Monoclonal antibodies produced against chick embryo limb bud proteoglycan (PG-M) were selected for their ability to recognize determinants on intact chondroitin sulfate chains. One of these monoclonal antibodies (IgM; designated MO-225) reacts with PG-M, chick embryo cartilage proteoglycans (PG-H, PG-Lb, and PG-Lt), and bovine nasal cartilage proteoglycan, but not with Swarm rat chondrosarcoma proteoglycan. The reactivity of PG-H to MO-225 is not affected by keratanase digestion but is completely abolished after chondroitinase digestion. Competitive binding analyses with various glycosaminoglycan samples indicate that the determinant recognized by MO-225 resides in a D-glucuronic acid 2-sulfate-containing determinant. A tetrasaccharide trisulfate containing D-unit at the reducing end is the smallest chondroitin sulfate fragment that can inhibit the binding of the antibody to PG-H. Decreasing the size of a D-unit-rich chondroitin sulfate by hyaluronidase digestion results in progressive reduction in its inhibitory activity. The results suggest that the epitope has a requirement for a long stretch of a disaccharide-repeating structure for a better fit to the antibody.

In recent years a large number of apparently different chondroitin sulfate proteoglycans have been isolated. They are primarily located in extracellular matrices where they are secreted or those located in the secretory granules of mouse mast cells (5) and human natural killer cells (6), appear to have different, more specialized functions. In early studies two types of chondroitin sulfate were distinguished, chondroitin sulfate proteoglycans have been isolated. They have different, more specialized functions. In early studies two types of chondroitin sulfate were distinguished. Chondroitin sulfate proteoglycans have been isolated. They have different, more specialized functions. In early studies two types of chondroitin sulfate were distinguished. Chondroitin sulfate proteoglycans have been isolated. They have different, more specialized functions. In early studies two types of chondroitin sulfate were distinguished. Chondroitin sulfate proteoglycans have been isolated. They have different, more specialized functions. In early studies two types of chondroitin sulfate were distinguished.
**TABLE I**

Inhibition of binding of MO-225 to PG-H by chondroitin sulfates of different disaccharide unit compositions

| Source of chondroitin sulfate | IC_{50}* | Nonsulfated | A-unit | C-unit | D-unit | E-unit |
|------------------------------|----------|-------------|--------|--------|--------|--------|
| Shark fin                    |          |             |        |        |        |        |
| Fraction III                 | 0.004    | 0.8         | 27.2   | 43.4   | 21.4   | 6.7    |
| Fraction II                  | 0.02     | 1.6         | 26.2   | 52.5   | 17.5   | 1.3    |
| Fraction I                   | 0.08     | 2.0         | 23.7   | 58.6   | 13.9   | 0.8    |
| Shark scapular cartilage     | 0.43     | 2.0         | 14.8   | 73.4   | 8.4    | 0.9    |
| Whale cartilage              | 4.3      | 2.7         | 74.2   | 19.4   | 3.0    | 0.4    |
| Squid cartilage              |          |             |        |        |        |        |
|                            | 30       | 0           | 21.2   | 9.8    | 0      | 61.0   |
| Chick embryo cartilage (PG-H)| 170      | ND*         | 29.4   | 68.1   | 2.0    | 0.5    |
| Human umbilical cord         | 430      | 12.3        | 12.4   | 74.6   | 0.5    | 0.1    |
| Sturgeon notochord           | >3300    | 1.3         | 92.5   | 3.3    | 0      | 0.2    |
| Squid skin (chondrocoitn)    | >3300    | 100         | 0      | 0      | 0      |
| Swamn rat chondrocoitn       | >3300    | 10.5        | 89.4   | 0.1    | 0      |

* Concentration (µg/ml) giving 50% inhibition in ELISA on PG-H.

### Notes
- **IC_{50}**—Immunosorbent assay concentration giving 50% inhibition of binding.
- **ND**—Not determined because 35S would not label nonsulfated disaccharide units.
- **A**—A-unit, GlcA-GalNAc; **B**—B-unit, GlcA-GalNAc 6-SO_{4}.
- **C**—C-unit, GlcA4alNAc 6-SO_{4}; **D**—D-unit, GlcA 2-SO_{4}-GalNAc 6-SO_{4}; **E**—E-unit, GlcA-GalNAc 4,6-bis-SO_{4}.
- **ND**—Not determined because 35S would not label nonsulfated disaccharide units.
Monoclonal Antibody to Intact Chondroitin Sulfate

Methods

Immunization, Fusion, and Cloning—Three 6-week-old female BALB/c mice were injected intraperitoneally with either PG-M or PG-H solutions (50 µg of the antigen/mouse, emulsified in complete Freund's adjuvant). Injection of immunogen in incomplete Freund's adjuvant was repeated twice in 2-week intervals and the formation of antibodies was monitored by ELISA (see below). Two days after the final injection, the spleens of two mice showing high antibody titers were removed and the cells from spleen were fused with NS-1 myeloma line by limiting dilution. From these, a clone that reacted with PG-H but not with keratanase-treated PG-H was cloned by passive adsorption overnight at 4 °C. The plates were then rinsed as above and horseradish peroxidase-conjugated goat anti-mouse IgG (γ- and L-chain specific), peroxidase-conjugated goat anti-mouse IgM (µ-chain specific), and peroxidase-conjugated goat anti-mouse IgG1 + IgM (IgG, µ-, and L-chain specific) were from Tago Co.; polyclinyl ELISA plates (96-well) were from Sumitomo Bakelite (Osaka, Japan); mouse monoclonal isotyping kit was from Zymed Laboratories (South San Francisco, CA); and Seraquad G-100 and Protein A-Sepharose were from Pharmacia Japan (Tokyo).

ELISA for Characterization of the Monoclonal Antibodies—ELISA was done as described by Biber and Muir (24) with a slight modification. 1.0 µl (0.5 µg of protein/ml in carbonate buffer/supernatant, 200 µl/well) were coated on the plastic surface of the microtiter well by passive adsorption overnight at 4 °C. The plates were then rinsed three times with phosphate-buffered saline, pH 7.4, containing 0.05% (w/v) Tween 20 and appropriate culture supernatant (50 µl diluted with 100 µl of phosphate-buffered saline, pH 7.4) or ascites fluids (0.1 µl diluted with 100 µl of phosphate-buffered saline, pH 7.4) were added and incubated for 2 h at room temperature. The plates were then rinsed as above and horseradish peroxidase-conjugated goat anti-mouse IgG (or IgG + IgM) diluted 1:500 in phosphate-buffered saline were added as the second antibody. After incubation, o-Phenylenediamine (0.1 mg/ml in methanol diluted 1:100 into 0.03% (v/v) H2O2) was added and color was allowed to develop for 15 min, after which the reaction was stopped by adding 50 µl of 0.1 M H2SO4. The brown color produced was measured spectrophotometrically at 490 nm.

To test the effects of chondroitinase or keratanase treatment on proteoglycan antigens, appropriate enzyme (5 units of chondroitinase ABC, 0.5 unit of chondroitinase AC II A. aureus, or 1 unit of keratanase/ml in enriched buffer containing protease inhibitors; (15) 100 µl/well) was added to the antigen-coated plates and incubated for 1 h at 37 °C. The plates were then rinsed and subjected to ELISA as above.

For competitive inhibition tests, test samples were serially diluted into phosphate-buffered saline, pH 7.4, and 100-µl aliquots were added to the antigen-coated plates. Culture supernatants containing antibodies were then added and allowed to react with the antigens as above. The concentration of inhibitor required for 50% inhibition (IC50) was calculated from plots of A492nm against inhibitor concentration.

RESULTS

Characteristics of Monoclonal Antibodies Produced against PG-M and PG-H—The monoclonal antibody (MO-225) characterized in this paper originates from a group of hybridoma clones prepared against PG-M, the chondroitin sulfate present in cartilage of newborn bovines (16) and PG-H, a proteoglycan with similar chemical characteristics. As one of a number of hybridoma culture supernatants with significant reactivity to the immunogen in ELISA, at least two did not cross-react with chondroitinase ABC-digested PG-M, suggesting that the chondroitin sulfate chains of PG-M are antigenic. From these, we cloned a hybridoma producing an IgM (µ, κ) and injected the clone into mice. The resulting ascites fluid contained the same monoclonal antibody that was found in the culture supernatant, as judged by specific activity toward various proteoglycans and glycosaminoglycans (see below). In the present study the antibody in culture supernatant was used unless otherwise indicated.

When a purified PG-H preparation from 12-day-old chick embryo epiphyseal cartilage (18) was used as an immunogen,
a number of positive hybridomas producing antibodies to the immunogen were obtained but all the hybridoma culture supernatants so far tested recognized either the core protein or a keratanase-sensitive part of PG-H (note that PG-H differs from PG-M in possessing keratan sulfate side chains (16, 17)). It appears that the keratan sulfate moiety of PG-H is more immunogenic than the chondroitin sulfate moiety. In this study a monoclonal IgG (\(\gamma_1, \kappa\)) (designated HM-110) prepared from one of these keratan sulfate-positive clones was used as a control.

Reactivity of MO-225 to Native, Chondroitinase-treated, and Keratanase-treated Proteoglycans—Using ELISA, MO-225 was shown to react with the following native proteoglycans from different sources: chick embryo limb bud PG-M (17), chick embryo cartilage PG-H (16), chick embryo cartilage PG-Lb (18), chick embryo cartilage PG-Lt (19), and bovine nasal cartilage proteoglycan (A1D1 fraction, see Ref. 20) (Fig. 1). In contrast, Swarm rat chondrosarcoma proteoglycan monomer (Agg-Dl fraction, see Ref. 21) showed no significant reaction with this antibody.

In control experiments, HM-110 was examined for its reactivity to the above six proteoglycans. The keratan sulfate-containing proteoglycans, PG-H and A1D1, showed strong reaction but the other four (lacking keratan sulfate) showed no significant reaction. Consistent with the notion that the antigenic determinant for HM-110 resides in the keratan sulfate chains, the difference between MO-225 and HM-110 in specificity was further illustrated by radioimmunoprecipitation analyses. Thus, when 5000 cpm each of \([^{35}S]\)sulfate-labeled PG-M and PG-H were subjected to immunoprecipitation with either MO-225 or HM-110, about 25% of the added PG-H was recovered in both of the MO-225 and HM-110 immunoprecipitate, whereas the labeled PG-M was found only in the MO-225 immunoprecipitate.

As Fig. 2 shows, enzymatic removal of the keratan sulfate side chains from PG-H did not alter its immunoreactivity to MO-225. However, the removal of its chondroitin sulfate side chains with chondroitinase ABC (or AC II A. aurecens) completely abolished the binding activity of the antigen to MO-225. In control experiments with HM-110 the reverse was true; i.e. keratanase could abolish the binding activity of PG-H to HM-110, whereas chondroitinase ABC (or AC II A. aurecens) could not.

Together these results indicate that the determinant recognized by MO-225 resides in the chondroitin sulfate side chains. The failure of MO-225 to react with Agg-D1 could have arisen from a absence in the chondroitin sulfate of this particular proteoglycan of the determinant to be recognized by the antibody (see below for further evidence).

Competitive Binding Analysis with Glycosaminoglycans—Various glycosaminoglycans and related anionic polymers from different sources were tested as competitive inhibitors of the binding of MO-225 to PG-H. Of these, the following compounds were inactive at the highest concentration tested, 3.3 mg/ml; heparin (porcine intestinal mucosa), heparan sulfate (porcine lung), dermatan sulfate (pig skin), keratan sulfate (shark cartilage), keratan sulfate (bovine cornea), hyaluronic acid (cokcscomb), dextran sulfate, and DNA. In contrast, many if not most chondroitin sulfate preparations gave significant inhibition (Table I). It appears that the inhibitory activity of chondroitin sulfate, expressed as a concentration (microgram/ml) giving 50% inhibition (IC\(_{50}\)), reflected the content of GlcA2-SO\(_4\)-GalNAc 6-SO\(_4\) disaccharide unit (designated D-unit), i.e. the higher the D-unit content the higher the inhibitory activity. Thus, the activity of shark fin chondroitin sulfate with the highest D-unit content (21.4% of the total hexuronate) was about 42,500-fold higher than that of chick embryo cartilage chondroitin sulfate (the side chains of PG-H) with a low D-unit content (2% of the total hexuronate).

None of the D-unit-lacking polysaccharides, i.e. sturgeon notochord chondroitin sulfate, Swarm rat chondrosarcoma chondroitin sulfate, and squid skin chondroitin, gave any significant inhibition at the highest concentration tested, 3.3 mg/ml. Since the chondroitin sulfate from Swarm rat chondrosarcoma represents the chondroitin sulfate moiety of Agg-D1, its failure to inhibit the binding of MO-225 may explain why the antibody did not bind to Agg-D1 (Fig. 1). It is also noteworthy that the inhibitory activity of whale cartilage chondroitin sulfate, which contains 3% D-units, was completely abolished after chemical desulfation (data not shown).

In contrast, squid cartilage chondroitin sulfate, the preparation that did not release D\(_1\)-diS\(_2\) after chondroitinase ABC (or AC I F. heparinum) digestion, gave significant inhibition (IC\(_{50}\) = 30 \(\mu\)g/ml). The structural basis for this apparently divergent finding is not known. Since, however, this chondroitin sulfate has been shown to differ from ordinary chondroitin sulfates in containing disaccharide repeat units with glucose branches linked by \(\beta-D-(1\rightarrow6)\) to the hexosamine residues (30), the observed inhibition activity may reflect the existence of GlcA 2-SO\(_4\) residues in some of those glucose carrying repeat units. The available data (30) suggest that many glucose carrying repeat units are adjacent to each other to form glucose branch-rich domains which are released after chondroitinase digestion as sulfated oligosaccharides larger than pentasaccharides.
Inhibition of Binding of MO-225 to PG-H with Oligosaccharides Derived from Chondroitin Sulfate—As Table II shows, the tetrasaccharide trisulfate and tetrasaccharide tetr sulfate obtained by enzymatic digestion of shark fin chondroitin sulfate Fraction III gave significant inhibition with MO-225, whereas a tetrasaccharide fraction obtained by digestion of whale cartilage chondroitin sulfate was inactive at the highest concentration tested, 10,000 µg/ml. Also inactive were a series of unsaturated disaccharides (ΔDi-OS, ΔDi-4S, ΔDi-6S, ΔDi-diSO₄, and ΔDi-diSO₂) obtained by chondroitinase ABC digestion of shark cartilage chondroitin sulfate. In control experiments, neither of the shark fin tetrasaccharides inhibited the binding of HM-110 to PG-H at 10,000 µg/ml. Between the shark fin tetrasaccharides, the disaccharide sequence, GlcA2-SO₄-GalNAc6-SO₄, is common. Thus, the results are compatible with the notion that MO-225 specifically recognizes D-unit. The inability of ΔDi-diSO₂ to inhibit the binding may be due to the structural difference in hexuronicyl residue. Since glycosides or disaccharides consisting of D-glucuronic acid 2-sulfate and an aglycone other than N-acetylgalactosamine 6-sulfate were not available for testing, it is not yet known whether the N-acetylgalactosamine 6-sulfate residue in D-unit is an obligate requirement for recognition by the antibody.

Factors Affecting the Binding of PG-H to MO-225—Physicochemical studies (38, 39) have shown that divalent cations have significant effects on the conformation of sulfated glycosaminoglycans and proteoglycans. Using the ascites fluid containing MO-225, effects of 5 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl, 1 mM EDTA, and 1 mM EGTA on the binding of the antibody to PG-H were tested. In no case, however, was the binding activity significantly influenced by these added reagents.

When shark cartilage chondroitin sulfate (D-unit content = 8.4%) was digested with testicular hyaluronidase, its ability to inhibit the binding of MO-225 to PG-H was progressively decreased with digestion time (Fig. 3). The results indicate that not only the D-unit content but also the chain length is important in determining the binding activity of antigenic chondroitin sulfate.

The binding of MO-225 to the PG-H substrate in ELISA was competitively inhibited by PG-H itself added to the medium (Fig. 4). The inhibitory activity of PG-H was considerably reduced after treatment of the proteoglycan with either TPCK trypsin or Pronase, indicating that the epitope has a requirement for protein core for a better fit of chondroitin sulfate to the paratope of the antibody.

**DISCUSSION**

Christner et al. (40) reported that degradation of bovine nasal cartilage proteoglycan with chondroitinase ABC produced new antigenic determinants bearing nonreducing Δ¹-glucuronic acid end groups. Autoantibodies to degraded glycosaminoglycans, including degraded chondroitin sulfate bearing Δ¹-glucuronic acid, were also reported in preimmune rabbits by Poole et al. (41). Some of these sera reacted with intact hyaluronic acid and chondroitin but never with intact chondroitin sulfate. It could be speculated then that chondroitin sulfate side chains of native proteoglycans are non-antigenic. In two other studies, however, monoclonal antibodies directed to either intact chondroitin 4/6-sulfates (15) or saturated chondroitin 6-sulfate oligosaccharides (42) have been prepared by immunization with ventral membranes of cultured chick fibroblasts or testicular hyaluronidase-treated chick embryo cartilage proteoglycans, respectively. This would indicate that GlcA-GalNAc 4-SO₄ and/or GlcA-GalNAc 6-SO₄, the common repeat units of many avian and mammalian chondroitin sulfates, can be the focus of an immune response in mice. Alternatively, the apparent antigenicity of the avian chondroitin sulfate preparations could be due to the fact that the preparations contained a small proportion of some other structural units (such as A-unit) which are foreign to mice. In view of the results of the experiments bearing Δ¹-glucuronic acid, no analysis with chondroitinases was performed. 3

**TABLE II**

| Tetrasaccharide | IC₅₀ (µg/ml) |
|-----------------|-------------|
| ΔGlcA-GalNAc-GlcA-GalNAc | 1,400 ± 200 |
| 4-SO₄ 2-SO₄ 6-SO₄ | 1,200 ± 200 |
| ΔGlcA-GalNAc-GlcA-GalNAc | 1,400 ± 200 |
| 4,6-bis-SO₄ 2-SO₄ 6-SO₄ | >10,000 |

*Our analysis of a chondroitin [³⁵S]sulfate prepared from the whole body of a newborn BALB/c mouse (which had been metabolically labeled with inorganic [³⁵S]sulfate) indicated the composition (percent of the total [³⁵S] of labeled disaccharide units to be 88.5 (A-unit), 7 (C-unit), and 3 (E-unit). No trace of D-unit was detected by the analysis with chondroitinases.*
reported here, we feel a need of reliable information on the
fine structure of the chondroitin sulfates used for specificity
studies.

The occurrence of sulfated glucuronosyl residues in shark
chondroitin sulfate was demonstrated many years ago (9, 10)
and the position of sulfate determined to be C-2 (11). Since,
however, this residue is present at low levels in most of the
chondroitin sulfate preparations so far isolated from avian
and mammalian sources, the significance of such a residue in
the structure and function of proteoglycan glycosaminoglycan
chains has been discounted. Recently an unusual species of
which contains a high proportion of GlcA%so4 residues (43).
Furthermore, a sulfatase which specifically hydrolyzes the
sulfate group from GlcA 2-SO4 residues but not from t-iduronic acid
2-sulfate has been observed in human skin fibroblasts and
chick embryo chondrocytes (44), suggesting that GlcA 2-SO4
residues in glycosaminoglycans might be of greater signifi-
cance than previously believed.4

We previously showed that a dermatan sulfate preparation
from pig skin contains IdoA2-SO4-GalNAc 4-SO4 units (5-
6% of the total disaccharide units) (9). In the present study,
this dermatan sulfate preparation was shown to give no in-
hibition to the binding of MO-225 to PG-H. Also shown to
be inactive were porcine intestinal mucosa heparin and por-
cine lung heparan sulfate which contain IdoA 2-SO4 residues
linked to N- and/or O-sulfated glucosamine units at consid-
erably high levels. The results indicate that the IdoA2-SO4-
containing disaccharide sequences involved in these glyco-
saminoglycans cannot be recognized by MO-225.

The occurrence of GlcA3-SO4-GalNAc 4-SO4 disaccharide
units has been suggested in king crab chondroitin sulfate (11).
In view of its high sulfate to glucuronic acid ratio (~1:6.4),
this polysaccharide must contain a high proportion of GlcA
3-SO4 residues. It was of interest therefore to test the anti-
genicity of this polysaccharide to MO-225. Our competitive
inhibition tests have shown that the inhibitory activity of a
king crab chondroitin sulfate preparation is far less inhibitory
than sturgeon notochord chondroitin sulfate (IC50
5.0 pg/ml) than the activities of shark cartilage chondroitin
sulfates. Also shown to inhibit to the binding of MO-225 to
PG-H. Also shown to inhibit to the binding of MO-225 to
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5.0 pg/ml) than the activities of shark cartilage chondroitin
sulfates. Also shown to inhibit to the binding of MO-225 to
PG-H.

Therefore the importance of this polysaccharide preparation
is unlikely to be recognized by MO-225, since squid cartilage
chondroitin sulfate contains IdoA2-SO4-GalNAc 4-SO4 residues.

Monoclonal Antibody to Intact Chondroitin Sulfate

A small amount of the nuclear heparan sulfate was kindly given
by Dr. N. Seno (Ochanomizu University). and was tested for its
effect on the binding of MO-225 to PG-H. At a concentration of 10
µg/ml, the sample had no effect on the binding.

Kindly donated by Dr. N. Seno (Ochanomizu University, Tokyo).

M. Yamagata, unpublished observation.

Well characterized antibodies are now available which rec-
ognize epitopes present in chondroitin sulfate, keratan sulfate,
and core protein structures characteristic of different subtypes
of chondroitin sulfate proteoglycans. The use of monoclonal
antibodies directed to the different parts of proteoglycans
may offer great potential in the immunohistochemical char-
acterization of the proteoglycan subtypes distributed in vari-
ous tissues. Furthermore, it can be surmised that glycosami-
nglycan chains of native proteoglycans from various sources
may contain different antigenic structures to which specific
monoclonal antibodies can be prepared. It is certainly desir-
able to have monoclonal antibodies which can detect differ-
ences in fine structures among glycosaminoglycan chains.

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