INDUCTION OF ANTIBODIES TO HYALURONIC ACID BY IMMUNIZATION OF RABBITS WITH ENCAPSULATED STREPTOCOCCI

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Hyaluronic acid was first described by Meyer and Palmer (1) in mammalian tissue and by Kendall et al (2) in group A streptococci. A number of investigators (2-11) have attempted to show that hyaluronate is immunogenic. In general, these studies have failed to show the immunogenicity of hyaluronate, despite the variety of methods used for immunization, including the use of hyaluronate conjugated to BSA as a hapten-carrier immunogen (6). These studies primarily used immunoprecipitation and agglutination reactions to detect hyaluronate antibodies. Recent studies (12) using more sensitive techniques have indicated that naturally occurring antibodies to hyaluronate may be found in the sera of a number of species of animals. Other investigators (13) have suggested that antibodies to DNA, which occur in the sera of patients with systemic lupus erythematosus, may crossreact with hyaluronate. We have obtained data (14) suggesting that antibodies to hyaluronate may be present in the sera of patients with poststreptococcal glomerulonephritis.

In this study, we report data indicating that hyaluronate is immunogenic in rabbits. Upon immunization of rabbits with formalinized encapsulated streptococci, antibodies are produced that react specifically with hyaluronate derived from both streptococci and from mammalian tissue. By an ELISA using a biotinylated conjugate of hyaluronidase-digested hyaluronate as antigen, and using a number of different inhibitors, we have determined that the immunodominant site of hyaluronic acid recognized by these antisera is the carboxyl group of a terminal glucuronic acid residue. Furthermore, hidden antigenic sites of hyaluronate, containing terminal glucuronic acid, are exposed by mammalian (testicular) hyaluronidase treatment.

Materials and Methods

Biochemicals. Purified mammalian hyaluronate was purchased from Miles Laboratories (Elkhart, IN), Sigma Chemical Co. (St. Louis, MO), and Worthington Biochemical Corp. (Freehold, NJ). Chondroitin-4-sulfate and heparin were obtained from Sigma Chemical Co. Heparan sulfate was obtained from Miles Laboratories. N-acetylglucosamine, N-acetylgalactosamine, and glucuronic acid (sodium salt) were obtained from Sigma Chemical Co. Galacturonic acid and glucuronamide were obtained from Aldrich Chemical Co., Milwaukee, WI. Testicular hyaluronidase was obtained from Worthington Biochemical Corporation.

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Corp. Type III pneumococcal polysaccharide was prepared from a stock type III strain (A66).

Preparation of Streptococcal Hyaluronate. Group C streptococcal strain D181 was grown in a fermenter in 60 liters of dialysate medium (15) at pH 7.5, with extra glucose. When growth ceased, the capsular hyaluronate was released from the surface of the bacterial cells by saturating the culture medium with chloroform. The bulk of the bacteria were then removed by Sharples centrifugation, although the process was inefficient because of the viscosity of the hyaluronate-containing medium. The hyaluronate was precipitated by the addition of Cetavlon at a final concentration of 0.5%, and the precipitate was washed repeatedly with distilled water. Resolution of the precipitate in 6 liters of 1 M CaCl₂ yielded an almost solid gel, and this property was retained after reprecipitation with two volumes of alcohol and resolution in 6 liters of 0.9% NaCl. This fraction is referred to as IA, and represented 60 g hyaluronate, as estimated by glucuronate analysis.

To render the hyaluronate manageable for further manipulation, it was partially hydrolyzed enzymatically. Two successive treatments with testicular hyaluronidase (Worthington Biochemical Corp.) were carried out at room temperature for 24 h at 20 and 100 mg/liter, respectively, using a total of 60 gm (10 gm/liter) of hyaluronate. The solution was now markedly reduced in viscosity and was filtered with suction through a bed of filter cel to remove residual bacteria and insoluble material. Fraction IA₁ was recovered by precipitation with one volume of alcohol, and presumably represents the larger oligosaccharide fragments remaining after enzymatic hydrolysis. This fraction contained 4.25 g of material, which on analysis showed N-acetylglucosamine, 49.0% (16) and glucuronic acid 47.7% (17). Fraction IA₂ was a crystalline fraction that appeared in the supernatant fluid of fraction IA₁; 10.1 g of this fraction was obtained, which on analysis contained 50% N-acetylglucosamine and 49.9% glucuronic acid.

Biochemical Modifications of Hyaluronate. To determine the immunodominant site of reactivity of hyaluronic acid, two methods for the biochemical modification of IA₁ were used. (a) Periodate oxidation and borohydride reduction: a modification of a previously described method (18) was used. 20 mg IA₁ was dissolved in 10 ml 0.1 M sodium metaperiodate (Sigma Chemical Co.) and rotated in the dark at room temperature for 4 h. The reactivity was monitored at 225 nm. After 4 h, a moderate decrease in absorbance was noted in the reaction solution compared to control solutions. The material was dialyzed against distilled water and lyophilized. The lyophilized material (5 mg) was dissolved in distilled water (1 mg/ml). 250 mg of sodium borohydride was slowly added at room temperature, and the mixture was stirred for 4 h. The solution was then neutralized with 4 N HCl, dialyzed against distilled water, and lyophilized. The final material was labelled as IA₁-periodate. (b) Carboxyl reduction: carboxylic groups of IA₁ were reduced according to the method of Taylor and Conrad (19). 30 mg of IA₁ were dissolved in 10 ml of distilled water. 191.7 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was slowly added, and the solution was titrated to pH 4.75 with 0.1 N HCl. The solution was stirred for 2.5 h, with the pH constantly titrated to pH 4.75. 20 ml of 2 M sodium borohydride was slowly added, with the pH maintained at 8.0 with 4 N HCl. The reaction mixture was stirred for 30 min. Finally, the mixture was dialyzed against distilled water and lyophilized. This material was labeled as IA₁-carboxyl.

Immunization of Rabbits with Encapsulated Streptococci. Vaccines were prepared by formaldehyde treatment of streptococci harvested in the log phase of growth in an attempt to promote retention of the capsule. The strains used were S23op (group A, type 14) and D181 (group C). The organisms were grown in Todd-Hewitt broth, recovered by centrifugation and resuspended in a solution containing 1.5% formaldehyde and 7.5% sucrose. After refrigeration for 3 d, the cells were washed twice with phosphate buffer, pH 7.5, and resuspended in the buffer. Capsules were readily demonstrable by the India ink method, although those on the S23op cells appeared much smaller than before formaldehyde treatment. Chemical analysis for glucuronic acid in the capsular material released from an aliquot of the vaccine by chloroform indicated the presence of 60 μg/ml hyaluronate in the S23op preparation and 300 μg/ml in D181. The vaccines were
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distributed in a series of plastic tubes and stored at -80°C until use. Freezing and thawing had no adverse effect on the capsules.

Rabbits were given a series of intravenous injections with the vaccines, two with S23op (rabbits 4511 and 4512) and two with D181 (rabbits 4513 and 4514). 1 ml was given on three successive days, followed by a test bleeding 1 wk later. After the fifth injection series, the animals were challenged intravenously with a suspension of living D181 organisms. Only one (4514) survived, and two further test bleedings were obtained from this rabbit.

Conjugation of Streptococcal Hyaluronate to BSA. Intact streptococcal hyaluronate (fraction IA) was conjugated to BSA (Gohn fraction V; Sigma Chemical Co.) This was done to assure binding of the hyaluronate to the ELISA plates via the protein portion of the conjugate. 20 mg of fraction IA were placed in 10 ml 0.1 M sodium phosphate, pH 8.0, and briefly sonicated until solubilized. 2.5 ml of 250 mM benzoquinone (Pierce Chemical Co., Rockford, IL) in absolute ethanol were added to the IA solution and rotated at room temperature for 20 min. 100 ml of ethanol/sodium acetate was added, and the mixture was centrifuged at 12,100 g for 15 min. The pellet was retained and washed twice with ethanol/acetate. BSA was acid-inactivated to destroy associated alkaline phosphatase activity as follows: 11 mg of BSA was added to 2 ml of distilled water, brought to pH 2.0 with 5 N HCl, and incubated for 20 min at room temperature. After incubation, the BSA solution was brought to pH 8.0 with 2 ml of 0.1 M sodium phosphate. Finally, the solution of acid-inactivated BSA was added to the pellet of hyaluronate-benzoquinone and rotated overnight at 4°C.

The conjugated material was then chromatographed on a 2.5 × 180 cm. column of Sephacryl S2000 in 0.1 M Tris buffer, pH 8. Three peaks were obtained when the column was analyzed by absorption at 280 nm. A plateau of absorbance was noted in fractions 34–37 and 38–40. Finally, a peak was noted in fractions 61–70. An analysis of the column for glucuronic acid (20) revealed that the majority of glucuronic acid eluted in the fractions from 10–45, with decreasing amounts of glucuronic acid from fraction 10 to fraction 45, and little or no glucuronic acid in fractions beyond 55. SDS-PAGE analysis, using a 10% gel, revealed that fractions 35–40 contained material that chromatographed as a diffuse, high molecular mass smear, with a sharper band appearing at 68 kDa, while fractions 65–67 contained only a sharp band at 68 kDa. Dot immunoblotting analysis of the column fractions showed that the fractions containing protein all reacted with a rabbit antiserum against BSA (Cappel Laboratories, Cochranville, PA). These data indicate that fractions 34–37 contained a significant amount of high molecular mass material containing both glucuronic acid and protein, with the highest ratio of glucuronic acid/protein in fractions 34–36. Fraction 35 was chosen for use as antigen in the ELISA, and is referred to as hyaluronate-BSA. Total protein was assessed by a colorimetric assay (Pierce Chemical Co.). The material was diluted to 10 μg/ml in Tris buffer and added to ELISA plates kept overnight at 4°C. After washing, rabbit sera in PBS with 0.5% Brij (PBSB) were added for 1 h, and subsequent steps were performed as described above.

Preparation of Biotinylated Streptococcal Hyaluronate. A second method for binding the hyaluronate to the ELISA plates was used (21) with some modifications for the introduction of a spacer arm (22). Biotinylated hyaluronate was prepared using the fraction IA. 25 mg of fraction IA, in 20 ml PBS was added to 5 ml of 250 mM benzoquinone (Aldrich Chemical Co.) in 100% ethanol. The mixture was rotated for 1 h at room temperature in the dark. 100 ml of absolute ethanol with 0.05 M sodium acetate was added, the mixture brought to 4°C, and centrifuged at 17,700 g for 20 min. The pellet was resuspended in 100 ml of ethanol/acetate and centrifuged a second time. After this, the pellet was resuspended in 18 ml of 0.5 M NaCl and dialyzed against 1 liter of 0.5 M NaCl at 4°C. 2.25 ml of 1 M diaminodipropylamine was added to the reaction mixture, and the mixture was rotated overnight at 4°C. The mixture was then dialyzed against 0.1 M NaHCO3 at 4°C, and centrifuged at 1,500 g to remove precipitated material. The conjugated hyaluronate in the supernatant was tested for free amino groups (23). 25 mg N-hydroxysuccinimide biotin (Sigma Chemical Co.) was dissolved in 5 ml of DMSO and

1 Abbreviation used in this paper: PBSB, PBS with Brij.
added to 20 ml of the supernatant containing free amino groups. This mixture was rotated overnight at 4°C and then dialyzed against distilled water and lyophilized. The biotinylated hyaluronate (IAI-B) was tested for glucuronic acid by the carbazole reaction (20) using d-glucuronolactone (Sigma Chemical Co.) as a standard. ~48% of the total weight of the IAI-B was glucuronic acid, indicating that 96% of the sample was probably hyaluronic acid, while 4% of the sample represented biotin and the linking peptides. Group C meningococcal polysaccharide obtained as previously described (24) was biotinylated in the same manner as described above.

Enzyme-linked immunosorbent assay. ELISA were performed essentially as we previously described (14), with some modifications for the use of streptavidin and biotin-labelled polysaccharides (25). ELISA plates (Immunoplate II; Nunc, Roskilde, Denmark) were coated with hyaluronate-BSA conjugate in various concentrations in 50 µl 0.1 M Tris buffer (pH 9.8) with 0.3 mM MgCl₂ overnight at 4°C. For experiments employing streptavidin (Sigma Chemical Co.) (10 µg/ml Tris buffer), streptavidin was first bound to the ELISA plates overnight at 4°C, the plates were washed, and biotinylated hyaluronate in PBSB was added for 2 h at room temperature. After this incubation, the plates were washed in 0.9% NaCl/0.05% Brij for a total of five washes. Subsequent incubations were performed at room temperature with material diluted in 0.1 M PBSB, with intervening washes performed as above. Finally, after an incubation with alkaline phosphatase–conjugated goat anti–rabbit IgG antisera (Sigma Chemical Co.) at 1:500 in PBSB for 1 h, the plates were washed, and developer containing 1 mg/ml of nitrophosphatase (Sigma Chemical Co.) in 10 ml of diethanolamine buffer (97 ml diethanolamine, 1 ml 1 M MgCl₂, 1 ml 0.1 M zinc acetate, 0.2 gm sodium azide, and 800 ml distilled water, pH 9.8) was added. The plates were incubated at 37°C, and after 1 h, were read at 405 nm in an automated ELISA reader (Titertek Multiscan; Flow Laboratories, McLean, VA).

Results

Immunologic Studies of Streptococcal Hyaluronate Using the Precipitin Reaction and Other Methods. Sera from the four rabbits were tested, during the course of the immunization, for the presence of precipitating antibodies. We used both the capillary method and the Ouchterlony method. No precipitating antibodies were detected over a range of concentrations of the streptococcal hyaluronate preparations. In preliminary experiments, microagglutination reactions were attempted using formalinized group C streptococci. Streptococci were diluted in PBS to form a dilute suspension in 96-well microtiter plates (Costar, Cambridge, MA). The results were read using an inverted microscope (Carl Zeiss, Thornwood, NY). Some agglutination was seen with immune sera from rabbit 4512 at titers of <1:40. In an attempt to show that such agglutination was due to specific reactions with the hyaluronate capsule, inhibition experiments were performed in which the sera were preincubated with IA or IAI (up to 100 µg/ml) for 2 h at room temperature, and overnight at 4°C before agglutination tests. However, no reproducible inhibition of agglutination could be detected.

Immunologic Studies of BSA-conjugated Hyaluronic Acid. Rabbit preimmune sera and sera from the fourth bleeding after immunization were chosen for testing by ELISA. Results showed that all four rabbits had a significant increase in the titer of reactivity to hyaluronate-BSA after immunization. Table I summarizes these results. In general, a greater than four-fold rise in titer was noted in the reactivity to hyaluronate-BSA after the fourth immunization series. Similar experiments using BSA (Sigma Chemical Co.) as antigen for the ELISA showed that all sera tested had a reciprocal titer of <20 to BSA, except for rabbit 4514,
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TABLE I

ELISA Studies of Preimmune and Peak Immune Rabbit Sera to Hyaluronate-BSA and to Biotinylated Hyaluronate

| Rabbit number | Preimmune titer | Immune titer |
|---------------|-----------------|--------------|
| Hyaluronate-BSA |                 |              |
| 4511          | <100            | 400          |
| 4512          | <50             | 400          |
| 4513          | <400            | 1,600        |
| 4514          | <200            | 800          |
| Biotinylated Hyaluronate |     |              |
| 4511          | <100            | 12,800       |
| 4512          | <100            | 3,200        |
| 4513          | <100            | 25,600       |
| 4514          | <100            | 12,800       |

Immune sera were taken from rabbits after the fourth immunization series. A titer is defined as the dilution of serum at which an optical density (at 405 nm) of 1.0 is obtained after 1 h of development. Results are expressed as reciprocal titer.

FIGURE 1. ELISA studies using hyaluronate-BSA (10 μg/ml) as antigen and immune rabbit serum (4514, fourth bleeding) at a 1:100 dilution. The broken line represents the results of the serum incubated alone without inhibitor. The inhibitors were used at varying dilutions, and were preincubated with the rabbit serum for 4 h at room temperature and overnight at 4°C before assay. Final concentrations are presented for all data. (Hs, heparan sulfate; ChS, chondroitin-4-sulfate; IA, fraction IA; HuHy, human hyaluronate from umbilical cord; BSA, bovine serum albumin).

which had a titer of <40. These results indicated that the reactivity to hyaluronate-BSA observed was not due to reactivity to BSA.

To further confirm that the reactivity of the rabbit sera was directed against hyaluronate, inhibition experiments were performed. For these experiments, sera were preincubated with inhibitors at room temperature with rotation for 2 h and then overnight at 4°C. In Fig. 1, the results of a representative inhibition experiment are shown. The fourth bleeding of rabbit 4514 (1:100) was prein-
cubated with fraction 1A, human hyaluronate, heparan sulfate, chondroitin sulfate, and BSA in varying concentrations. As can be seen from the figure, fraction 1A (streptococcal hyaluronate) and human hyaluronate inhibited the reactivity of sera 4514 (bleeding four) to hyaluronate-BSA in a dose dependent manner, while BSA inhibited the reactivity to hyaluronate-BSA only in the largest dose (250 μg/ml). No effect was seen when heparan sulfate or chondroitin sulfate were used as inhibitors. Since these glycosaminoglycans both contain hexosamine and uronic acid and are highly anionic, similar to hyaluronate, this result confirms the specificity of the inhibition reaction to the hyaluronate polysaccharide. To eliminate further the possibility that hyaluronate might have some nonspecific effect in the inhibition reaction, the reactivity of a rabbit antiserum to BSA (Cappel Laboratories) was tested in the ELISA using hyaluronate-BSA. This antiserum was preincubated with either fraction 1A, human hyaluronate, or BSA. Only BSA significantly inhibited the reactivity of the rabbit anti-BSA serum with hyaluronate-BSA in a dose-dependent manner (Fig. 2).

Immunologic Studies of Biotinylated Hyaluronic Acid. Streptavidin was coated in ELISA plates at 10 μg/ml in Tris buffer at room temperature for 4 h. After washing, IA₁-B in PBSB was added and incubated for 2 h at room temperature, and then overnight at 4°C. After washing the plates, rabbit sera were added at

FIGURE 2. ELISA studies of hyaluronate-BSA using a rabbit antiserum to BSA. The studies were performed and are presented as in Fig. 1.

FIGURE 3. ELISA studies using biotinylated IA₁ (IA₁-B) (10 μg/ml) as antigen. All bleedings of each rabbit (4511, 4512, 4513, and 4514) before and during immunization with encapsulated streptococci were tested at a final concentration of 1:200.
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Fig. 4. ELISA studies employing IA1-B as antigen. The plates were preincubated with biotin (1 mg/ml, biotin/PBSB) before the addition of IA1-B to specifically block the attachment of IA1-B. Peak immune rabbit serum was then added in varying dilutions. In control wells, only PBSB and no biotin was added.

Fig. 3 shows the results of a study in which each bleeding from each rabbit was studied at a final dilution of 1:200. All four rabbits clearly developed increased reactivity to the IA1-B during the immunization period. Rabbits 4513 and 4514, which were immunized with group C D181 streptococci containing more extensive capsules, developed generally higher titers earlier during the course of immunization than rabbits 4511 and 4512, which were immunized with group A S23op streptococci possessing less abundant capsules. In titration experiments comparing the titer of preimmune sera with those obtained after the fourth immunization, a >32–256-fold increase in titer was noted (Table I). The observation that the IA1-B is a much better antigen in the ELISA than the hyaluronate-BSA may be related, in part, to the fact that long-chain hyaluronate was used for conjugation with BSA, while hyaluronidase-digested hyaluronate was used for conjugation with biotin.

To show that the sera were indeed specifically reacting with the biotinylated hyaluronate, the following experiment was performed. After coating the plates with streptavidin and washing, the plates were incubated with either PBSB alone or biotin (1 mg/ml) in PBSB, for 1 h. The plates were washed, IA1-B was added, followed by the addition of sera (1:1000) and other reagents according to the protocol above. In this way, the free biotin should prevent the binding of the biotinylated hyaluronate. As shown in Fig. 4, this was indeed the case. The plate was read at 2 h to accentuate the differences. While the preimmune sera showed only background activity, the immune sera reacted with a titer of >12,800. However, the addition of free biotin abolished this reactivity almost completely, indicating that the sera were indeed reacting with the biotinylated hyaluronate. Similar results were noted with all four rabbit sera.

To confirm that the ELISA reliably measures antipolysaccharide antibody,
group C meningococcal polysaccharide prepared according to methods previously described (24) was biotinylated in the same manner as described above for the hyaluronate. Human and rabbit sera shown (26) to contain antibody to the group C meningococcal polysaccharide by a passive hemagglutination method were used for testing. Results in the ELISA were identical to those found with RIA. Titers of group C meningococcal antibody in immune rabbit sera were found to be $>2.5 \times 10^6$ (reciprocal titer). Biotin inhibition experiments similar to those described above completely eliminated the rabbit sera reactivity.

The specificity of the immune rabbit sera were investigated by inhibition experiments. These were performed as described above, by preincubating the sera with inhibitors overnight before assay. These experiments showed that the reactivity of all four rabbit sera was directed against hyaluronic acid (representative data shown in Fig. 5). Only preparations of hyaluronate were effective as inhibitors, including both mammalian and streptococcal hyaluronate. Inhibition of reactivity was shown using fraction IA$_2$, which was the most purified preparation of hyaluronate available, containing 50% glucuronic acid and 50% N-acetylglucosamine. This result made it highly unlikely that contaminating substances accounted for such reactivity, particularly since 50% inhibition of reactivity was noted with 1 µg/ml of IA$_2$. Further, the same hypothetical contaminant would need to be present both in the streptococcal and the mammalian hyaluronate preparations, a situation that seems highly unlikely.

The specificity of the epitope that accounted for the observed immunoreactivity was studied by specific inhibition experiments (Fig. 5). Other charged polysaccharides containing uronic acid, including the type III pneumococcal polysaccharide and glycosaminoglycans (including heparan sulfate, heparin, chondroitin sulfate) were completely ineffective inhibitors. This result indicated, as was shown previously, that the immunoreactivity was directed against specific epitopes of the hyaluronate polysaccharide.

The results of these experiments also showed that while intact, long chain hyaluronate was a relatively poor inhibitor of the serum reactivity to IA$_1$-B, hyaluronidase-digested preparations of streptococcal hyaluronate were ~100-fold more effective as inhibitors (Fig. 5). To confirm this hypothesis, 2 mg of mammalian hyaluronate (from human umbilical cord; Miles Laboratories), at a concentration of 1 mg/ml in 0.15 M NaCl, pH 6, was digested with 0.2 ml
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FIGURE 6. ELISA studies using I\(_A\),B as antigen. Inhibitors were preincubated with peak immune rabbit serum. HuHYA digest, human hyaluronate digested with hyaluronidase.

FIGURE 7. ELISA studies using I\(_A\),B as antigen. Peak immune rabbit serum was preincubated with various monosaccharides.

testicular hyaluronidase (1 mg/ml in 0.15 M NaCl, pH 6, 400 U/mg; Worthington Biochemical Corp.) and rotated for 4 h at room temperature. The material was centrifuged and the supernatant was precipitated three times with absolute ethanol. Finally, the precipitate was washed in ether and dried in air. The partially digested mammalian hyaluronate was approximately five times more effective as an inhibitor than the original starting preparation (Fig. 6). This result suggested that a determinant of the hyaluronate that was partially exposed on the long chain was further exposed after hyaluronidase treatment. Since hyaluronate is a repeating disaccharide, either of the monosaccharides, N-acetylglucosamine or glucuronic acid, could be the primary immunodeterminant of serum reactivity. Inhibition reactions performed using these monosaccharides showed that glucuronic acid (sodium salt) was an effective inhibitor of serum reactivity to I\(_A\),B, while N-acetylglucosamine and N-acetylglactosamine were ineffective inhibitors (Fig. 7). Galacturonic acid was also an effective inhibitor of serum reactivity, though generally somewhat less so. All monosaccharide solutions had a pH of 7.2. These results indicated that a terminal glucuronic acid unit is probably the primary epitope of the major immunodeterminant responsible for the immune response to hyaluronic acid in these sera, and that this epitope was shared by galacturonic acid.

To investigate further the hypothesis that an epitope on a terminal monosaccharide of the polysaccharide chain was responsible for the serum reactivity to
IA₁-B, the IA₁ was treated with periodate and borohydride, as described in Materials and Methods, to preferentially destroy the terminal monosaccharide of the polysaccharide chain. As seen in Fig. 8, these results show that periodate oxidation and borohydride reduction effectively reduced the antigenicity of the IA₁ polysaccharide.

Finally, we investigated the hypothesis that the determinant shared by glucuronic acid and galacturonic acid which was the immunodominant site of serum reactivity to hyaluronate was the carboxyl group of the terminal glucuronic acid. IA₁ was treated with carbodiimide and then subjected to borohydride reduction, destroying the carboxyl group. This procedure significantly reduced the antigenicity of the IA₁ polysaccharide (Fig. 8). This was also shown using glucose and glucuronamide as inhibitors. The differences among these hexoses are illustrated in Fig. 9. As seen in Fig. 9, only glucuronic and galacturonic acid were effective inhibitors of serum reactivity. In summary, these data provide evidence that the
carboxyl group of the terminal glucuronic acid of hyaluronate is the primary focus of the immune response to hyaluronic acid in rabbits immunized with encapsulated streptococci.

Discussion

This study reveals the presence of antibodies to hyaluronate in the sera of rabbits immunized with formalinized, encapsulated group A and group C streptococci. These same sera were tested earlier for precipitating antibodies, and none were found. One possible explanation for the discrepancy is that the concentration of specific antibody is too low to yield visible precipitates, and that positive reactions can only be detected with tests of much higher sensitivity, such as the ELISA. However, a second possibility is raised by the inhibition experiments, which suggest that the antibodies recognize only the terminal nonreducing residues of the hyaluronate molecule. Since the hyaluronate is an unbranched, linear molecule, antibodies of this type could not build a precipitating lattice. An analogy to this situation has been described by Braun (27) in relation to the antibody response to the cell wall carbohydrate of group A and A variant streptococci. 90% of the antibodies mice produced against these antigens recognize only the terminal nonreducing end of the molecule (N-acetylglucosamine and rhamnose, respectively), and are thus nonprecipitating. Braun has confirmed this with the isolation of mAbs to the carbohydrate, 90% of which are of the nonprecipitating type.

The results of the experiments reported here indicate that the terminal glucuronate of the hyaluronate molecule is the dominant determinant of specificity, with the carboxyl group playing a significant role. This is supported by the inhibitory effect of sodium glucuronate (and to a somewhat lesser extent, sodium galacturonate) and the lack of inhibition by N-acetylglucosamine. The fact that increasing the concentration of terminal glucuronide residues by partial enzymatic hydrolysis markedly enhanced the inhibitory effect of hyaluronate preparations is consistent with the possibility that the antibodies are only recognizing the nonreducing terminus of the hyaluronate molecule. When IA1 was treated with periodate oxidation and borohydride reduction, preferentially destroying the terminal glucuronide (since there are no other cis-hydroxyl groups in the polysaccharide), the antigenicity of IA1 was destroyed, providing more evidence that a terminal glucuronide is the primary immunodeterminant of hyaluronate. The specificity of the reaction for the carboxyl group of the glucuronide was revealed by the demonstration that galacturonic acid was also an effective inhibitor of serum reactivity, while glucose and glucuronamide had minimal inhibitory effect. Finally, elimination of the carboxyl groups of IA1 by reduction with carbodiimide followed by borohydride reduction eliminated the antigenicity of the IA1, further demonstrating that the immunodominant site of antibody reactivity to hyaluronate is the carboxyl group of the terminal glucuronide. Kozel and Gotschlich (28) have previously shown that rabbits can form carboxyl-specific antibody against polysaccharides containing uronic acid. Although the antibodies produced in response to immunization with hyaluronate are directed against a carboxyl group of glucuronate as the primary immunodeterminant, the lack of inhibition by other polysaccharides also containing glucu-
ronate (such as other glycosaminoglycans and the pneumococcal polysaccharide) indicates that this epitope is only part of the complete antigenic determinant of the hyaluronate polysaccharide.

Previous attempts to identify antibodies to hyaluronate have been unsuccessful despite the use of a variety of methods for immunization and detection of antibody (2–11). Although Lowenthal (7) suggested that antibodies may be induced to hyaluronate by using young cultures that are carefully heat killed, this work has been subsequently criticized (11) because of the possibility that contaminants from culture broth might have been responsible for the presence of precipitins in immune sera. Sandson et al. (10) also suggested that antibodies to hyaluronate could be induced in rabbits by immunization with connective tissue components. However, the presence of protein and galactosamine in their preparations of hyaluronate suggests the presence of components other than hyaluronate that might account for the observed immunoreactivity of their immune sera. Recent studies using more sensitive techniques have shown the presence of antibodies reacting with hyaluronate in the serum of various species (12) including humans (13).

Although polysaccharides vary greatly in their immunogenicity, hyaluronate appears to be a poor immunogen. Among polysaccharides containing uronic acid, some polysaccharides are highly immunogenic, such as the pneumococcal polysaccharides (29) and cryptococcal polysaccharides (28). The apparent poor immunogenicity of streptococcal hyaluronate has been attributed to the fact that hyaluronate is a normal constituent of many mammalian tissues, and therefore normal suppressive immune mechanisms prevent the recognition of a hyaluronate immunogen as foreign. However, many common host components may be the focus of an immune response, including other glycosaminoglycans (30–32). An alternate possibility to explain the apparent lack of immunogenicity of hyaluronate is that naturally occurring antibodies to native host molecules may occur, but may be difficult to detect because of their absorption by excess host antigens (33).

The biologic significance of immune reactions to hyaluronate in the pathogenesis of streptococcal infections and autoimmune disease is not known. It is conceivable that immune reactions to hyaluronate could contribute to host defenses against streptococcal infections. The induction of autoimmunity to mammalian hyaluronate by streptococcal infections with encapsulated organisms could be a mechanism for the induction of autoimmunity to tissue hyaluronate in man. We have shown that a number of extracellular matrix components, including proteoglycan, laminin, and type IV collagen may be the focus of an immune response in humans (14, 34, 35). However, further investigation is required before these experimental results can be related to the pathogenesis of human disease.

Summary

The immunogenicity of hyaluronic acid was investigated. Rabbits were immunized with encapsulated group A and C streptococci. Intact long-chain hyaluronate was conjugated to BSA for use as antigen in an ELISA. Antibodies to the hyaluronate-BSA conjugate were detected in peak immune sera. The speci-
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Specificity of the antibodies for both mammalian and streptococcal hyaluronate was shown by inhibition studies. To further confirm the presence of antihyaluronate antibodies, hyaluronidase-digested streptococcal hyaluronate was conjugated to biotin and used as an antigen in the ELISA. A clear immunization effect was shown for each rabbit by the study of preimmune and postimmunization bleedings. Titers for each rabbit increased by >32–256-fold. Inhibition studies using hyaluronidase-digested hyaluronate and periodate-treated hyaluronate showed that the immunodominant site of antibody reactivity was a terminal glucuronic acid residue. Further studies showed that the carboxyl group of the terminal glucuronide was the major immunoreactive site. Both mammalian and streptococcal hyaluronate inhibited the immune rabbit sera reaction to streptococcal hyaluronate, demonstrating crossreactivity of these molecules. Thus, hyaluronate was shown to be immunogenic in rabbits.

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