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The Selective Suppression of Immunogenicity by Hyaluronic Acid*

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ABSTRACT

A hyaluronidase-sensitive component of human peritoneal fluid from a patient with Wilms' tumor when injected into rabbits has been shown to suppress the formation of humoral precipitating antibodies to certain major classes of proteins present in the fluid.

Furthermore, it has been found that hyaluronic acid, when included with certain test antigens (serum albumin, fetuin) or antigen mixtures (tumor isolates or mixtures of albumin, immunoglobulin G and immunoglobulin M), produces a marked distortion or complete blockage of immunoelectrophoresis precipitin arcs, as well as altered gel chromatography elution profiles.

These findings that hyaluronic acid can interfere profoundly with both the elicitation of a complete antibody response and the formation of "normal" patterns of antigen-antibody precipitates in laboratory tests supports the possibility that this polysaccharide may play an immuno-regulatory role by masking potential immunogens. Consideration of the mechanisms for these *in vivo* and *in vitro* effects suggests that there may be some common basis in an "excluded volume" property of the hyaluronate, but this does not appear sufficient to explain the complexity and selectivity of the observed phenomena.

Introduction

Among the major classes of vertebrate glycosaminoglycans (acid mucopolysaccharides), the hyaluronic acids are rather distinctive because of (a) their high molecular weights, (b) their ubiquitous distribution among many tissues, and (c) their occurrence as unbranched and unsulfated polysaccharide chains generally free of covalently bound protein.[17] Increased levels of hyaluronic acid, and

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of the other glycosaminoglycans, have been noted in association with a variety of tumors.[7] In recent years, many reports have appeared comparing the glycosaminoglycan profiles of neoplastic cells and tissues with those of their normal counterparts.[10,14] Although the differences observed are often striking, it has been difficult to interpret them because the biological roles of these substances in normal and aberrant developmental processes are not fully understood.[1,3,13,20,25,27]

This study was initiated by our discovery that a sample of a viscous peritoneal fluid from a patient with Wilms' tumor elicited an incomplete antibody response in rabbits, i.e., antibodies were not produced against protein antigens known to be present in the specimen. Subsequent experiments implicated hyaluronic acid as the likely suppressive agent. Other tests with conventional immunoelectrophoresis procedures showed that hyaluronic acid is capable of altering immunoprecipitin arcs (and in some cases preventing their formation) when it is present in test antigen sources. Studies with similar mixtures of hyaluronate and these test proteins were performed with molecular sieve column chromatography in order to examine the selectivity and the extent of binding between the polysaccharide and the protein antigens.

Materials and Methods

Human peritoneal fluid was obtained from a patient (J.M.) during an operation for surgical removal of a primary Wilms' tumor. Human umbilical cord hyaluronic acid, crystalline bovine albumin, and human immunoglobulins (IgG, IgM) were purchased* and Spiro's purified calf fetuin and antiserum to whole human sera were obtained.† Electrophoretically pure human mercaptalbumin was prepared from fresh plasma by ethanol precipitation and treatment with cysteine according to the methods of Allerton et al.[2] Varidase (streptokinase, streptodornase) was obtained‡ as was Freund's complete adjuvant.§

Preparation of Antisera

Two groups of four young female New Zealand white rabbits that were used to develop antisera were inoculated with injectates consisting of 1.5 ml antigen solution containing 15 to 50 mg protein mixed with 1.5 ml Freund's complete adjuvant. This preparation was thoroughly emulsified and was injected subcutaneously in 0.5 ml portions to six sites at the base of each animal's neck. Four booster injections were given at approximately 20-day intervals, and the animals were bled via cardiac puncture within 15 days of the last booster. All of the blood samples were collected in sterile Vacutainer tubes and allowed to clot. Following centrifugation, the serum was tested for specificity against whole human serum with Ouchterlony and immunoelectrophoresis techniques. For the experiments presented here, the antisera samples from animals within each group were found to be qualitatively indistinguishable and were therefore pooled. The antisera were aliquotted and stored frozen.

Immunoelectrophoresis

The Millipore-Worthington Phoroslide electrophoresis system was used for all immunoelectrophoresis analyses as recommended by the manufacturer,

* Sigma Corporation, St. Louis, MO.
† Gibco Grand Island, NY.
‡ Lederle Laboratories, American Cyanamid Company, Pearl River, NY.
§ Calbiochem Corporation, San Diego, CA.

except that Coomassie Brilliant Blue was substituted for the Buffalo Black stain. A sample volume of two μl and an antisera volume of 50 μl were used. Samples were electrophoresed at 100 volts for 35 minutes, incubated with antisera, extensively washed, and finally stained with 0.4 percent Coomassie Brilliant Blue in five percent methanol-acetic acid solution.

"Rocket" immunoelectrophoresis plates were used to examine the effect of hyaluronic acid on the interactions between bovine serum albumin (BSA) and rabbit anti-BSA. These plates were prepared by mixing 0.5 ml of antisera with liquefied Ionagar‖ at 45°C followed by cooling to room temperature. Sample wells of 1.5 mm were introduced into the gels to accommodate 15 μl of sample. Following an equilibration electrophoresis at 100 volts for 15 minutes, specimens with and without added hyaluronic acid were electrophoresed for two hours at 100 volts. After washing, the gels were stained as indicated.

Immunodiffusion

Ouchterlony double diffusion agar gels¶ were filled with either 10 μl of antigen or antisera, incubated for 48 hours, then deproteinized and stained as indicated for the immunoelectrophoresis.

Digestions with Varidase

Human peritoneal fluid and other test samples were enzymatically degraded with Varidase containing potent hyaluronidase activity (0.1 g per dl in calcium and magnesium-free phosphate buffered isotonic saline) for 24 hours at 37°C.[26] Control specimens were incubated in parallel without Varidase.

Gel Filtration

Molecular sieve chromatography, used to explore the interactions between hyaluronate and selected serum proteins, was conducted in glass columns (2.5 cm × 50 cm) packed with Sephacryl S-200.* The columns were equilibrated with isotonic buffered saline at pH 7.2, and the samples were loaded in a like buffer. The columns were maintained at a flow of 0.5 ml per minute, monitored with a spectrophotometer† (at 280 nanometers), and the effluent was collected in 0.7 ml samples.

Results

The immunoelectrophoresis profile of peritoneal fluid from the patient with Wilms' tumor produced with antisera against this material (generated in our rabbits) is shown in figure 1. For comparison, the profile produced with this same antigen source and antisera made against Varidase-treated peritoneal fluid is illustrated in figure 2. It is apparent from these examples that after four to six weeks post-inoculation, the animals treated with the "intact" peritoneal fluid generated antibodies only against five proteins of beta electrophoretic mobility, whereas the digested samples generated a wide spectrum of antibodies which reacted with antigens of the test specimens. The latter of these provided an immunoelectrophoresis profile analogous to that made by commercial antisera to whole human sera (figure 3). Of particular interest was the demonstration of precipitin arcs against major protein antigens such as albumin and IgG only with the commercial antisera or our antisera to the digested material. Also worthy of note was the distortion and elongation of precipitin lines in all of the

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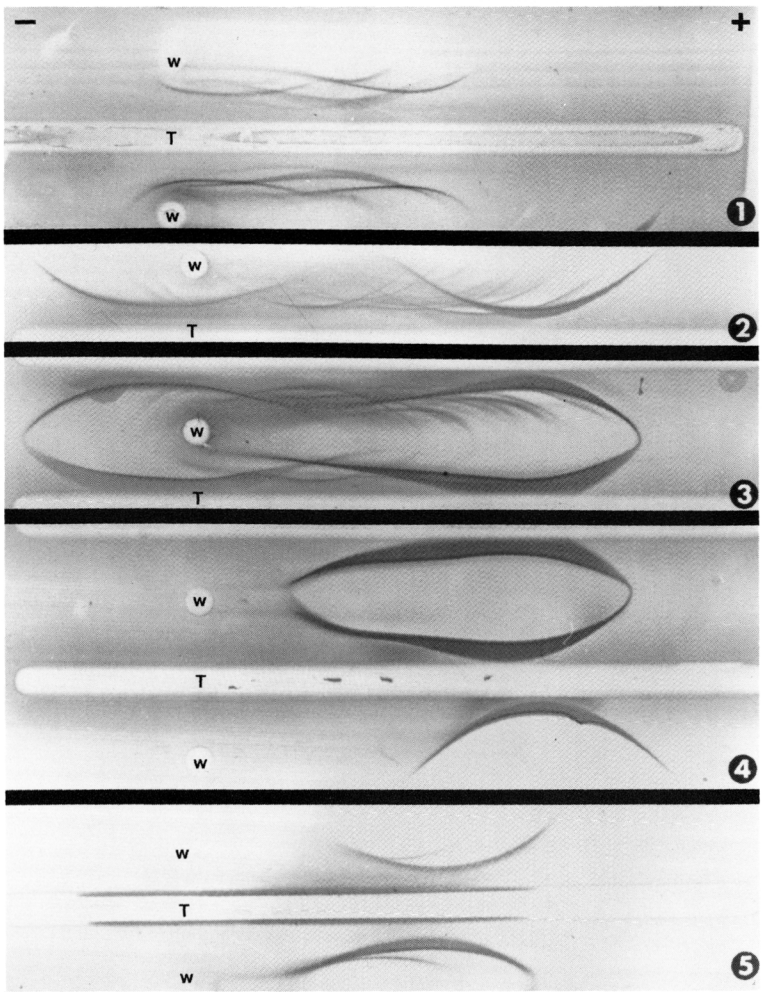
tests having "intact" peritoneal fluid as the antigen source (see the albumin band, figure 3). This aberration was eliminated by pre-treatment of the peritoneal fluid with Varidase.

The "band distortion" effect of hyaluronic acid was also studied with two purified antigens, human serum mercaptalbumin and calf serum fetuin. With these test proteins, significant elongation of the precipitin arcs could be generated by electrophoresing the antigens in the presence of umbilical cord hyaluronic acid (figures 4 and 5, respectively); as indicated, the observed distortion could be eliminated by Varidase digestion of the hyaluronate in the test samples. Additional abnormal precipitin bands were noted in a survey of immunoelectrophoresis patterns obtained with ethylene diamine tetracetic acid (EDTA) extracts of specimens of Wilms' tumor or of cells cultured from Wilms' tumors. In all of these cases (14 specimens), the



FIGURES 1 TO 5. Gel immunoelectrophoresis patterns, with antigen loading wells at left and antiserum troughs extending horizontally above and/or below the wells. "W" and "T" indicate wells and troughs respectively.

1. Peritoneal fluid from the Wilms' tumor patient vs. rabbit antiserum to the peritoneal fluid.

2. Peritoneal fluid from patient with Wilms' tumor vs. rabbit antiserum to Varidase-treated peritoneal fluid (cf. with fig. 1).

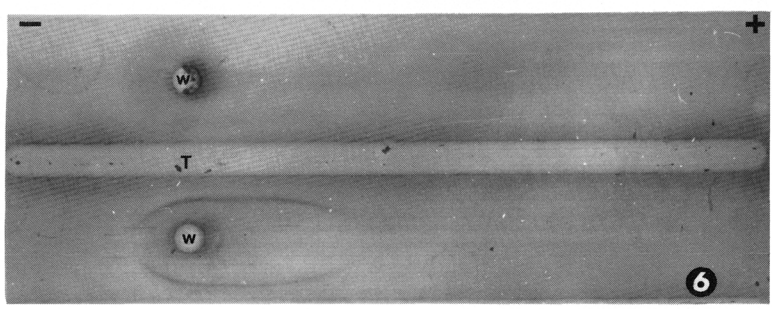
3. Peritoneal fluid from patient with Wilms' tumor vs. commercial rabbit antiserum to whole human serum.

4. Effect of added hyaluronic acid on immunoelectrophoresis profile of plasma albumin. Top well: human mercaptalbumin (25 mg/ml) + hyaluronic acid (0.5 mg per ml). Central trough: rabbit anti-human serum. Bottom well: human mercaptalbumin + hyaluronate sample after Varidase treatment.

5. Effect of added hyaluronic acid on immunoelectrophoresis profile of bovine fetuin. Bottom well: fetuin (5 mg/ml) + hyaluronic acid (0.5 mg per ml). Central trough: rabbit antiserum to Spiro's fetuin. Top well: fetuin + hyaluronate sample after Varidase treatment.

"abnormalities" disappeared after treatment of the antigen mixtures with Varidase. It was of particular interest that, in some samples, additional precipitin bands were disclosed as a consequence of Varidase treatment of the test samples (figure 6). It appears, therefore, that hyaluronic acid can not only alter the immunoelectrophoretic behavior of the antigens, but can also mask their appearance entirely (for certain proteins). This effect can be quantitated by "rocket" immunoelectrophoresis (figure 7): increasing amounts of hyaluronate in a test antigen sample (human mercaptalbumin) causes reductions in the apparent available content of the antigen until, at a ratio of 0.4 μg of hyaluronate to 1.0 μg mercaptalbumin, the "rocket" precipitin bands are fully abolished.

Gel filtration studies with serum proteins were also conducted to examine the hyaluronate-antigen mixtures for direct evidence of bonding interactions. This was accomplished by generating elution profiles of protein standards (albumin, IgG, and IgM) chromatographed on Sephacryl S-200. The resulting profiles, shown in figure 8, demonstrate that in the presence of the polysaccharide, the protein components were not resolved; this was verified by Ouchterlony immunodiffusion tests of each of the column fractions (after Varidase treatment of each). Furthermore, the hyaluronic acid caused a marked increase in the elution volume (retention) of the proteins, suggesting that these substances were excluded from the mobile phase by the hyaluronate.



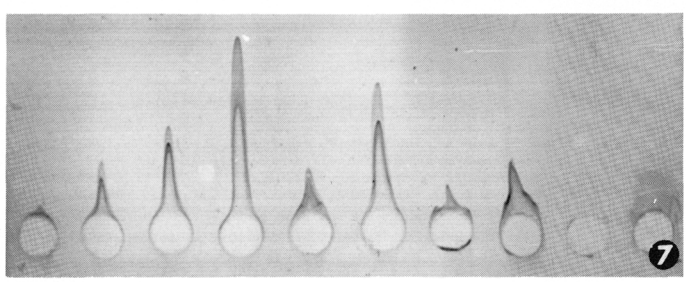


FIGURE 6. Effect of Varidase treatment on the detection of antigens by immunoelectrophoresis. Top well: isotonic EDTA extract of Wilms' tumor tissue. Bottom well: same extract after incubation with Varidase. Central trough: antiserum to peritoneal fluid of Wilms' patient, after absorption with normal human serum.

FIGURE 7. Rocket immunoelectrophoresis of bovine serum albumin (BSA) with rabbit anti-BSA in the support agar. Initial contents of test wells (from left to right):

1. 0.25 μg BSA
2. 0.50 μg BSA
3. 1.00 μg BSA
4. 2.00 μg BSA
5. 0.50 μg BSA + 0.1 μg hyaluronic acid
6. 1.00 μg BSA + 0.1 μg hyaluronic acid
7. 0.50 μg BSA + 1.0 μg hyaluronic acid
8. 1.00 μg BSA + 1.0 μg hyaluronic acid
9. 0.50 μg BSA + 10.0 μg hyaluronic acid
10. 1.00 μg BSA + 10.0 μg hyaluronic acid

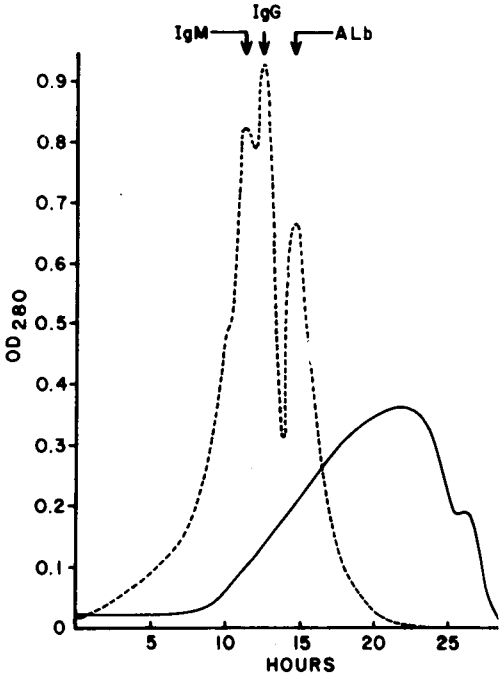


FIGURE 8. Sephacryl S-200 chromatography profiles of human Igg, IgM, and albumin with ( ) and without (—) hyaluronate. (Sample: 2 ml containing 100 mg of each protein and 20 mg of human umbilical cord hyaluronic acid).

Discussion

To date, relatively little is known concerning the biological roles of glycosaminoglycans. For example, it is thought that hyaluronic acid, by virtue of its high molecular weight, polyanionic nature, and random coiled configuration, binds considerable water, acts as a lubricant, and "excludes" other substances in its environment.[4,22] In recent years, a more specific role of hyaluronate has been found in cartilage, wherein proteoglycan units are assembled onto strands of hyaluronic acid, thus creating large proteoglycan aggregates.[11,17]

Our knowledge concerning the immunological reactivity of the glycosaminoglycans is rather sparse. It is known that, with few exceptions, the hyaluronic acids are not very immunogenic, although other glycosaminoglycans, such as chondroitin sulfates, can alter the antigenic determinants of protein moieties in proteoglycans.[8,9] *In vivo,* the glycosaminoglycans are found not only in extracellular spaces but also at cell surfaces,[17,19] a fact which prompted Lippman to suggest that the promotion of mouse tumor development in allogenic hosts by mucopolysaccharide treatment might be due to blocking of cell surface antigens.[18] In the same vein, Burger and Martin have proposed that hyaluronic acid can mask agglutinin (plant lectin) receptors of transformed fibroblast surfaces.[5]

The results of our current studies suggest both an additional systemic biological function of hyaluronic acid (the modulation of a humoral immune response) and indicate the need for added caution when conducting experiments in the presence of glycosaminoglycans.

Specifically, it has been shown by us that hyaluronate, when included with known protein immunogens, selectively suppresses the formation of circulating precipitating antibodies directed toward some of these substances. At this time, the mechanism of this effect is not known nor why it should operate in a selective fashion for proteins such as albumin, alpha-globulins, and gamma-globulins. It could be argued that the relatively high levels of protein in our injectates caused a type of immunosuppression that was reversed by Varidase treatment. The amount of antigen used, however, is not unreasonable considering our observation that the peritoneal fluid was similar in composition to dilute whole plasma (which is known to cause strong immune responses with similar protein concentrations). Of course it is possible that the observed *in vivo* differences reflect responses to a combination of enzyme activities, since Varidase generally contains some protease and oligosaccharidase activities (in addition to hyaluronidase activity).

Our supporting *in vitro* experiments, though support the belief that these responses are hyaluronate-mediated.

Collectively, our observations appear to indicate that hyaluronic acid interacts with albumin and other proteins so as to alter their immunogenicity. One possible explanation for such interaction is a so-called "excluded volume" effect described by Balazs,[4] whereby the large effective volume of hyaluronate in solution reduces the domain of water available to other solutes (which in turn, become less soluble and tend to seek accessible compartments of higher water activity). An example of such a reduced solubility consequence was described in Hellsing's study of the effect of dextrans which increased the amount of precipitate formed in an albumin-antialbumin reaction.[12]

Laurent[15,16] also has reviewed many examples of the compartmental redistribution phenomenon, including the classical study of Ogston,[23] in which albumin was preferentially distributed to the hyaluronic acid-free chamber in a transfilter experiment. Our own gel chromatography study may further exemplify this phenomenon; the hyaluronate increased the partitioning of the test proteins toward the gel phase, thereby causing delays in elution and mixing of protein components. This same kind of phenomenon may account for distortion of immunoelectrophoresis bands, although it does not seem adequate to explain the selective loss of antigen and immunogenicity. Hyaluronic acid-mediated electrophoretic band distortion has also been reported in association with the production of hyaluronic acid by neoplastic cells.[1,13,28] A second, not mutually exclusive, mechanism might involve preferential binding, as described by Pigman and his associates.[21,24] In their studies, new moving boundaries and increased mobilities were observed when intact hyaluronic acid was present with proteins of synovial fluids in moving-boundary electrophoresis. Such associations of hyaluronic acid with various proteins may be too weak to detect by conventional means such as gel chromatography or sedimentation velocity studies, but they could have marked effects on immunogenicity, shapes of immunoprecipitin bands and solubility of antigen-antibody complexes.

Additionally, the formation of charge-mediated complexes between glycosaminoglycans and particular proteins could significantly alter the immunogenicity of the proteins in question. The possible ability of hyaluronate to mask the immunogenicity of particular proteins combined with the observation that certain neoplasms produce excess amounts of hyaluronate[1,3,13,20,25,27] suggests a functional role. Indeed, it is tempting to postulate that neoplastic cells may produce glycosaminoglycans as an immunogenic mask to reduce or eliminate the recognition of tumor cell surface proteins by circulating antibodies. This might be particularly relevant in virus-induced neoplasms where viral proteins are integrated into the cell membrane upon virus release. Although our current understanding of the possible role glycosaminoglycans play *in vivo* is speculative, our studies do affect the way in which experiments should be designed.

Hyaluronate clearly affects the availability of particular proteins to the surrounding environment. Specifically, it is apparent that the search for and the characterization of "tumor-associated" protein antigens[6] must consider the potential interfering effects of hyaluronic acid and possibly other glycosaminoglycans.

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