IMMUNOGENICITY OF LIPOSOME-BOUND HYALURONATE IN MICE

At Least Two Different Antigenic Sites on Hyaluronate Are Identified by Mouse Monoclonal Antibodies

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Hyaluronate (HA)[1] is an ubiquitous component of mammalian tissue with many important structural and functional roles (1). HA also comprises the capsular material of the group A *Streptococcus* (2). These presumably identical polysaccharides have been of considerable interest as a model for the role of microbial mimicry in autoimmune disease (3). Theories have proposed that microbial crossreactions may play an essential role in the induction and expression of autoantibody (4), possibly via genomic mechanisms (5). In addition, immunity to streptococcal capsular HA could conceivably play a role in protection against streptococcal infections (6, 7), but little evidence has accumulated to support this possibility.

Indeed, HA immunogenicity and antigenicity remain poorly understood. HA has been thought to be a nonimmunogenic, nonantigenic molecule (8, 9). However, recent studies demonstrated that naturally occurring antibodies to HA are present in several species (10). Some investigators have demonstrated autoantibodies to HA that are "broadly crossreactive" (11), and therefore, may be of low affinity and of little pathogenetic significance.

We have previously shown HA to be immunogenic in rabbits immunized with formalinized, encapsulated group A *Streptococci* (12). We now further demonstrate the immunogenicity of HA by induction of antibodies to HA in mice after immunization with HA bound to liposomes. At least two different antigenic immunodeterminants of the HA polysaccharide were identified.

Materials and Methods

Biochemicals Purified mammalian HA was purchased from Miles Laboratories Inc., (Naperville, IL) Sigma Chemical Co. (St. Louis, MO), and Worthington Biochemical Corp. (Free-

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[1] *Abbreviations used in this paper:* HA, hyaluronate; HS, heparan sulfate; IA, intact HA; IaI, hyaluronidase-digested HA; IA1-FA, IA1 in Freund's adjuvant; PBSB, PBS with 0.5% Brij.

hold, NJ). Chondroitin 4 sulfate and heparin was obtained from Sigma Chemical Co. Heparan sulfate was obtained from Miles Laboratories Inc. *N*-acetylglucosamine, *N*-acetylgalactosamine, and glucuronic acid (sodium salt) were obtained from Sigma Chemical Co. Glucuronamide was obtained from Aldrich Chemical Co. (Milwaukee, WI). Testicular hyaluronidase was obtained from Worthington Biochemical Corp. Biotin and streptavidin were obtained from Sigma Chemical Co. Synthetic lipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL).

Preparation of Streptococcal Hyaluronate. Group C streptococcal strain, D181, was grown, harvested, and the capsular hyaluronate (fraction IA) released and purified, as previously described (12). After partial hydrolysis with testicular hyaluronidase (Worthington Biochemical Corp.), fraction IA1 was recovered by precipitation with alcohol.

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, as previously described (12). Rabbits were given a series of intravenous injections with the vaccines, followed by a test bleeding 1 wk after each course of injections.

Preparations of Liposomes and the Attachment of Hyaluronate. 80 mg of synthetic phosphatidylethanolamine and 20 mg of synthetic phosphatidylcholine were dissolved in chloroform and dried on an acid-cleaned glass test tube by rotary evaporation. 1 ml PBS was then added, and the sample was thoroughly sonicated in a water bath sonicator. The liposomes were then centrifuged (12,100 *g*) and washed three times in PBS. The final pellet of liposomes was resuspended in 2× volume of PBS and stored at 4°C.

Benzoquinone-derivitized IA1 was prepared as previously described (12). 25 mg IA1 in 20 ml PBS was added to 5 ml of 250 mM benzoquinone in 100% ethanol and 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, centrifuged at 17,700 *g* for 20 min, resuspended in 100 ml ethanol/acetate, and again centrifuged. 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. After this, the material was dialyzed against 4 liters of PBS. Equal volumes of the benzoquinone-derivitized IA1 and liposomes were mixed by rotation overnight at 4°C and washed five times by centrifugation in PBS. The final pellet (2.5 ml) was resuspended in a 2× volume of PBS, aliquoted, and stored at 4°C for use. This material is referred to as IA1-liposomes. Approximately 15 mg of HA was recovered in the final preparation of IA1-liposomes, as approximated by the measurement of uronic acid in the sample (13). For immunologic experiments, the amount of uronic acid (μg/ml) in a given volume of IA1-liposomes was measured and used for quantitation of the amount of HA in the sample.

Immunization of Mice with HA. Four BALB/c/SJL × F_1 mice were immunized with IA1-liposomes. Preimmune sera were taken intraorbitally. 100 μl of IA1-liposomes in PBS (containing 1 mg/ml of HA determined, as described in the preceding section) were injected intraperitoneally. 2 wk later, mice were bled and immunized. This was repeated a third and final time 2 wk later. Another group of five mice initially received 100 μl of a solution containing 50 μl IA1 in PBS (2 mg/ml) and 50 μl of CFA, which was mixed to form an emulsion. Subsequent immunizations used IFA. The same immunization protocol as described above for IA1-liposomes was used.

Production of Hybridomas and mAbs. Standard methods for the production of hybridomas were used (14). Two fusions were performed using the pooled spleen cells from each group of mice. Briefly, spleens were dissected, minced, and the cells from the spleens of each group of mice were separately pooled for fusion with NS-1 myeloma cells. After fusion, cells were plated in DME media containing 10% CPSR-3 supplement (Sigma Chemical Co.), hypoxanthine, aminopterin, and thymidine (HAT) and maintained in this medium throughout subcloning. 14 d after fusion, the hybridoma supernatants were removed and tested for antibody activity using the ELISA described below. ELISA-positive clones were replated for subcloning, and were retested at confluence. A total of three subclonings were performed, at which time the subclones were presumed to be monoclonals. Subsequent limiting dilution experiments confirmed that these were indeed single clones. The clones were grown to 2-liter volumes in DME plus CPSR-3 and the supernatant fluid concentrated by precipitation with 18% sodium sulfate. The precipitate was resuspended in 20 ml 0.01 M sodium phosphate, pH 7.0, to which 20 ml of a 30% PEG in sodium phosphate buffer was added. After centrifu-

gation, the precipitate was resuspended in sodium phosphate buffer and applied to a hydroxylapatite (Calbiochem-Behring Corp., La Jolla, CA) column equilibrated in sodium phosphate buffer. A linear gradient between 10 and 300 mM of sodium phosphate was applied, and the fractions were assayed for reactivity by ELISA. The reactive fractions were collected and pooled.

ELISA. This assay was performed as we previously described (12) using streptavidin and biotin-labeled IA1 (IA1-B). ELISA plates (Immunoplate II; Nunc, Roskilde, Denmark) were coated with streptavidin at 10 μg/ml in 0.1 M carbonate buffer, pH 9.1, at room temperature for 1 h and then overnight at 4°C. After washing, IA1-B (5 μg/ml) in 0.1 M PBS with 0.5% Brij (PBSB) was added for 1 h. After washing, rabbit sera or mAbs were added at varying dilutions in PBSB for 1 h. For competitive immunoinhibition experiments, diluted sera and inhibitor (in PBSB) were rotated for 1 h at room temperature, and overnight at 4°C, centrifuged in a microfuge (12,100 *g*), and the supernatant was used in the ELISA. Finally, after an incubation with alkaline phosphatase–conjugated goat anti-rabbit IgG (1:1,000) or anti-mouse IgG antisera (1:350) (Sigma Chemical Co.) in PBSB for 1 h, the plates were washed, and developer was added. The plates were incubated at 37°C and read at 1 h at 405 nm in an automated ELISA reader (Physica, Long Island City, NY).

Alterations of Hyaluronate for Immunochemical Studies. Mammalian and testicular HA were treated with testicular hyaluronidase as described previously (12). HA was treated with ascorbic acid according to modifications of previous methods (15). 10 mg IA was dissolved in 1 ml PBS. To this solution was added 5 mg of ascorbic acid and 5 μl of 1 M copper sulfate. The mixture was intermittently rotated manually for 3 h at room temperature. An additional 5 mg of ascorbic acid and 5 μl of 1 M copper sulfate was added, rotated, and the mixture incubated for 3 d at 4°C. Finally, HA was also treated by ultrasonication according to modifications of previous methods (16). 2 ml of a 10 mg/ml solution of IA in PBSA was placed in a 2.5-cm-thick pyrex tube. The tube was placed in an ice bath. A probe sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY; Ultrasonic processor W-380) was used with the output control at "4". The material was intermittently sonicated with power on for 1 s and power off for 3 s (to prevent heating) for 4 h.

Chromatography of Treated HA. Hyaluronidase-treated HA, sonicated HA, and ascorbate-treated HA in PBSA were chromatographed on Sephacryl S-200, using a 100 × 1.5-cm column at a flow rate of 10.5 ml/h, with PBSA as the running buffer. 2-ml fractions were collected and tested for the presence of uronic acid (13). Peaks containing HA were pooled, dialyzed against distilled water, lyophilized, and stored at 4°C for use in immunologic assays.

Results

Immunogenicity of HA in Mice. To demonstrate that the IA1-liposomes contained immunoreactive HA antigen, immune rabbit sera were used in competitive immunoinhibition experiments (Fig. 1). IA1-liposomes effectively inhibited immune rabbit sera reactivity to IA1, while untreated liposomes had little effect. Liposomes could not be reacted at concentrations >100 μg/ml, since thick emulsions were formed at these concentrations. The inhibitory capacity in immune rabbit sera of IA1-liposomes was intermediate between intact HA (IA) and hyaluronidase-digested HA (IA1). These results confirmed that IA1-liposomes contained immunoreactive HA antigen.

Four mice were immunized with IA1-liposomes, and five mice were immunized with IA1 in Freund's adjuvant (IA1-FA). Serial bleedings were taken from each immunized mouse. While the mice immunized with IA1-liposomes developed serum antibody to IA1 by ELISA, the mice immunized with IA1-FA did not develop measurable antibody to IA1 (Fig. 2).

Pooled spleens from three mice immunized with IA1-liposomes were used for production of hybridomas. Supernatants from culture wells of microtiter plates were tested for production of antibody to IA1 by ELISA. Four hybridomas were identified

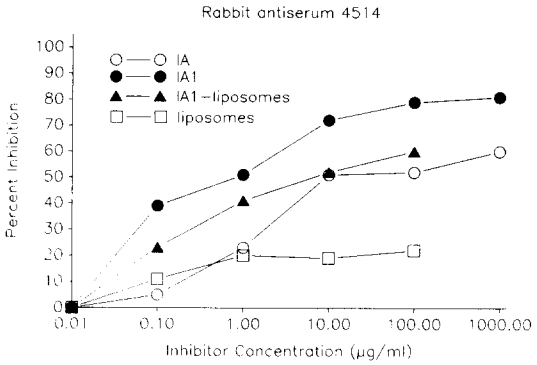

FIGURE 1. Competitive inhibition ELISA using biotinylated IA1 (IA1-B) as antigen, immune rabbit sera (4514 at 1:10,000 dilution), and various hyaluronate and liposome inhibitors (represented by symbols: see text for abbreviations). Results for competitive inhibition experiments are expressed as percent inhibition of serum reactivity to IA1-B, calculated as the OD of the uninhibited serum minus the OD of the inhibited serum, divided by the OD of the uninhibited serum (× 100%).

that produced antibody to IA1. Hybridomas producing antibody to IA1 were subcloned by limiting dilution. Of the initial four clones, two hybridomas ceased antibody production, and two were stable after three subclonings (10G6 and 5F11).

Immunochemical Characterization of Mouse mAbs to HA. Monoclonals 10G6 and 5F11 were characterized immunologically. Both hybridomas produced IgG antibody (data not shown) and recognized human and streptococcal HA by competitive ELISA inhibition (Fig. 3).

The effects of various treatments on HA antigenicity were explored (Fig. 3). As we have previously demonstrated in immune rabbit sera, testicular hyaluronidase digestion enhanced the antigenicity of streptococcal and human HA for 10G6. Hyaluronidase treatment also enhanced the antigenicity of streptococcal HA for 5F11. Thus, testicular hyaluronidase treatment exposes a specific terminal HA antigenic site on IA recognized by both mAbs. Ascorbic acid treatment markedly reduced IA antigenicity for 5F11, but had little effect on IA antigenic recognition by 10G6, suggesting that the antigenic site on IA recognized by 5F11, but not 10G6, is destroyed by oxidation. Sonication of intact human and streptococcal HA had little effect on the reactivity of the mAbs. As determined by Sephacryl S-200 chromatography (data not shown), all three treatments had similar effects in reducing the chain length of the IA to an approximate molecular weight of 1.5×10^4 kD. These data

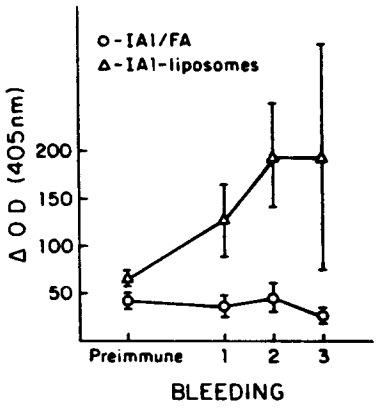

FIGURE 2. ELISA titers of antibody to IA1 in sera from mice immunized with IA1 either conjugated to liposomes (*IA1-liposomes*), or in Freund's adjuvant (*IA1/FA*). Results (mean +/− SE) are expressed as the observed OD, 405 nm) in mictotiter wells containing antigen (IA1) minus OD in wells without antigen as background control.

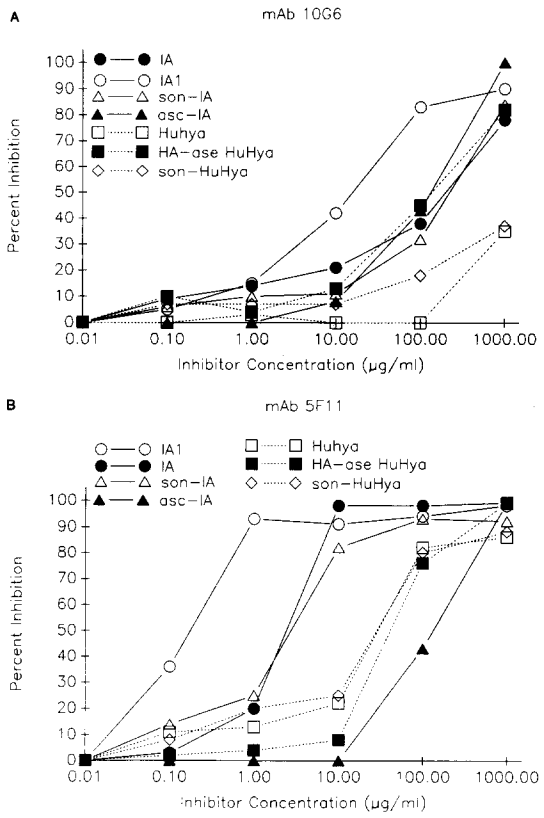

FIGURE 3. Competitive inhibition ELISA studies of mAbs 10G6 (*A*) and 5F11 (*B*) using various hyaluronate preparations as inhibitors. Results are expressed as in Fig. 1. *son-IA*, sonicated IA; *asc-IA*, ascorbate-treated IA; *HuHya*, intact human hyaluronate; *HA-ase HuHya*, hyaluronidase-treated HuHya; *son-HuHya*, sonicated HuHya.

demonstrate that HA immunogenicity is not simply a function of chain length, but rather, is dependent on immunospecific epitopes that are demonstrable by specific treatments and differentially recognized by the two mAbs.

Differences in the exact HA epitope that the mAbs recognized were also found when the mode of presentation of HA was explored (Fig. 4). 10G6 recognized an immunodeterminant that was most effectively presented when IA1 was bound to liposomes, rather than free in solution. In contrast, 5F11 reacted most effectively with free IA1. Essentially no reactivity was noted with unconjugated liposomes used as control. These data clearly indicate that the two anti-HA mAbs recognize different sites on HA that are differentially exposed when free in solution or on liposomes.

The immunochemical specificity of the epitope recognized by the mAbs was explored by using specific competitive immunoinhibitors. 10G6 was effectively inhibited by as little as 0.5 M glucuronic acid. However, none of the monosaccharides tested inihibited reactivity to IA1 by 5F11 (Fig. 5). 10G6 did not crossreact with other glycosaminoglycans tested (Fig. 6). However, 5F11 did crossreact with heparan sulfate, although at a considerably lower affinity than IA1. In addition, while 10G6 did not crossreact with polyanionic phosphorylated molecules (Fig. 7), including DNA, 5F11 showed crossreactivity with phosphorylcholine, again at a considerably lower affinity than IA1.

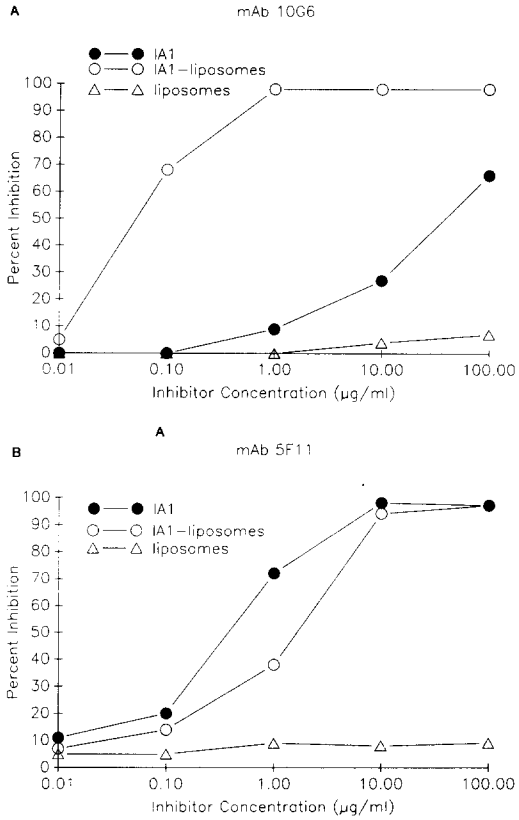

FIGURE 4. Competitative inhibition ELISA studies of mAbs 10G6 (*A*) and 5F11 (*B*) using various hyaluronate inhibitors.

These data suggested that electrostatic forces might play a role in the immunodominant site recognized by 5F11. To test this hypothesis, titrations of the mAbs reactivity to IA1 in the presence of varying dilutions of NaCl were performed (Fig. 8). Increasing concentrations of NaCl had no effect on the reactivity of 10G6 to IA1. However, the addition of 0.5 M NaCl reduced the reactivity of 5F11 for HA, indicating that electrostatic charge does play a role in the immunodominant site of 5F11.

Discussion

The immunogenicity of HA in rabbits has been previously demonstrated using encapsulated group A streptococcal cells as immunogen (12). To further demonstrate that HA is immunogenic, we attempted to induce an immune response to HA in another animal species using a different HA immunogen. We speculated that HA on the streptococcal cell may exist as a polymer extending radially outward from the membrane, and that this configuration might be important to the immunogenicity of encapsulated streptococcal cells. We hypothesized that HA linked to the exterior of liposomes might present terminal HA immunodeterminants in a manner similar to streptococcal cells. The presence of HA on the exterior of the liposomes was confirmed by biochemical data showing uronic acid in IA1-liposomes, and the demonstration that immune rabbit sera, known to contain antibodies to HA ter-

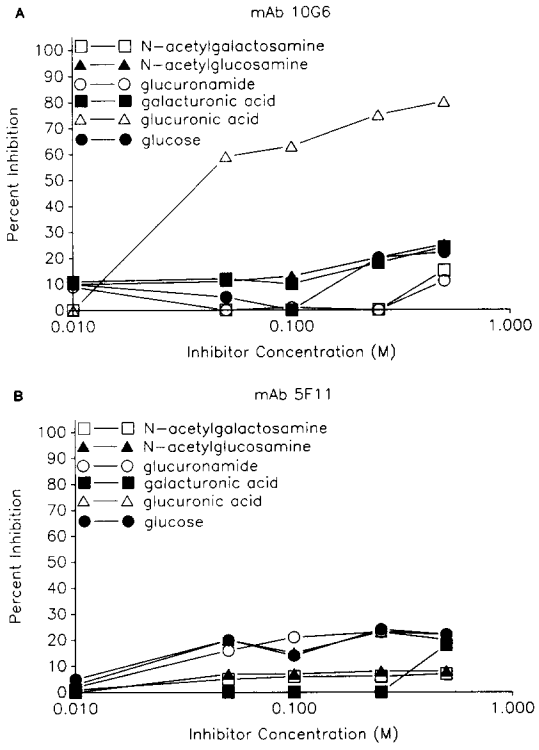

FIGURE 5. Competitive inhibition ELISA studies of mAbs 10G6 (*A*) and 5F11 (*B*) using various monosaccharides as inhibitors.

minal immunodeterminants (12), effectively reacted with IA1-liposomes. Serial bleedings of mice immunized with IA1-liposomes demonstrated a significant increase in antibody titer to HA, while mice immunized with free IA1 in an emulsion (Freund's adjuvant) did not. These data confirm the immunogenicity of HA, and indicate that the mode of presentation of HA is important in its immunogenicity. The significance of radially arrayed, multiple terminal immunodeterminants in the immunogenicity of vaccines has been previously discussed in regard to the streptococcal group polysaccharides (17).

Immune rabbit sera and mAb 10G6 appear to recognize a similar terminal HA immunodeterminant specifically exposed by mammalian hyaluronidase digestion, and containing glucuronic acid in the immunodominant site. Neither sonication nor ascorbic acid treatment of HA had any significant effect on its reactivity with 10G6, indicating that the terminal site recognized by 10G6 is uniquely exposed by testicular hyaluronidase treatment. Furthermore, 10G6 showed no crossreactions with any glycosaminoglycans or phosphorylated compounds tested, and the addition of NaCl had no effect on 10G6 immunoreactivity. These data indicate that electrostatic forces do not play a major role in the immunodominant site recognized by 10G6. While immune rabbit sera recognized free IA1 more effectively than Ia1-liposomes, mAb 10G6 recognized IA1-liposomes far more effectively than free IA1. The explanation for these more subtle immunologic differences between immune rabbit sera and mAb 10G6 require further investigation. In summary, 10G6 mAb is im-

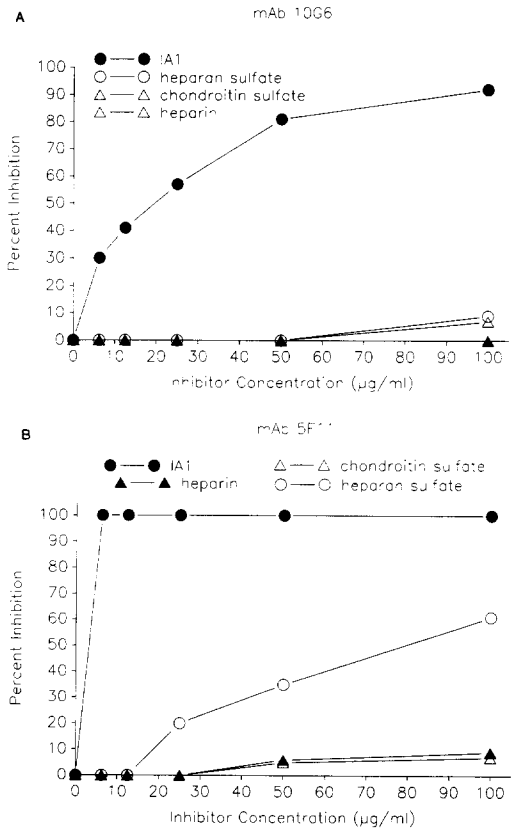

FIGURE 6. Competitive inhibition ELISA studies of mAbs 10G6 (*A*) and 5F11 (*B*) using various glycosaminoglycans as inhibitors.

munospecific for a unique terminal HA epitope, which is also recognized by immune rabbit sera.

The immunodominant site of HA recognized by mAb 5F11 was investigated. mAb 5F11 also recognized an immunodeterminant on IA exposed by hyaluronidase digestion. However, in contrast to immune rabbit sera and mAb 10G6, mAb 5F11 was not inhibited by glucuronic acid, indicating that this monosaccharide alone does represent the immunodominant site recognized by 5F11. The immunodeterminant recognized by 5F11 could be the complete terminal disaccharide unit, hyalobiuronic acid, or a larger oligosaccharide unit. In addition, again in contrast to 10G6, 5F11 was more effectively inhibited by free IA1 than IA1-liposomes. These data suggest that 5F11 most effectively recognizes a monovalent, rather than a polyvalent, terminal immunodeterminant, or that the immunodominant site recognized by 5F11 may be relatively hidden in the IA1-liposomes. The fact that ascorbic acid specifically destroyed the immunodominant site recognized by 5F11 suggests that the immunodominant site is susceptible to oxidation. Previous studies have explored the biochemistry of oxidative reactions of HA (15, 18, 19). Configuration may be important in the susceptibility of HA carboxylate groups to oxidation (20), and thus, conformational factors may be important in the antigenic sites of HA recognized by

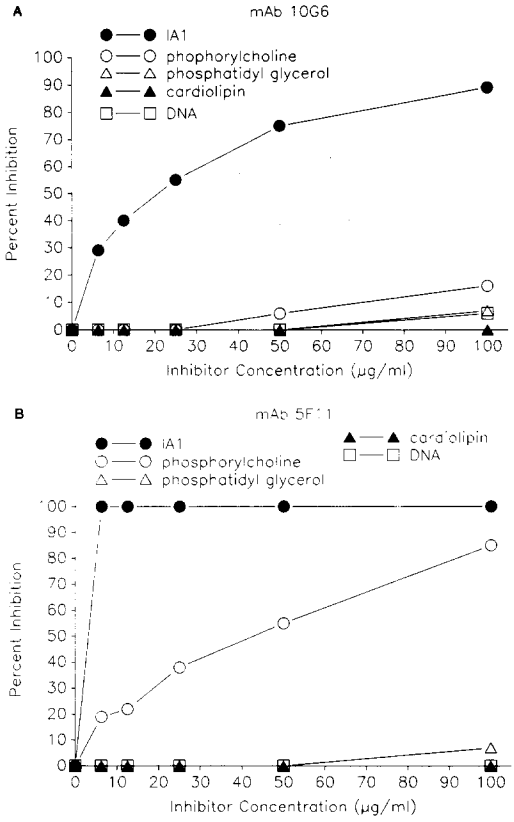

FIGURE 7. Competitive inhibition ELISA studies of mAbs 10G6 (*A*) and 5F11 (*B*) using various phosphorylated molecules as inhibitors.

5F11. However, the exact biochemistry of ascorbate-induced oxidative reactions of HA is not known.

Antibody crossreactions in which electrostatic forces play a role have been previously demonstrated (4, 21). It has been suggested (22) that electrostatic forces may also play a role in crossreactions between HA and DNA. Our mAbs did not crossreact with DNA, indicating the possibility of a third antigenic site on HA, in addi-

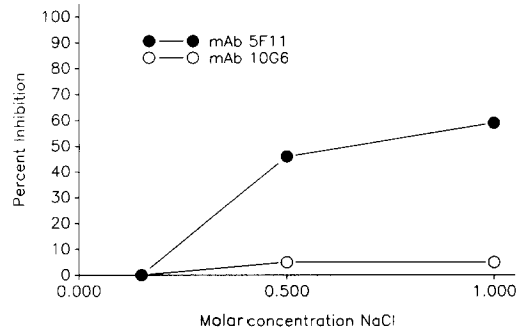

FIGURE 8. ELISA studies of mAbs 10G6 and 5F11 reactivity to IA1 in the presence of various concentrations of NaCl.

tion to the two we have demonstrated. Although electrostatic forces appear to contribute to the immunodominant site recognized by 5F11, electrostatic forces are only partially responsible for the immunospecificity of 5F11, since only specific glycosaminoglycans and phosphorylated compounds were effective as immunoinhibitors. As discussed above, the configuration or conformation of charged groups may be important in the immunodominant site recognized by 5F11.

It is particularly interesting to note that 5F11 recognizes a crossreactive site on HA and heparan sulfate (HS) in which electrostatic forces play an important role. Anionic sites on HS are critical to the maintenance of the normal charge-barrier function of the vascular premeability barrier (23). Thus, crossreactions between HA and HS anionic sites could be involved in the pathogenesis of autoimmune vascular disease. Such crossreactions could be initiated by mechanisms related to microbial mimicry (3). Antibodies to HS have been demonstrated to cause proteinuria in animal models (24) and to be present in the glomeruli of animals and patients with glomerulonephritis (11). We also demonstrated the presence of crossreactions between HA and HS in human sera from patients with glomerulonephritis (25).

Quantitative differences in the immunoreactivity of preparations of human HA and streptococcal HA were noted in several of our experiments. Based on our current knowledge of the biochemistry of HA, it seems unlikely that this represents true antigenic variation between these HA preparations. However, possible immunologic differences based on sequence variations cannot be completely ruled out. Differences in the chain length of the intact HA molecule obtained by different extraction methods, or naturally occuring differences in the chain length of HA produced by *Streptococci* and mammalian cells, more likely account for variations in antigenicity of different HA preparations. For example, the molar amounts of terminal antigenic determinants in a given preparation of HA would depend on the mean chain length of the HA chains in the preparation. However, it is also clear that chain length alone does not determine the antigenicity of a given HA preparation. For example, although sonication, ascorbate oxidation, and hyaluronidase treatment reduced the chain length of HA to a similar degree, hyaluronidase enhanced the antigenicity of intact HA, sonication had no effect on intact HA antigenicity, and ascorbate oxidation destroyed the site on intact HA recognized by 5F11.

The current investigations not only confirm the immunogenicity of HA, but further demonstrate the presence of at least two different antigenic sites on HA. mAbs represent an important method for immunological studies of hyaluronic acid. The occurrence of HA in normal serum (1), and the ubiquity of the molecule in mammalian tissue, probably absorbs most naturally produced antibody and prevents its detection. Hybridoma technology circumvents this problem, and allows the full array of immune responses to HA immunodeterminants to be detected and studied.

Summary

Hyaluronate (HA) was previously demonstrated to be immunogenic in rabbits. The immunogenicity of HA in mice was studied. Hyaluronidase-digested streptococcal HA (IA1) covalently linked to liposomes (IA1-liposomes) were produced for immunization. Mice immunized with IA1-liposomes developed measurable serum antibodies to IA1, while mice immunized with IA1 in Freund's adjuvant did not. mAbs produced by two stable hybridomas (10G6 and 5F11) from mice immunized with

IA1-liposomes produced IgG antibody reactive with HA in ELISA. 10G6 had a much higher avidity for liposome-bound IA1 than free IA1, while 5F11 did not, suggesting that the mode of presentation of IA1 is important in HA immunogenicity and antigenicity. Both mAbs recognized terminal HA immunodeterminants exposed by hyaluronidase treatment. Sonication had no effect on HA reactivity for either mAb. However, ascorbic acid treatment significantly reduced the antigenicity of HA for mAb 5F11, but not 10G6. Only 10G6 was inhibited by glucuronic acid. Electrostatic forces appear to play a role in the binding site of 5F11, but not 10G6. 5F11 crossreacts with heparan sulfate and phosphorylcholine, while 10G6 did not crossreact with any glycosaminoglycans or phosphorylated compounds tested.

These results confirm that HA is immunogenic. They suggest that the mode of presentation of HA is important for the induction of the immune response, and in HA antigenicity. At least two different antigenic sites on HA were demonstrated. 10G6 recognizes a terminal HA antigenic site expressed on IA1-liposomes that contains glucuronic acid in its immunodominant site. 5F11 recognizes an HA antigenic site in which electrostatic forces appear to play a role, is sensitive to ascorbic acid treatment, and is crossreactive with heparan sulfate. The use of mAbs should facilitate immunologic studies of HA.

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