations of Beylinson and Shufer, who conducted the comparative investigations of the therapeutic effect of native diphtherial serums and serums concentrated by different methods with respect to guinea pigs with artificially induced diphtheria of the eye. According to the data of these authors the most active were serums concentrated by the Diaferm-3 method.

These serums possess five to twenty-fold higher therapeutic effect in comparison with native serums. It should be noted

that Beylison and Shufer did not conduct any special study of the changes of the therapeutic activity of serums as a function of the method of their concentration, but only compared the therapeutic activity of the native serum and of the serums concentrated by different methods.

We investigated forty-six antidiphtherial serums, and fifty-two antitetanus antitoxic serums before and after concentration. The first experiments were conducted with antidiphtherial serums.

Method

The guinea pigs, weighing 250 to 300 g were injected with one Dlm of the serial toxin in four ml of physiological solution subcutaneously in the sternum region. Three hours after the injection of the toxin, the guinea pigs were injected with 20, or 10 AE, 5 and 1 AE of the tested serum subcutaneously into the hind leg. The avidity of these serums was checked earlier. The therapeutic doses were calculated on the basis of the antibody titer, determined in the neutralization reaction according to Remer (Table).

Table

Avidity and therapeutic properties of the concentrated antidiphtherial serums.

Avidity	Number of serums	Therapeutic dose (in AE/ml)		
		20 or 10	5	1
High	36	22	9	5
Medium	10	3	4	3
Low	-	-	-	1

In order to analyze our data more conveniently, we arbitrarily divided our serums into three groups, guiding ourselves by the levels of their avidity. Serums with indices of the completeness of compound K, equal to or greater than 7.05, were placed in the group of serums with high avidity, we grouped serums with avidity index equal to 0.5 to 0.75 as serums with intermediate avidity and serums, the indices of which were less than 0.5 we grouped as low avidity serums.

It is obvious that such a subdivision was purely arbitrary and we made out merely for convenience of the comparison of the therapeutic activity of serums with different avidity.

The high avidity concentrated antidiphtherial serums were generally found active in smaller doses than serums with low avidity. In therty-seven our of forty-six investigated serums, the avidity increased as a result of pre-concentration. Twenty-three out of these thrity-seven serums also showed increased therapeutic activity. These data indicate a definite parallelism between the changes of the avidity and the therapeutic activity as a result of the pre-concentration of the antidiphtherial serums.

Conclusions

1. The investigations conducted on the purification and pre-concentration of antidiphtherial serums show that there is a definite relationship between the avidity and the therapeutic activity of serums. Serums with high avidity in the majority of cases display a higher therapeutic effect than serums with low avidity.

2. The relationship between the avidity and the therapeutic activity of serums is also supported by the fact that during the concentration of antidiphtherial serums, for the majority of serums the change in the avidity coincides with changes of the therapeutic activity.

AVIDITY AND THERAPEUTIC ACTIVITY OF NATIVE ANTIDIPHTHERIAL AND ANTITETANUS SERUMS*

by

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* The work was conducted in the Perm Scientific Research Institute of Vaccines and Serums.

Up to the present time the therapeutic strength of antitoxic serums was measured by determining the number of antitoxic units in them.

This situation is a natural extension of the teachings of the great German scientist P. Erlich on the subject of immunity. According to his theory the strength of the immune serums corresponds totally and wholly to the number of antibodies which they contain.

However, since the beginning of the twentieth century when the immune serums, primarily antidiphtherial serums, were widely utilized for therapeutic purposes and prevention of disease, cases of noncorrespondence were noted between the antibody titer of the serums and their therapeutic effects.

This was first pointed out by a French scientist, E. Rue. According to his data in a number of cases serums with lower antibody content possess higher therapeutic strength than certain serums containing a larger number of antibodies.

Analogous data were later obtained by Dreyer and Madsen, Shwoner and Becher, Kraus, Barykin and others (1903-1913).

In order to explain this noncorrespondence between the antibody titer and the therapeutic strength of the serum, Kraus proposed the hypothesis on the different relationship of the serum to the toxin, as a result of which the reaction between different serums and toxins proceeds with a different intensity.

This property of the serum was given the name "avidity."

At present, the word avidity designates the intensity with which a reaction proceeds between the antibody and the antigen.

Certain investigators, for instance(Frize, Madsen and Shmidt, Makarova-Tarasevich and Levkovich) indicate the advantage of highly avid serums as compared with low avidity serums in the therapeutic respect. Others (Kolle and Wigge, Feygina) do not find any relationship between the avidity and the therapeutic activity of serums.

In order to clarify this question we conducted several series of experiments in which we compared the therapeutic effect of high and low avidity native antidiphtherial and antitetanus serums.

Avidity and Therapeutic Activity of Antidiphtherial Serums

We investigated 136 diphtherial equine serums. We determined the avidity and therapeutic activity of the serums as they were obtained from the horses.

The evaluation of the therapeutic activity of antidiphtherial serums was conducted immediately after the determination of their avidity: both avidity indices were taken into account: the index of the completeness of the binding of the antitoxin with toxin and the index of the strength of the obtained compound. The selected serums were as fresh as possible. They were tested not later than one month after the sample was taken from the animal.

The experiments were conducted with guinea pigs. The avidity was determined on the basis of the completeness of the combination of the antitoxin with the toxin and on the basis of the strength of this bond.

As the basis of the method for the determination of the

avidity of serums from the completeness of the binding of antitoxin with toxin multiple observations on the frequent noncorrespondences in antidiphtherial serums of the magnitude of the antibody titer determining in vitro in the flocculation reaction according to Ramon and in vivo in the neutralization reaction according to Remer and Erlich were taken into account. The experiments of Lenni and others (1925), verified by our data, show that the ratio of antibody titer $\frac{\text{in vivo}}{\text{in vitro}}$ for a given serum in a definite interval of time is a constant. According to the data of Lenni, this number corresponds to the results on the determination of avidity according to the Madsen method.

The method for the determination of the strength of the compounds in antitoxins and toxins consists of the addition of different amounts of anatoxin to the neutral antitoxin plus toxin mixture. The serum which requires a large amount of anatoxin for the dissociation of the bond with toxin is avid. If the compound in the serum with toxin is split upon the addition of small doses of anatoxin the serum has low avidity. It is quite understood that the avidity of different serums may be intercompared only if the same anatoxin is used.

The experiments on the treatment of animals and low avidity serums were conducted according to the following method. Guinea pigs weighing 250 to 300 grams were injected with one Dlm of diphtheria toxin in four ml of physiological solution subcutaneously in the chest region. The preliminary experiments which we conducted have shown that during the intravenous injection of toxin it is very difficult to determine the minimum lethal dose, apparently due to the individual sensitivity of the animals to the toxin. Therefore, we resorted to subcutaneous injection.

Six hours after the injection of the toxin, the guinea pigs were injected subcutaneously with two, five and ten AE of the investigated serum respectively. Each dose of the

serum was injected into six guinea pigs. The control animals were injected with normal equine serum subcutaneously. The death of the animals was observed in the course of four to five days after the injection of the toxin. Since the titers in the flocculation reaction according to Ramon and neutralization reactions according to Remer display a discrepancy, in many serums, the therapeutic activity of antidiphtherial serums in the first series of experiments were determined parallel in two experiments.

In the first experiment the serum dose was injected on the basis of the antibody titer, determined by means of the flocculation reaction, while in the second experiment the serum dose was injected which was calculated on the basis of the antibody titer determined by the neutralization reaction according to Remer.

In computing the therapeutic doses, basing them on the antibody titer, determined in the flocculation reaction, a definite advantage of the highly avid serums was observed in comparison with low avidity serums (Table 1).

Table 1.

The relationship between the avidity and the therapeutic effect of native antidiphtherial serums in computing doses and taking into account the antibody titer, determined by the flocculation reaction.

Avidity index (K)	Number of serums	Therapeutic dose (in AE)		
		2	5	10
>1.0	48	27	19	2
0.75 - 1.0	49	12	26	11
<0.75	39	-	17	22

A somewhat different relationship between the avidity and the therapeutic property of the serums was observed when the dose was computed on the basis of the antibody titer determined in the neutralization reaction according to Remer.

Such sharp differences in the therapeutic activity of high and low avidity serums as was shown in choosing the therapeutic doses of serums on the basis of the antibody titer (determined in the flocculation reaction) was not observed in this case. However, in these cases one would also note a definite advantage of high avidity serums over low avidity serums (Table 2).

Table 2.

The relationship between avidity and the therapeutic effect of native antidiphtherial serums in computation of doses on the basis of antibody titer determined in the neutralization reaction according to Remer.

Avidity index (K)	Number of serums	Therapeutic dose (in AE)		
		2	5	10
>1.0	48	17	22	9
0.75 - 1.0	49	15	20	14
<0.75	39	4	15	20

The explanation for this phenomena we believe may be given on the basis of the difference in the strength of compounds with toxin in high and low avidity serum. It is known that the formation of a compound between antitoxin and toxin is reversible and a certain fraction of toxin may be liberated as a result of the dissociation of the compound. In low avidity serums, the strength of the compound with toxin is significantly lower than in high avidity serums. Consequently, in using low avidity serums as a result of the dissociation of antitoxin plus toxin compound, a larger amount of toxin is

liberated than in the case of highly avid serums.

In order to produce a therapeutic effect it is necessary to neutralize the liberated toxins. The lower the avidity of the serum the larger the amount of toxin which is in the free state and consequently, the amount of serum which is required for the additional neutralization is larger.

It is unclear, why there is not a complete parallelism between the magnitude of the avidity of the serums and the degree of their therapeutic effect. We believe that the following explanation is most appropriate. The effect of immune serum in the organism of an animal cannot be reduced to a simple reaction between the serum and the toxin, by equating a living organism to a test tube with reacting substances. Without any foubt a whole combination of complex reactions, both chemical and physiological between the serums and the organism, the organism and the toxin, between different intermediate products of these reactions among themselves, as well as with the organism in turn, etc. take place.

As a result, the organism is subjected to a colossal number of possible impulses and irritations. The various therapeutic or prophylactic effects result from all of the interactions on the organism.

In order for the immune serum to be highly effective it must possess many properties. One of the properties, as our experiment has shown, is the avidity -- the intensity of the reaction of the serum with toxin. However, while avidity is determined qualitatively the reaction aspect between the antibody and the antigen cannot, and does not determine all interrelationships between the serum, the toxin and the organism.

Avidity and Therapeutic Activity of Native Antitetanus Serums

It is known that in view of the very characteristic interaction between tetanus toxin and the nervous system, the

specific serotherapy of tetanus intoxication is much less effective than seroprevention. We have considered that the difficulty of the serotherapy of tetanus may hinder the differentiation of antitetanus serums with respect to their therapeutic effectiveness. On the other hand, it would be very valuable to determine the relationship between the therapeutic effectiveness and the avidity for antitetanus serums.

We investigated eighty-one serums from thirty horses. In order to obtain more pronounced results we selected for the experiments, serums with the highest and the lowest avidity.

The experiments were conducted with white mice. The experimental method was as follows. The mice were injected with two Dlm of the toxin subcutaneously into the hind leg. On the following day when the mice displayed the symptoms of the first degree tetanus, they were injected with 100, 300 and 500 AE of the tested serum respectively. The survival results were observed in the course of four days (Table 3).

Table 3.

Therapeutic activity and avidity of native antitetanus serums.

Avidity index (K)	Number of serums	Therapeutic dose (in AE)			
		100	300	500	without any therapeutic effect
>1.0	45	19	14	8	4
<0.5	36	2	10	13	11

The highly avid serums were more advantageous in the therapeutic respect than the serums with low avidity.

Conclusions

1. As a result of the investigation of the therapeutic activity of native antidiphtherial serums it was found that out of forty-eight highly avid serums twenty-seven displayed a therapeutic effect in 2, 19 and 5 AE dose. Out of thirty-nine low avidity serums not one showed a therapeutic effect in a two AE dose, seventeen serums were found to be effective in five AE doses and twenty-two serums in ten AE doses.

2. Analogous data were obtained in testing the therapeutic activity of the native antitetanus serums. Out of forty-five serums with high avidity nineteen serums possessed high therapeutic effectiveness, twenty-two serums displayed a therapeutic effect in relatively large doses and four serums did not show any therapeutic effect at all. Out of thirty-six low avidity serums only two serums possessed high therapeutic effect, twenty-three serums displayed therapeutic effects in high doses and eleven serums were found not too effective at all in the investigated doses.

3. The obtained results give basis for the belief that there is a definite relationship between avidity and the therapeutic activity of the native antitoxic serums. In the majority of cases, the serums with high avidity possess higher therapeutic activity than serums with low avidity.

A MULTICOLOR MACHINE FOR LABELING AMPULES AND BOTTLES WITH MEDICAL AND MICROBIOLOGICAL PREPARATIONS

(Communication I)

by

B. V. Sizov and V. A. Nikitin, Bottling and Packaging Laboratory

The labeling of medical preparations cannot be considered insignificant. Until recently, ampules and bottles were labeled with paper labels. This method has a number of significant drawbacks. Paper labels interfere with the visual control of the preparation both in the production process, as well as prior to use.

Therefore, direct printing on the glass, i.e., printing of the labels on ampules and bottles, is being used more and more frequently.

The appearance of ampules and bottles with labels put directly on the glass is definitely superior than of the ampules and bottles with paper labels. In this case, as it was stated above, not only the external appearance of the ampules and bottles is improved, but also the macroscopic control of the preparations both in the production process as well as prior to use is also easier.

The conversion to labeling directly on the glass also eliminates the necessity of conducting a number of operations associated with the printing of additional information on the labels (for example, printing of the date of preparation, printing of the date beyond which the preparation should not be used, the number of the series, capacity, etc.), and glueing and the step involving the printer is also eliminated which is very important.

These advantages necessitate the construction of labeling machines for direct printing on the glass.

The presently existing labeling machines have a host of drawbacks: poor quality of the printing, printing with one color, bulkiness of the machines, etc.

This situation detracts from the wide usage of these machines for printing on glass.

As a result of multiple experiments, a multicolor printing machine for direct labeling of ampules and bottles was developed in our institute. A great deal of work was also conducted in our institute on the selection and making of paints* and solvents, which have a significant effect on the quality of the printing.

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* The formula for the paints will be discussed in a separate communication.

The machine which was introduced into production meets all of the production requirements and is used for marking of ampules and bottles with preparations issued for the Moscow Scientific Research Institute of Vaccines and Serums in Mechnikov.

The general view of the machine in the working position during the printing of three color labels is shown in Fig. 1.

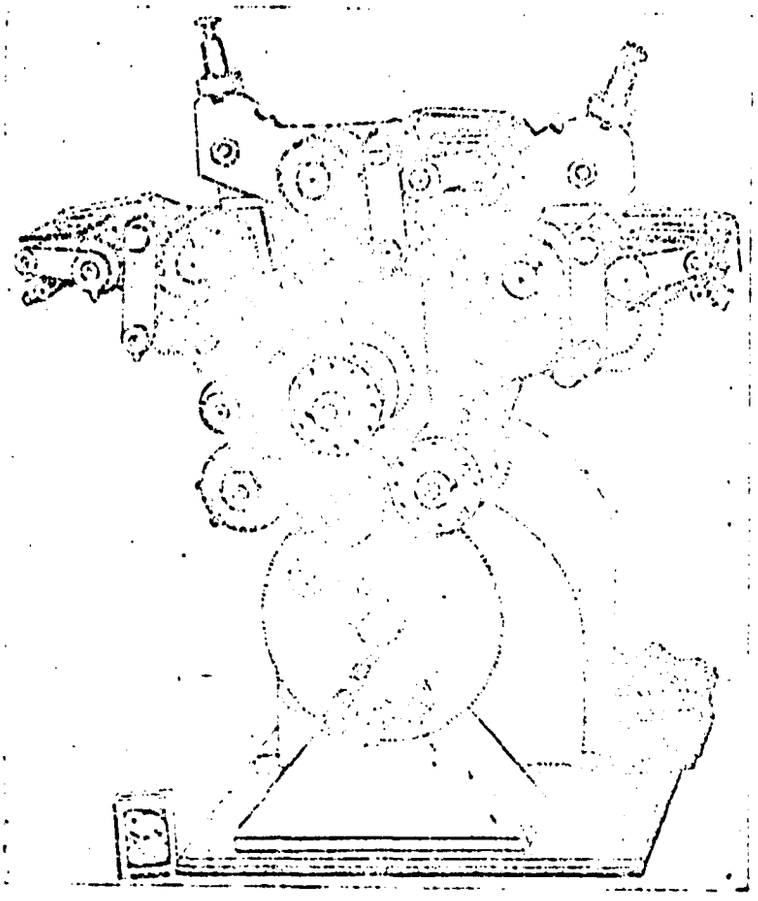

Figure 1.

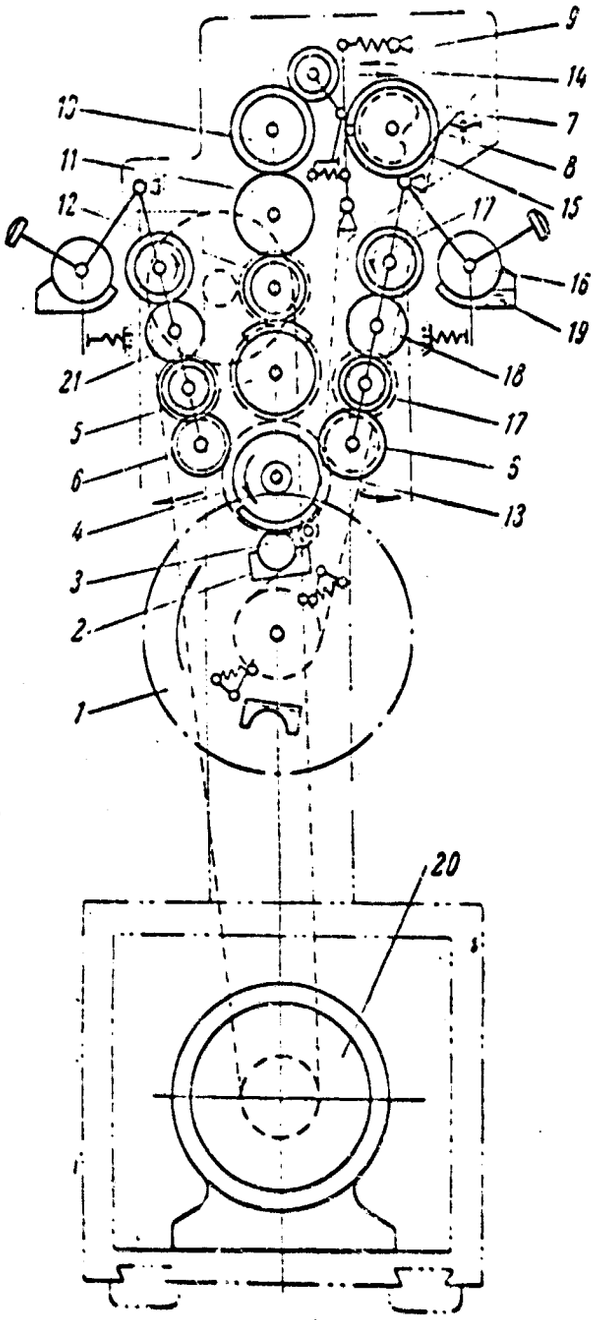

Figure 2

Machine Specifications

Capacity of ampules (Bottles) which the machine can handle ..... 1-50 ml
Production capacity ..... 1640 items/hr
Number of revolutions of the transported disk .... 14 rpm
Dimensions of the machine: length ..... 400 mm
width ..... 450 mm
height ..... 550 mm
Weight of the machine with electric motor... 40 kg
Characteristics of the electric motor:
number of rpm ..... 1400 rpm
capacity ..... 0.25 kw
voltage requirements..... 220-380 v

The schematic diagram of the machine is represented in Figure 2.

Construction of the Machine

The machine consists of a rotating transporter disk (1) with easily replaceable sockets (2) for ampules (3) and a printing device. The latter consists of an offset cylinder (4), three form cylinders (5,6) and three paint apparatus. The paint is fed to the form cylinder (5) by the paint distributing apparatus, consisting of ducted shaft (7) with a steel knife (8), elastic transmission roller (9), elastic roller (10), steel cylinder (11) and the elastic roller (12). The Ducted shaft (7) is rotated continuously by means of the transporter disk (1) and a belt drive (13). The transmission roller is moved by means of a spring-loaded bar with a roller, which interacts with the cam (15) on the ducted shaft. The remaining rollers of the paint apparatus are driven by each other as a result of the forces of friction.

The paint is fed to the form cylinders (6) by two paint

apparatus on the side, each of which consists of a ducted shaft (16), elastic rollers,(17) and a steel cylinder (18). The thickness of the layer of paint on the ducted shaft is regulated by positioning it to the appropriate level in the paint box (19).

The machine is driven by an electric motor (20). The side paint apparatus with form cylinders (6) are mounted on swivel frames (21) which can be moved away from the offset cylinder (4). The sockets (2) for ampules or bottles (3) consist of two elastic supporting rods (22) with semicylindrical grooves (23) and a support (24) for rigidly fixing the ampule in the axial direction (Figure 3). The elastic construction of the sockets permits handling of nonstandard containers (ampules, bottles), the diameter of which can range from 1 to 2.5 mm. The well is mounted on a pin of the transported disk with two nuts which enables one to replace it easily and rapidly when different capacity ampules are being handled.

The ampule for example (3) is placed into the notches (23) in the sides (22) of the socket (2) when the latter is moved towards the offset cylinder (4), on which the printing has been placed by means of three form cylinders(5, 6). The drawings and the writing are reproduced by means of an offset cylinder (4), on the surface of the container(3), while the container is rotated in the socket due to the friction with the offset cylinder. After this the container is removed from the socket (2).

The Operation of the Machine

For the operation with the machine a bronze engraving plate containing the text to be printed on the label, in straight thick characters is prepared. In preparing the engraving plate the dimensions of the text, the size of the ampules and bottles must be taken into account in order to make an optimum placement of the label on the surface of the ampule. Those parts of

the ampule (bottle) label which are frequently changed depending on the lot number of the preparation, the data of the preparation, the capacity, the series number, etc. are made replaceable in the plate. Windows are made in the plate to provide these changeable designations.

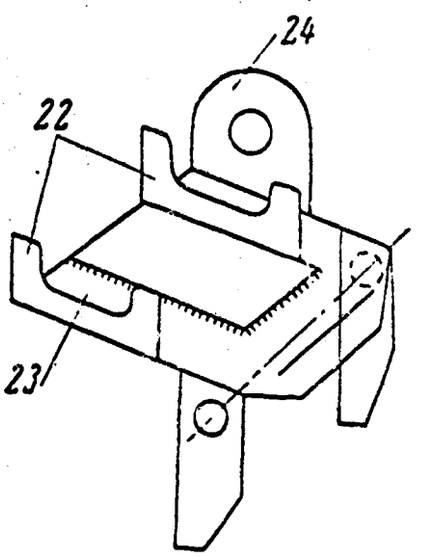

Figure 3. (A more detailed description of the machine is given in the instructions issued by the Moscow Scientific Institute of Vaccines and Serums im. I. I. Mechnikov in 1960).

To produce a three-color label, the plate is made of three parts, each of which is placed in a separate stereotyped block and they are mounted on the appropriate form cylinder (5,6). If necessary these three parts of the engraving may be placed into a single stereotyped block which is mounted on the form cylinder (5) and printed with one color. In the case of necessity, the label may also be printed in two colors.

For printing with one color, the two end paint apparatus are separated from the offset cylinder by simply moving them away to the side and fixing them in a neutral position with pins (Figure 4). The end paint apparatus are mounted on the sliding frame which enables rapid and easy conversion of the

machine to a single, double or triple color printing.

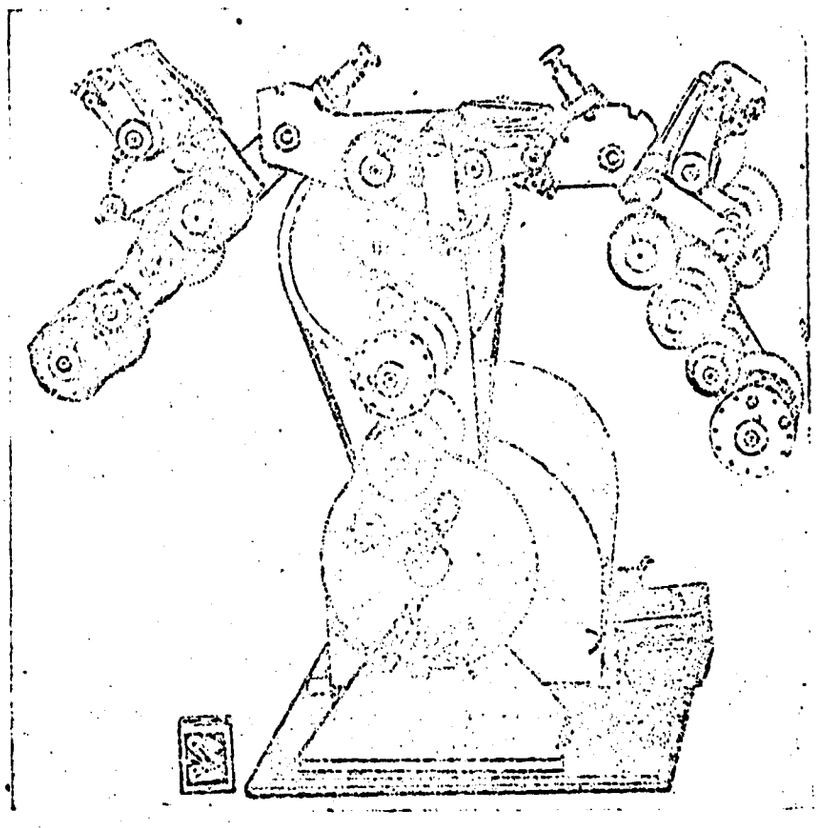

Figure 4.

The machine utilizes elastic rollers from a roller composition. The roller composition consists of gelatin and glycerine, which can easily be prepared in place and cost into the shape of the rollers in the appropriate forms, to replace the old worn out rollers. This process does not require vulcanization and subsequent accurate machining of the rollers. This again makes the operation of the machine independent of the supply plants and creates a corresponding convenience of operation.

Prior to the beginning of work, the appropriate color paints are put into the paint boxes. On the left of the machine a tray with ampules is placed and on the right, an empty tray for finished ampules. The machine is turned on, the operator takes the ampule with his left hand and places it into the socket. After printing, he removes the ampule with the printed label using his right hand, checks the quality of the printing and places it into the empty tray at the right etc.

The immediate packing of the printed containers into the packaging tares is also done.

As it can be seen, the machine has a number of significant advantages over the earlier known machines, namely: 1) portability, small dimensions and small weight; 2) simplicity of construction and operation; 3) printing with one, two and three colors; 4) regulation of the pressure between the cylinder and the roller of the printing device; 5) it practically insures printing of labels of any content within the system of bureaus of the Ministry of Health of the USSR and RSFSR; 6) it is a universal machine due to the replaceable sockets, it prints on containers (ampules, bottles) of different capacity (1-50 ml), and as a result of the replacement of the transporter disk and the wells, it can print on containers, the capacity of which is in excess of 50 ml; 7) printing on ampules and bottles of the same capacity which vary in diameter by as much as 1 to 2.5 mm is possible due to the elastic construction of the sockets.

Conclusions

1. The direct printing on the glass ensures macroscopic control of the preparation both in the production process as well as prior to use by the comsumer.

2. The machine may be used for small and large series production of the industrial establishments producing galenicals

pharmaceuticals, institutes and sanitary epidemological stations and other establishments which issue medicines and microbiological preparations in ampules and bottles.

3. The machine can print one, two and three color labels.

4. The machine can print on ampules and bottles which have not been accurately calibrated.

5. The machine improves the external appearance of the ampules and bottles and creates conditions for further improvement in the preductivity of labor as a result of the mechanization of the feeding and packaging of ampules and bottles.

Bibliography

[1] Sizov, B. V. and Nikitin, V. A. "Multicolor Printing Machines for Printing Markings and Labels on the Surface of Cylindrical Objects, Made of Glass for Example." Author's certificate No. 122485. Byulleten' Izobreteniy (Bulletin of Inventions), No. 18. 1959.

A MULTICOLOR SEMIAUTOMATIC MACHINE FOR PRINTING LABELS ON AMPULES AND BOTTLES WITH MEDICAL AND MICROBIOLOGICAL PREPARATIONS

(Communication II)

by

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The need for increasing the productivity of labor and improvement of the quality of printing forced the further improvement in the construction of the existing labeling machines. We have described earlier, a multicolor label printing machine for printing labels on ampules and bottles with microbiological preparations. That machine was loaded and unloaded manually.

On the basis of the experience with the operation of that machine in our institute we have now developed a more advanced labeling machine, a semiautomatic type with automatic wiping of the ampules and bottles prior to printing of the label.

In printing machines the productivity is generally limited by the manual feeding of ampules and bottles into the machine. In the case of manual operation, one must make the necessary pauses, which disrupt the continuous operation of the machine and lowers its productivity. Therefore, to increase the productivity of the machines, it is first of all necessary to automate the feeding and the removal of the ampules. This was the principal factor which limited the productivity of the machine.

To improve the quality of the printing and also to ensure clear and complete printing, a new section was added to the machine for wiping the surface of the ampules prior to printing, since even on the surface of ampules which were washed, small traces of grease are found which sharply decrease the quality

of the printing.

With the introduction of preliminary wiping it becomes unnecessary to have a thorough quality control of the printing and retain only an occasional random control, which again enables one to increase the productivity of the machine.

Thus, the productivity of the machine may be increased due to the increase of the rate of operation of all of its units, correspondingly increasing the rate of processing of the ampules (during passage through the machine) up to any magnitude.

However, for microbiological preparations such acceleration above certain limits leads to foaming of the contents of the ampules, which has an undesirable effect on their quality and therefore is not permissible.

So, foaming is the final factor which limits the productivity of the machine.

In the case of semiautomatic loading of the machine, the ampules are placed into the bunker in cartridges, which have been loaded a priori. The finished product is manually removed from a wide transporter. The elastic construction of the socket has been retained which permits the printing of the ampules for which the outside diameter varies within 1 to 2.5 mm limits.

This enables printing of labels on ampules of nonstandard production.

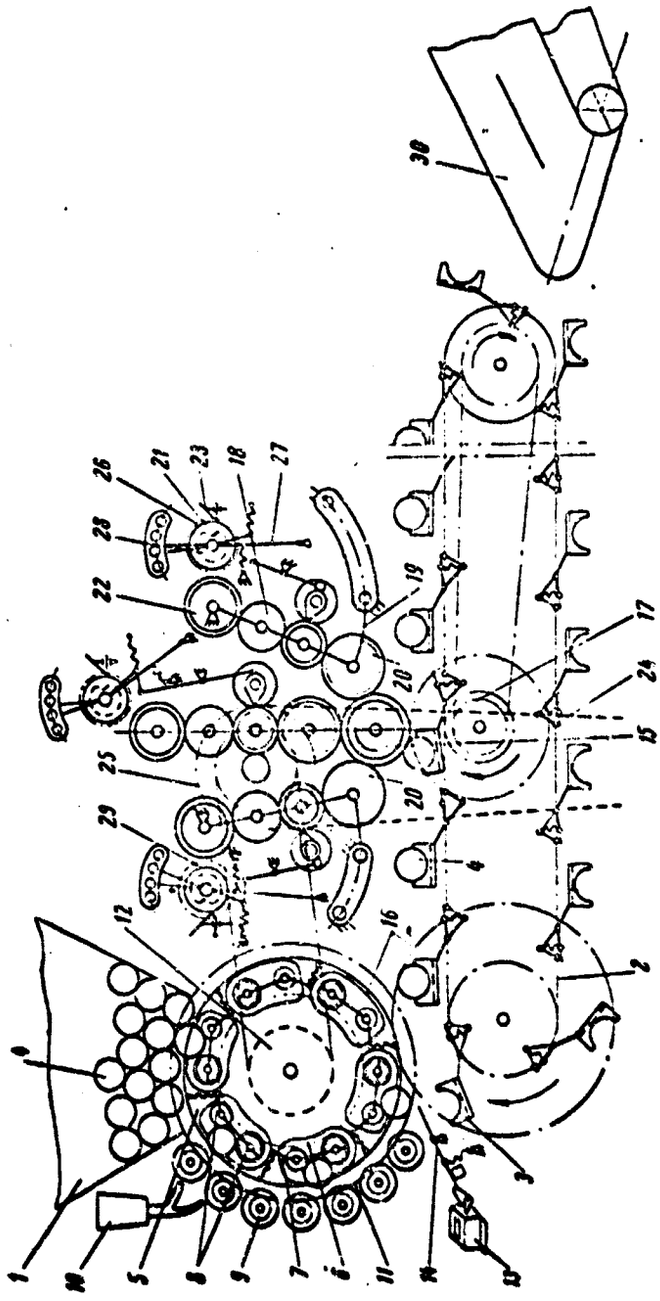
Technical Specifications of the Machine

Productivity --- 4000 items/hour.

Capacity of the ampules (bottles) which can be handled -- 1 to 50 ml.

The electric drive motor -- 0.5 kw, 220/380 v, 140 rpm.

The general schematic diagram of the machine is shown in the Figure.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The machine consists of a hopper (1), chain transporter (2), with sockets (3) for the ampules (4) and the printing apparatus. The bottom of the hopper (1) is a drum (5) with sockets (6). In each socket a pair of rollers (8) is placed on a hinged spring loaded lever (7), and along the periphery of the drum wiping rollers (9) are placed which are wet periodically by means of a dropper (10) and rotated by means of a gear (11), which is connected to a pulley (12).

Between the drum (5) and the transporter (2), there is a counter (13) for counting the number of objects. The counter acts when the ampule or bottle presses upon the lever (14).

The transporter (2) is made in the form of two chains with sockets mounted on them (3). The sockets are made elastic for pressing the objects to the offset cylinder (15). The drum (5) is driven by the transporter (2) through a gear (16), and the transporter is driven by the offset cylinder (15). To prevent sagging of the chain of the transporter during contact of the object with the offset cylinder (15) support sprockets (17) are used.

The three-color printing apparatus consists of three analogous systems, where the central system is mounted rigidly, while the two end systems are mounted on sliding frames (18) with retainers (19), which enables the easy changeover from three to two or one color printing or visa versa.

The paint is fed to the form cylinders (20) by means of the ducted shaft (21) and a system of spreading rollers (22). The required amount of paint is controlled by a knife (23).

The machine is driven by an electric motor (not shown in the figure) through a belt drive (24) to a pulley (25) and subsequently to one of the spreading rollers (22) and the offset cylinder (15). The end systems of the printing apparatus are driven by the cylinder (15). Each of the ducted shafts (21) is moved pariodically by means of a ratchet mechanism and is rocked on a spring-loaded lever (27), which presses it

periodically to the spreading roller (22) during the interaction of the notch in the cam (28) with the stationary roller (29).

From the hopper (1), the objects (4) enter one in each socket (6) in the drum (5) and are placed on the spring loaded rollers(8), which during the rotation of the drum press them to the wiping rollers (9) for cleaning. Subsequently, the drum passes them into the sockets (3) of the transporter (2). The transporter moves them to the offset cylinder (15), which places the label on their surface after which the object is removed by the carryout transporter (30).

In the proposed semiautomatic machine the printing apparatus is analogous to the multicolor printing machine of our institute which was described earlier.

Therefore, the printing part of the semiautomatic machine is prepared for the operation in much the same manner as the printing part of the previously described machine of our institute. Prior to the beginning of operation, the appropriate color paints are put into the paint boxes, the magazine with ampules is placed into the hopper, the appropriate engraving plates are placed on the form cylinders, and the cleaning liquid is put into the dropper. Following this,one can proceed with the operation of the semiautomatic printer. During operation the magazines are occasionally changed: empty magazines are removed and magazines loaded with ampules and bottles are inserted. In order to change over to operation with different capacity, ampules change of the transporter (2) and regulation of the rollers (8) of the wiping drum are required.

The replacement of the transporter is associated with certain inconveniences. Therefore, in the new design of the semiautomatic machine, a disk transporter is anticipated which will be more convenient and simple to produce, and also simple to interchange during operation. At the same time, the relative placement of the individual units making the machine

more compact and convenient has been made. Thus, the printing apparatus placed at the bottom end is closed by the body of the machine.

Conclusions

1. The automation of the feeding and removal of individual ampules during the magazine loading of the machine increases the productivity of the machine significantly.

2. The device for wiping the ampules and bottles in the machine ensures an improved quality of printing.

3. The significant advantage of the machine is its ability to operate with nonstandard containers, which have significant scattering in their external diameters.

4. The machine is capable of marking vessels with one, two or three colors.

5. The machine is simple to construct and to use.

Bibliography

[1] Sizov, B. V., Nikitin, V. A. and Polyak, Yu. D. "A Multicolor Printing Machine for Printing Markings and Labels on the Surface of Cylindrical Objects, Made of Glass for Example." Author's certificate No. 122486. Byulleten' Izobreteniy (Bulletin of Inventions), No. 18, 1959.

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