SERUMS FROM PATIENTS WITH POSTSTREPTOCOCCAL
GLOMERULONEPHRITIS CONTAIN ANTIBODIES TO
GLOMERULAR HEPARAN SULFATE PROTEOGLYCAN

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Proteoglycans (PG) are important tissue components, both in the extracellular
matrix (1) and as cell membrane-associated components (2). They are composed
of protein and glycosaminoglycans (GAG), containing glucosamine or galactosa-
ine and (generally) uronic acid. About fifty years ago, Goebel (3) demonstrated
the antigenicity of protein-bound uronic acid and, further, showed that rabbit
antisera could distinguish protein-bound glucuronic from galacturonic acid (4).
More recently, the presence of immunity to heparan sulfate and chondroitin
sulfate PG has been demonstrated in animals (5–7). Both protein core and GAG
determinants are antigenic. Studies of systemic lupus erythematosus (SLE) sera
(8) suggest that antibodies to DNA may cross-react with hyaluronate and chon-
droitin sulfate on a nonspecific charge basis because of the repeating anionic
sites that DNA and GAG have in common.

Previous studies (9–13) suggest that autoimmunity to a number of kidney
antigens may exist in acute and chronic glomerulonephritis (GN). Our own work
(13, 14) suggests that the carbohydrate antigens of the glomerular basement
membrane (GBM) may be antigenic. Since one of the major carbohydrate-
containing antigens of the GBM is heparan sulfate PG (1), we studied the sera of
patients with acute poststreptococcal glomerulonephritis (PSGN) for the presence
of antibodies to glomerular PG. These studies demonstrate the presence, in
PSGN sera, of antibodies to glomerular PG. Heparan sulfate is the primary
target of immune reactivity. Mammalian hyaluronate and streptococcal hyalu-
ronate inhibited the observed reactivity, suggesting cross-reactivity in the im-
une response to heparan sulfate, mammalian, and streptococcal hyaluronate.
A second site of immunoreactivity contains N-acetylgalactosamine, possibly rep-
resenting autoimmunity to chondroitin or dermatan sulfate PG.

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Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; GAG, glycosami-
nerglycan; GBM, glomerular basement membrane; GN, glomerular nephritis; PBS, phosphate-buff-
ered saline; PBSB, PBS-Brij buffer; PEG, polyethylene glycol; PG, proteoglycan; PSGN, poststrept-
ococcal GN; SLE, systemic lupus erythematosus.
Materials and Methods

Reagents. DEAE-Sepharose CL-6B and Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Chondroitinase ABC and heparitinase were purchased from Miles Laboratories Inc., Elkhart, IN. DEAE-cellulose, pepstatin A, benzamidine-HCl, 6-aminohexanoic acid, guanidine-HCl (grade 1), phenylmethanesulfonyl fluoride, N-ethylmaleimide, N-acetylgalactosamine, N-acetylmannosamine, D-glucuronic acid, and diatomaceous earth (grade 1) were obtained from Sigma Chemical Co., St. Louis, MO. Papain (twice crystallized) was from Worthington Biochemical Corp., Freehold, NJ. Urea (Fisher Scientific Co., Pittsburgh, PA; certified American Chemical Society), as an 8 M solution, was passed through a Fisher high-capacity deionizing cartridge shortly before use. Standard GAG were provided by Dr. Allen Horwitz, University of Chicago, including chondroitin-4-sulfate and chondroitin-6-sulfate, hyaluronic acid, dermatan sulfate, heparin, and heparan sulfate. Group A streptococcal hyaluronate, and group A, A variant, and C carbohydrates were obtained from Dr. Maclyn McCarty, The Rockefeller University.

Human sera were obtained from normal controls and patients with acute and chronic PSGN in San Fernando, Trinidad. All patients had appropriate clinical studies, including antistreptolysin O titers and streptococcal cultures, as well as long-term evaluations of renal function. Sera from normal controls were also obtained in New York.

Extraction of Proteoglycans. Glomeruli were isolated from fresh bovine or human kidneys by a sieving method (15) and stored frozen. After thawing, the suspension was centrifuged at 2,000 g for 10 min, and the pellet was suspended in 100 vol distilled water containing protease inhibitors (0.1 M 6-aminohexanoic acid, 0.01 M EDTA, and 0.005 M benzamidine-HCl), stirred for 3 h, and centrifuged at 17,700 g for 10 min. The pellet was resuspended in 4 M guanidine-HCl, 0.05 M sodium acetate, pH 5.8, with protease inhibitors, in 10 times the volume of the original pellet; stirred for 48 h, and centrifuged as above. A second extraction in the same solvent was performed for 18 h. The combined supernatants were dialyzed against three changes of 10 vol deionized 7 M urea, 0.01 M EDTA, 0.05 M Tris-HCl, pH 6.5. The extract was then filtered through diatomaceous earth, and the cake washed well with 7 M urea buffer. The combined solution was passed through a column (10 ml bed vol) of DEAE-Sepharose CL-6B that had been equilibrated with 7 M urea buffer. Elution was done stepwise with five bed-volumes each, of increasing concentrations of NaCl in 7 M urea buffer (0.0–1.0 M NaCl in 0.1 M increments). All the above steps were carried out at 4°C. Each eluate was dialyzed against water, and lyophilized. The materials that eluted at 0.4 M, 0.5 M, and 0.6 M NaCl were redissolved in 0.5 M sodium acetate, pH 5.8, and each was chromatographed at room temperature on a column (0.78 × 110 cm) of Sepharose CL-4B in 0.5 M sodium acetate, pH 5.8. The fractions were analyzed, and pooled zones were thoroughly dialyzed against water and lyophilized.

Enzyme-linked Immunosorbent Assay (ELISA). ELISA was performed essentially according to the method of Engvall and Perlmann (16), with Nunc immunoplates (Intermed, Denmark). Various amounts of antigen in 50 μl of 0.1 M Tris-HCl buffer, pH 9.8, containing 0.3 mM MgCl₂, were kept overnight, in wells of the plates, at 4°C. The plates were washed once with phosphate-buffered saline (PBS) containing 0.5% Brij (PBSB). Non-specific sites on the plates were blocked by two further incubations with PBSB for 30 min each. Serum was diluted in PBSB, and 50 μl were added to the plates for 4 h. The plates were washed with 250 μl of 0.9% NaCl containing 0.5% Brij. An alkaline phosphatase–conjugated goat anti–human IgG (gamma chain–specific) antiserum (lot 88F 9350; Sigma Chemical Co.) was added at a dilution of 1:1000 in 50 μl of PBSB for 4 h. The plates were washed in saline-Brij. A developing reagent containing 10 mg of nitrophenyl phosphate (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 g sodium azide, and 800 ml distilled water, pH 9.8) was added. The plates were read at 405 nm in a Titertek Multiskan (Flow Laboratories, McLean, VA) at intervals of up to 4 h.

Rabbit Antisera to GBM. Adult New Zealand White female rabbits were immunized with either bovine or human GBM prepared by the Meezan (17) procedure. 1 mg of
GBM in complete Freund's adjuvant was injected intradermally into two rabbits at 3-wk intervals, for a total of three injections. Serum was harvested 1 wk after the final injection.

**Chemical Composition of Antigenic Preparations.** Uronic acid was measured by a variation of the borate/carbazole method (18). Hexosamines were quantitated on a Technicon TSM amino acid analyzer after hydrolysis in 4 M HCl at 100°C for 8 h (19). Protein was measured by a modified Lowry procedure (20), or by absorbance at 280 nm. Digestion of PG with chondroitinase ABC (0.1 U/mg) was done in 0.1 M sodium acetate, 0.1 M Tris-HCl, containing the following protease inhibitors: 10 mM EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, and 0.035 mM pepstatin A, pH 7.3, at 37°C, for 4 h. Digestion of PG with papain was carried out in 0.1 M potassium phosphate, 0.01 M EDTA, 0.01 M cysteine, pH 6.8, at 64°C for 18 h with a ratio of PG to papain of 100:1. Nitrous acid treatment of PG was carried out as described by Shively and Conrad (21). Heparitinase digestion was carried out as described by Hovingh and Linker (22). All digests were chromatographed on Sepharose CL-4B in 0.5 M sodium acetate, pH 5.8. Intact PG preparations used as controls were chromatographed under identical conditions.

**Results**

**Isolation of PG.** As described in Materials and Methods, the 4 M guanidine extraction of whole bovine glomeruli was fractionated by ion-exchange chromatography on DEAE-Sepharose CL-6B. Fig. 1 shows the distribution of the hexosamine-containing compounds (as measured by aminosugar analysis [19]). The glucosamine content of the unabsorbed and 0.1 M NaCl eluates probably represents noncharged glycoproteins and, possibly, low-sulfated GAG. The major anionic hexosamine-containing material emerged in 0.4 M, 0.5 M, and 0.6 M NaCl eluates. This distribution was probably due to varying degrees of sulfation, since all three fractions gave similar profiles after gel chromatography on

![Figure 1](image-url)
Characterization of PG. The materials in peak I and II, which contained protein, uronic acid, and hexosamine, and were highly anionic, were further identified as PG by their susceptibility to specific enzymatic and chemical treatments. This was tested by chromatography of the material on Sepharose CL-4B before and after treatment. Movement of the material to a chromatographic position with a much higher $K_v$, indicated susceptibility to enzymatic or chemical degradation. All fractions were degraded by papain, indicating their proteinaceous nature. The glucosamine-containing component present in peak I was susceptible to nitrous acid and heparitinase, as expected of a heparan sulfate. In peak II, glucosamine-containing material susceptible to nitrous acid and heparitinase, and galactosamine-containing material susceptible to chondroitinase ABC, were present. These results indicate that peak II contains a mixture of heparan sulfate and chondroitin/dermatan sulfate PG, or possibly a hybrid molecule with both kinds of GAG. However, rechromatography of peak II material on a Sepharose CL-4B column, under dissociative conditions, in the presence of 4 M guanidine-HCl, 0.5 M sodium acetate, pH 5.8, did not separate the two PG species.

Immunologic Studies of Glomerular PG. We tested the PG obtained after Sepharose CL-4B chromatography by immunoblotting and ELISA, using human
sera from controls and patients with PSGN. The Sepharose CL-4B–purified PG obtained from the 0.4, 0.5, and 0.6 M NaCl eluates from DEAE–Sepharose CL-6B all reacted similarly. Thus, the peak II PG obtained from the 0.4 M NaCl elution of DEAE–Sepharose CL-6B was most extensively studied and is reported in detail here.

In Fig. 3a, using ELISA, the peak II antigen was titrated from 10 to 0.1 μg/ml and tested with sera from a patient with acute PSGN, and a control. We saw a clear titration effect as the antigen concentration was changed, indicating that ELISA reactivity was directed against the antigen that was used to coat the plates. In a representative experiment (Fig. 3b), a clear titration curve of reactivity was seen as the serum was diluted and the peak II antigen concentration held constant, indicating that IgG in the serum being tested accounted for the observed antibody binding. Of 13 acute PSGN sera studied, 6 (46%) contained high-titered antibodies to peak II. Four of 11 (36%) chronic PSGN sera had moderate titers of anti–peak II antibody. Only 1 of 11 (9%) control sera contained low-titered anti–peak II antibody. Similar ELISA results with human sera were obtained when peak I antigen was used. Thus, immunoreactivity to isolated heparan sulfate PG (peak I) was demonstrated.
IMMUNITY TO PROTEOGLYCANS

Rabbit antisera made to both bovine and human whole GBM showed high-titered reactivity to the bovine peak II material. Because the rabbit antisera against human GBM reacted with bovine antigen as well, cross-reactivity of the bovine peak II material with the equivalent human antigen is apparent. We also tested human-DE-0.6M with ELISA. Sera from patients with acute PSGN reacted to the human antigen in titers approximating that found with bovine peak II antigen. Thus, the bovine and human glomerular PG preparations appear to be cross-reactive.

We performed control experiments to determine whether the PG immunoreactivity was due to nonspecific ionic binding of cationic IgG, or to specific binding of antibody to the antigenic sites of these anionic molecules. Inhibition studies, using the ELISA (Fig. 4), with high concentrations of NaCl (up to 1 M) to inhibit binding of the peak II material, showed essentially no inhibition. Inhibition of acute PSGN sera (1:40 in PBSB) with the peak II material (62.5 μg/ml) in equal volumes was performed for 1 h at room temperature and 30 min at 4°C, followed by centrifugation at 12,000 g, and use of the supernatant for immunologic testing by ELISA. This resulted in specific inhibition of 90% of the serum reactivity to the peak II material.

Inhibition experiments with purified GAG revealed inhibition of acute PSGN serum reactivity to peak II only with heparan sulfate, and not with other GAG (Fig. 5). However, further studies with peak II antigen revealed inhibition of acute PSGN serum reactivity with both streptococcal and bovine umbilical cord hyaluronate at ~10-fold higher concentrations than with heparan sulfate (Fig. 5).
FIGURE 5. ELISA studies of peak II (10 μg/ml). An acute PSGN serum (1:40) was inhibited with various purified GAG at varying concentrations. Ch4S, chondroitin-4-sulfate; Ch6S, chondroitin-6-sulfate; DeS, dermatan sulfate; Hep, heparin; Hya (Bov), bovine hyaluronate; and HeS, heparan sulfate.

FIGURE 6. ELISA studies of peak I I (10 μg/ml). An acute PSGN serum (1:40) was inhibited with heparan sulfate (HeS), bovine hyaluronate, [Hya (Bov.)], and streptococcal hyaluronate (Str. Hya.) at varying concentrations.
These studies suggest that human antibodies against heparan sulfate may cross-react with mammalian and streptococcal hyaluronate.

Inhibition experiments with N-acetylgalactosamine alone at concentrations of 0.5 M showed ~50% inhibition of acute PSGN serum (1:40) reactivity to peak II antigen, while N-acetylglucosamine, d-glucuronic acid, and N-acetylmannosamine showed no inhibition at this concentration. Group C streptococcal carbohydrate (5 mg/ml), in which the immunodominant carbohydrate is a terminal N-acetylgalactosamine disaccharide (23), also inhibited ~50% of acute PSGN serum (1:40) reactivity. Group A (immunodominant site containing terminal N-acetylgalactosamine) and A variant (immunodominant site containing primarily rhamnose) (24) streptococcal carbohydrates (5 mg/ml) showed little (9%) inhibition. These data indicate the possible presence of a second antigenic site of glomerular PG containing N-acetylgalactosamine.

Since we know (25) that acute PSGN sera contain high titers of immune complexes, it was possible that the observed serum reactivity to PG might be due to the presence of unrelated cationic immune complexes. This possibility was examined by polyethylene glycol (PEG) precipitation of the serum to remove immune complexes. We added PEG 8000 (5.4%) in 0.1 M sodium borate buffer to acute PSGN sera (1:10) in equal volumes and incubated at 4°C for 1 h. We then centrifuged the sera at 12,000 g for 10 min. The supernatant was assayed by ELISA using the peak II material as antigen (10 μg/ml). No effect on serum reactivity, as judged by serum titer in the ELISA, was noted after removal of the immune complexes by this method. Finally, we checked the possibility that the serum immunoreactivity might be directed against anionic DNA contaminating the peak II antigen. The presence of anti-single/double stranded DNA antibodies was tested by radioimmunoassay in the laboratory of the late Dr. Henry Kunkel. No anti-DNA antibodies were detected in any of the sera that were reactive to the glomerular PG antigens.

Discussion

Biochemical studies show that the materials isolated from bovine and human glomeruli contain the components of PG: protein, uronic acid, glucosamine and galactosamine, and strong negative charge. Studies using both human and bovine antigenic preparations yielded similar results, and rabbit antisera made to human GBM cross-reacted with bovine glomerular PG. Peak I, which contains glucosamine, no detectable galactosamine, and is susceptible to nitrous acid and heparitinase, has the properties of a heparan sulfate PG. Enzymatic and chemical susceptibilities demonstrated the presence of heparan sulfate, and either chondroitin sulfate or dermatan sulfate PG in peak II. Since peak II contains both GAG, it may be a hybrid PG, or two molecules that cannot be separated even by dissociative chromatography in 4 M guanidine-HCl. Immunologic studies revealed the presence of antibodies in PSGN sera to peak I and peak II PG. Antibodies to peak I indicate specific immunoreactivity to glomerular heparan sulfate PG. The antigenic specificity of these sera was further investigated in ELISA inhibition experiments, and two carbohydrate immunodeterminants were identified: heparan sulfate and a second antigenic site containing N-acetylgalact-
tosamine. PSGN sera may also contain antibodies to PG protein core determinants; this will require further investigation.

Charge interactions could play an important role in antibody binding to the highly charged PG molecules. Faaber et al. (8) suggested that the anti-DNA antibodies in SLE sera, which cross-react with GAG, bind primarily on a charge basis, to polyanionic molecules in general. Our studies indicated that the heparan sulfate–antibody reaction was not due to nonspecific charge interactions. The GN sera we studied did not contain antibodies to single- or double-stranded DNA by radioimmunoassay, which would have been expected if broadly reactive antibodies with strongly cationic combining sites were present. Charge binding was further ruled out by the observation that 1 M NaCl had no effect on immunoreactivity in the ELISA. In addition, cationic immune complexes were not found to account for the observed reactivity. Finally, the finding of similar immunoreactivity to the 0.4, 0.5, and 0.6 M NaCl eluates of DEAE–Sepharose CL-6B that were further purified by Sepharose CL-4B also indicated that the heterogeneous charge nature of the PG had no detectable influence on the overall antigenicity of the molecule. Thus, our observations indicate that immune reactivity to PG in PSGN sera may be directed, in part, toward highly specific carbohydrate determinants, and is not due to nonspecific charge interactions.

We studied the nature of the specific carbohydrate antigenic sites responsible for immunoreactivity to glomerular PG in ELISA inhibition experiments. The specific antigenic site of polysaccharides may contain either a monosaccharide or multiple units of repeating disaccharides. Heparan sulfate is a repeating disaccharide containing glucuronic acid and N-acetylgalactosamine. Although these monosaccharides are individually antigenic (3, 24), it appears, from our data, that the antigenic site of heparan sulfate defined by PSGN sera contains multiple repeating disaccharide units. The monosaccharides, N-acetylgalactosamine and glucuronic acid, did not inhibit the binding of acute PSGN sera to glomerular PG, but the polysaccharide heparan sulfate was able to inhibit the observed immunoreactivity. These results regarding the nature of the heparan sulfate site are consistent with the observation of Kabat (26) that the optimum binding site of polysaccharide antigens may require several repeating disaccharide units for the best fit into the antibody-combining site.

Immune reactivity to a N-acetylgalactosamine site of peak II was shown by inhibition experiments using N-acetylgalactosamine monosaccharide and group C carbohydrate. Inhibitions with GAG containing N-acetylgalactosamine (such as chondroitin or dermatan sulfate) were unremarkable. Although the exact nature of the N-acetylgalactosamine site in the peak II PG remains unknown, it is possible that this site represents chondroitin/dermatan sulfate, which we have not been able to demonstrate by our inhibition experiments. Chondroitin/dermatan sulfate is present in our peak II material and is probably derived from the glomerular mesangium (7), which was likely included in our 4 M guanidine extract of whole glomeruli. Immune reactivity to chondroitin sulfate appears to be directed primarily against antigenic moieties that are exposed only after enzymatic treatment that reveals hidden antigenic sites (6, 27). Thus, our inability to detect chondroitin sulfate antibodies by inhibition with intact GAG may indicate that a hidden N-acetylgalactosamine determinant contains the immu-
nondominant site of antibodies in PSGN sera. Inhibition experiments with chondroitinase-digested chondroitin sulfate fragments (6, 27) will be necessary to determine if this hypothesis is correct.

The PSGN sera we studied contained antibodies to heparan sulfate that appeared to be cross-reactive with hyaluronate. This cross-reaction may have a molecular basis, since both hyaluronate and heparan sulfate have similar repeating disaccharide units of N-acetylgalactosamine and glucuronic acid. The differences in the linkages and sulfation of these GAG could account for the differences in ELISA inhibitory capability of these molecules on an equivalent weight basis. We noted inhibition with both mammalian and streptococcal hyaluronate, which are thought to be identical (M. McCarty, personal communication). The inhibition of immunoreactivity of PSGN sera with hyaluronate in our experiments suggests that antibodies to hyaluronate are present. Finally, the finding of antibodies to a PG site containing N-acetylgalactosamine that are inhibited by group C streptococcal carbohydrate may indicate the presence of another cross-reaction between PG and streptococci. It is possible that antibodies to the N-acetylgalactosamine site were induced by group C streptococcal infections in these patients.

In studies of carbohydrate immunity to artificial antigens, Avery's group (28) demonstrated the marked degree of specificity exhibited by antisera to carbohydrate antigens. Immune sera were capable of distinguishing synthetic protein-conjugated alpha or beta glucoside antigens (28), and synthetic protein-conjugated glucuronic or galacturonic acid antigens (4). These findings led Avery (28) to the conclusion that the immunological specificity of carbohydrates is determined by the subtleties of their chemical structure.

However, Goebel and Hotchkiss (4) eventually indicated that antibodies to anionic carbohydrates may be of two types, carbohydrate-specific antibodies and nonspecific antibodies binding anionic groups. In antipneumococcus horse sera, antibodies reactive with pneumococcal polysaccharide (containing uronic acid) cross-reacted with synthetic uronic acid-protein antigens. One group of these antibodies was specific for carbohydrate moieties, and were inhibited only by glucuronic acid and not by other anionic antigens, including galacturonic acid. A second group of antipneumococcal polysaccharide antibodies were precipitated nonspecifically by a number of anionic antigens, including glucuronic and galacturonic acids, as well as unrelated anionic molecules such as p-aminobenzoic, carboxylic and sulfonic acids. The broad reactivity to anionic groups could be absorbed by pneumococcal polysaccharide, and was not found in normal horse serum, indicating that these broadly reactive antibodies were of an immune nature, and presumably contained cationic groups in their combining sites. Fischetti (29) and others (30) later demonstrated that anionic antigens may elicit antibodies with cationic combining sites.

In light of these earlier observations, and based on our current findings and those of Faaber et al. (8), we conclude that the human immune response to anionic GAG apparently produces both nonspecific antibodies broadly reactive with anionic molecules, and antibodies that recognize specific carbohydrate immunodeterminants. The mechanism accounting for the induction of PG autoimmunity in disease is not known. After inflammatory injury, the exposure
of hidden or altered PG antigens to the immune system could result in the onset of PG autoimmunity in a genetically predisposed host. Autoimmunity to heparan sulfate in patients with PSGN may be induced by cross-reactive streptococcal capsular hyaluronate during infections. The resultant autoimmunity to heparan sulfate PG could cause the progression of acute PSGN to chronicity in some patients many years after the inducing streptococcal antigen has disappeared. The role of PG autoimmunity in pathogenesis requires further investigation. In autoimmune connective tissue diseases such as SLE, the broad range of organs involved could be related to the presence of nonspecific antibodies broadly reactive with anionic molecules, including PG, while in organ-specific diseases, such as GN, antibodies directed towards specific PG immunodeterminants involve only specific target organs. Since heparan sulfate PG contributes the majority of anionic charges to the filtration barrier of the basement membrane, and blocking these sites leads to proteinuria (31), autoimmunity to glomerular heparan sulfate PG could cause proteinuria in patients with GN.

Summary

Antibodies, found in human sera from patients with poststreptococcal glomerulonephritis, against proteoglycans (PG) derived from bovine and human glomeruli were investigated. PG were isolated by 4 M guanidine-HCl extraction of whole glomeruli, followed by DEAE-Sepharose CL-6B ion exchange chromatography. The anionic fractions were further purified by chromatography on Sepharose CL-4B. Biochemical analysis of the two resulting peaks revealed the presence of high molecular weight anionic material containing protein, uronic acid, glucosamine, and galactosamine. Enzymatic and chemical susceptibilities indicated the presence of heparan sulfate PG and a galactosamine-containing PG.

Immunologic studies revealed the presence of anti-PG antibodies to both PG peaks of the Sepharose CL-4B column in glomerulonephritis sera. Inhibition studies using an ELISA demonstrated that heparan sulfate was a major antigenic determinant. Cross-reactivity with both mammalian and streptococcal hyaluronate was noted. Inhibition studies also indicated the presence of a second antigenic site containing N-acetylgalactosamine, possibly representing chondroitin or dermatan sulfate PG.

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