Identification of a Monoclonal Antibody That Specifically Recognizes Corneal and Skeletal Keratan Sulfate

MONOCLONAL ANTIBODIES TO CARTILAGE PROTEOGLYCAN*

(Received for publication, March 29, 1983)

Bruce Caterson‡, James E. Christner§, and John R. Baker§

From the ‡Biochemistry Department, West Virginia University Medical Center, Morgantown, West Virginia 26506 and the §Institute of Dental Research, School of Medicine and Diabetes Research and Training Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

Monoclonal antibodies were raised against proteoglycan core protein isolated after chondroitinase ABC digestion of human articular cartilage proteoglycan monomer. Characterization of one of the monoclonal antibodies (1/20/5-D-4) indicated that it specifically recognized an antigenic determinant in the polysaccharide structure of both corneal and skeletal keratan sulfate. Enzyme immunoassay analyses indicated that the mouse monoclonal IgG1 recognized keratan sulfate in native proteoglycan aggregate and proteoglycan monomer preparations isolated from hyaline cartilages of a wide variety of animal species (human, monkey, cow, sheep, chicken, and shark cartilage). The 1/20/5-D-4 monoclonal antibody did not recognize antigenic determinants on proteoglycan isolated from Swarm rat chondrosarcoma. This finding is consistent with several biochemical analyses showing the absence of keratan sulfate in proteoglycan synthesized by this tissue.

A variety of substructures isolated after selective cleavage of bovine nasal cartilage proteoglycan (Heinegård, D., and Axelson, J. (1977) J. Biol. Chem. 252, 1971–1979) were used as competing antigens in radioimmunoassays to characterize the specificity of the 1/20/5-D-4 immunoglobulin. Substructures derived from the keratan sulfate attachment region of the proteoglycan (keratan sulfate peptides) showed the strongest inhibition. Both corneal and skeletal keratan sulfate peptides as competing antigens in radioimmunoassays showed similar inhibition when compared on the basis of their glucosamine content. Therefore, the 1/20/5-D-4 monoclonal antibody appears to recognize a common determinant in their polysaccharide moieties. Chemical desulfation of the keratan sulfate reduced the antigenicity of the glycosaminoglycan. The antibody did not recognize determinants present in dermanatan sulfate, heparin, heparin sulfate, or hyaluronic acid.

Proteoglycans are ubiquitous components of connective tissues. They are particularly abundant in tissue where there is greatest development of extracellular matrix material (e.g., cartilage, skin, bone, and teeth). They endow the extracellular matrix, and the tissue, with many of its characteristic biochemical and biophysical properties (1). Proteoglycans from bovine nasal cartilage and bovine tracheal cartilage have been the most thoroughly studied (2, 3) although in recent years studies on the Swarm rat chondrosarcoma have provided considerable information on the structure and biosynthesis of its constituent cartilage proteoglycans (4–7).

In cartilage, proteoglycans occur as aggregates (2, 3, 8, 9) which consist of three classes of components: proteoglycan monomers, hyaluronic acid, and link proteins (2, 3, 10, 11). A model for the cartilage proteoglycan aggregate has been proposed (12, 13). In this model, the proteoglycan monomers are pictured as asymmetric molecules. A region of the proteoglycan monomer core protein, the hyaluronic acid-binding region, specifically associated with a central strand of hyaluronic acid (2, 3). Beyond the hyaluronic acid-binding region of the proteoglycan monomer, the protein core bears a region containing predominantly keratan sulfate side chains and beyond that is the chondroitin sulfate attachment region of the molecule where a few keratan sulfate chains are found interspersed with the relatively predominant chondroitin sulfate chains. In addition, N- and O-linked oligosaccharide structures distributed on the polypeptide backbone of the proteoglycan monomers have been recently described (6, 14, 15). The link proteins of the proteoglycan aggregate stabilize the association of proteoglycan monomers with hyaluronic acid (2, 3, 16). This stabilization occurs through specific interactions of the link protein with both the proteoglycan monomer and the hyaluronic acid (20, 17, 18).

Determination of proteoglycan structure by conventional biochemical procedures has been complicated by the polydispersity and heterogeneity which appears common to all classes of connective tissue proteoglycan. Potentially, immunological methods may be particularly useful, as antibodies to proteoglycan may be used to determine specific antigenic determinants, regardless of polydispersity and heterogeneity of the proteoglycan. Substructures of cartilage proteoglycan monomer (2, 19) have distinct biochemical characteristics and can therefore, potentially, be recognized and quantitated by specific immunological means. Polyclonal antibodies recognizing link protein and several of the proteoglycan monomer substructures have been reported (20–24) and have been used to quantitate and immunohistochemically localize proteoglycans in connective tissue preparations. Monoclonal antibodies, raised by a technique based on that of Kohler and Milstein (25), recognize determinants on hyaluronidase-treated proteoglycan monomer from chick sternal cartilage (26, 27). One of these monoclonal antibodies has been used to isolate proteoglycan core synthesized in cell-free systems (26), and the other was shown to specifically recognize saturated oligosac-
chondroitin 6-sulfate attached to the protein core of hyaluronidase-treated proteoglycans (27). Recently, we have described the production and partial characterization of several monoclonal antibodies to link protein and to chondroitin ABC-treated proteoglycan monomer from bovine nasal cartilage (28). The immunoglobulins produced by the hybridomas were specific for many different determinants of proteoglycan monomer and link protein. In this paper, we describe the characterization of a monoclonal antibody that specifically recognizes a determinant in the characteristic polysaccharide of keratan sulfate. This monoclonal antibody can be used in radioimmunoassay procedures to specifically detect and quantitate keratan sulfate in proteoglycan preparations.

**Experimental Procedures**

**Materials**—The hydrochloric acid used for acid hydrolysis was Aristar grade (British Drug Houses). All other reagents used were of analytical reagent grade. Chondroitin ABC was purchased from Miles Laboratories. Ficin, pronase mucin (type II), bovine serum albumin, and the gene hydrolysis reagents were from Sigma. Nonidet P-40 was obtained from Gallard-Schlesinger and 6-aminohepoxic acid was purchased from Eastern Kodak. Freund's complete adjuvant and incomplete adjuvant were bought from Lederle Laboratories. Phycocyanine (CF) was purchased from Amersham. Tissue culture dishes (100 mm) were from Corning (cat. no. 20520). All other tissue culture dishes (6-, 24-, and 96-well culture dishes) were purchased from Costar. Medium (RPMI 1640), sterile Dulbecco's phosphate-buffered saline, media additives (fetal calf serum, glutamine, Pen-Strep, and Fungisone), Lindbro EIA plates and the Titertek Multiskan for reading the EIA plates were purchased from Flow Laboratories. Gels for gel chromatography were obtained from Pharmacia Fine Chemicals. CPG 2500 was a generous gift from Dr. Paul Goetinck (University of Connecticut, Storrs, CN) and was originally purchased from Electromedicals. CPG 200-Glycophase was obtained from Pierce. Formalin-treated, heat-inactivated *Staphylococcus aureus* was prepared by published procedures (29). Dermatan sulfate, heparin, and heparan sulfate were generous gifts from Dr. Richard Reynerton and Dr. Lennert Roden, University of Alabama in Birmingham, and were prepared by published procedures (30). Human and dog blood group glycolipids, prepared by published procedures (31), were generously provided by Dr. John McKibbin, University of Alabama in Birmingham.

**Column Chromatography**—Preparative columns of Sepharose CL-2B, CPG 2500, and CPG 200 were eluted with 0.05 M NaHCO3, pH 8.0. The occurrence of proteoglycan, peptides, and proteoglycan fragments in the eluted fractions was monitored by absorbance at 200 nm on a Ultroptic (Beckman Instruments) at 241 nm on a UV II (Pharmacia Fine Chemicals) ultraviolet monitor, respectively. Fractionated materials were recovered from pooled fractions by lyophilization.

**Anodicolysis Methods**—Uronic acid determinations were according to the carbazole method of Bitter and Muir (32). Amino acid analyses and hexosamine analyses were performed after 20- and 7-h hydrolyses, respectively, in 6 M HCl at 105 °C and quantitated on a Beckman 119C automatic amino acid analyzer (33). Norleucine was used as an internal standard in both analyses. Protein (50 μg) or glycosaminoglycan (20 μg) was hydrolyzed in Reacti-vials (Pierce Chemical Co.) under nitrogen (total hydrolysis volume, 400 μl). Sulfate analyses were performed using a modified Rhodizone procedure described by Silvestri et al. (34).

**Isolation of Cartilage Proteoglycans**—Proteoglycans from BNC, bovine articular cartilage, sheep nasal cartilage, sheep articular cartilage, RC, monkey nasal cartilage, monkey articular cartilage, chicken epiphysial cartilage, chick sternum cartilage, shark cranial cartilage, shark brachial cartilage, and HAC were extracted and isolated by published procedures (9, 35). In brief, the hyaline cartilage was cut into small pieces and extracted with 4 M guanidine HCl containing protease inhibitors (4) for 16 h at -10 °C. The extracts were separated from the cartilage residue by centrifugation at 1000 × g and the supernatant was dialyzed for 16 h at 4 °C against 0.05 M sodium acetate, pH 6.5, containing protease inhibitors (4). The proteoglycan aggregate fraction (A1) and proteoglycan monomer fraction (A1D1) were obtained after CsCl equilibrium density gradient centrifugation under "associative" and "dissociative" conditions, respectively. The proteoglycan fractions were dialyzed at 4 °C for 24 h against 0.1 M sodium acetate, pH 6.5, containing 0.1 M sodium azide, 0.15 M NaCl, and 0.02% NaN3, and then dialyzed against deionized water prior to their lyophilization and storage in a desiccator at room temperature. Stock solutions of proteoglycan A1 (2 mg/ml) and A1D1 (4 mg/ml) were prepared in PBS-azide and stored at 4 °C for analyses in the EIA and RIA procedures described below.

**Isolation of Human Articular Cartilage Proteoglycan Core Protein Used As The Antigen for Immunization of BALB/c Mice**—A pooled sample of human articular cartilage from the femoral and tibial condyles of six normal males (age range, 50–77 years) was used for extraction of human articular cartilage proteoglycan. In all cases, the cartilage was obtained at autopsy within 4 h of death, dissected from the bone surface, and stored at -80 °C until required for the study. The cartilage was diced into small pieces and homogenized on an ice-cooled dissection plate. The proteoglycan aggregate and monomer preparations were obtained by the procedures described above. The galactosamine to glucosamine ratios of aggregate and monomer preparations were 5.4 and 7.5, respectively, suggesting a high proportion of keratan sulfate (cf. proteoglycans from bovine nasal cartilage) in these preparations.

**Human articular cartilage proteoglycan core protein (HAC-PG-Core(A1)) was obtained after digestion of the proteoglycan monomer, A1D1 fraction, with chondroitin ABC. The core protein was pre pared from chondroitinase-degraded cartilage by chromatography on Sephacrose CL-6B, using conditions described previously (24). The void volume material was pooled, dialyzed exhaustively against deionized water, lyophilized, and stored desiccated at room temperature. A stock solution (800 μg/ml in sterile Dulbecco's phosphate-buffered saline, 5 × 0.5-ml aliquots) was prepared, stored at -20 °C, and used in the injection protocol described below.

**Immunization, Fusion, and Cloning**—Three 4–6-week-old female BALB/c mice were immunized with HAC-PG-Core(A1) and hybridoma fusion performed according to the protocol described by Kearney and co-workers (37, 38). The antigen (HAC-PG-Core(A1)) was administered to each animal at six injection sites (hind foot pads, dorsal thoracic and inguinal regions) of each leg, so that each antigen (0.5 ml of HAC-PG-Core(A1), 800 μg/ml) was mixed with 0.5 ml of Freund's complete adjuvant (day 1), 0.5 ml of Freund's incomplete adjuvant (day 3), and 0.5 ml of Dulbecco's PBS at days 6, 9, and 12, respectively, and 0.05 ml of the antigen mix was injected subcutaneously at the six injection sites of each leg. For the final injection (day 14), the draining lymph nodes from the regions nearest the sites of injection (axillary, brachial, inguinal, and popliteal lymph nodes) were removed aseptically under a laminar flow hood and placed in a 25-mm Petri dish containing 2 ml of Dulbecco's PBS, cooled at 4 °C in an ice bath. Lymphocytes were dispensed in the solution by teasing the lymph nodes with forceps and finally by repeated suction and aspiration through an 18-gauge needle using a 3-ml syringe. The lymphocytes were separated from adhering connective tissue by passing the cell suspensions through a sterile glass wool filter plug in a Pasteur pipette and the cells were washed twice with 40 ml of RPMI 1640 medium at 4 °C.

The characteristics of the hyoxanthine-aminopterin-thymidine-sensitive mouse myeloma cell line (×63 – Ag8.653) have been described elsewhere (37, 39). This cell line is available from the American Type Culture Collection. The mouse myeloma cell line (×63 – Ag8.653) was cultured in normal culture media (RPMI 1640 containing 15% fetal bovine serum, 0.1% L-glutamine, 0.05 ml PenStrep, 0.001 unit/ml Fungisone, and 0.001 ml 2-mercaptoethanol) in 100-mm tissue culture Petri dishes (Corning, 20520). Myeloma cells from eight Petri dishes growing at log phase (2–5 × 10° cells total) were washed twice with RPMI 1640 medium at 4 °C to remove fetal calf serum and medium components. Lymphocytes (×1
The procedures for isolating the BNC proteoglycan substructures are depicted in Fig. 1. BNC-PG-Core(ABC) (Fig. 1A) was prepared after chondroitinase ABC digestion of BNC-A1D1 (24). BNC chondroitin sulfate-oligosaccharide peptides (Fig. 1, F and H) and BNC keratan sulfate peptides (Fig. 1, E and G) were prepared after protease digestion of the BNC-PG-Core(ABC) according to the methods described by Heinig and Axelsson (19). A minor modification of this procedure was the use of CPG 200 column chromatography instead of Sepharose CL-6B chromatography which gave a more rapid separation of the keratan sulfate peptides from the chondroitin sulfate oligosaccharide peptides. BNC chondroitin sulfate-oligosaccharide peptides and BNC keratan sulfate peptides samples (Fig. 1) were obtained by pooling appropriate fractions after gel filtration and concentrated by lyophilization. Freeze-dried samples were redisolved in 0.15 M NaCl prior to amino acid analysis and hexosamine analysis or use in the competitive binding RIA described below.

BNC glycosaminoglycan fractions (Fig. 1, B, C, and D) were obtained from BNC-A1D1 after digestion with trypsin and fractionation on DEAE-cellulose using published procedures (30). Three fractions (Fig. 1, antigens B, C, and D) from the digest were obtained by stepwise elution at 0.3, 0.4, and 0.5 M NaCl, respectively from DEAE-cellulose. The glycosaminoglycan fractions accounted for 15, 69, and 16% of the dry weight of the glycosaminoglycan recovered from the digest, respectively. Eluted fractions were dialyzed exhaustively against deionized water and freeze-dried. Stock solutions (10 mg/ml in PBS-azide) were prepared and used for amino acid analyses, hexosamine analyses, and ELISA analyses of 1/20/5-D-4 immunoglobulin specificity. A corneal keratan sulfate glycosaminoglycan fraction was prepared from peptic/pronase digests of bovine corneas according to the procedures described by McCarthy and Baker (40). Desulfated keratan sulfate was isolated after methanolic HCl treatment of the corneal keratan sulfate fraction (40). Sulfate analyses (34) of this desulfated corneal keratan sulfate fraction indicated that 75% of the ester sulfate had been removed.

Enzyme Immunoassay for Detection and Characterization of the Clonal Hybrid Specificities—The method for preparing alkaline phosphatase-labeled purified goat antibodies (goat anti-mouse κ and λ light chains) has been described elsewhere (37). Antibody-secreting hybrids were detected by a modification of an ELISA procedure described in a recent publication (41). Antigens (1–5 μg/ml in PBS-azide, 200 μl/well) were coated on the plastic surface of the ELISA plate by passive adsorption overnight at 4°C. The unreacted sites on the ELISA plate were blocked by addition of 200 μl of ELA buffer (1.0% bovine serum albumin in PBS-azide) to each well and incubation for 1 h at 37°C. The plates were washed three times with PBS-azide and appropriate dilutions of the monoclonal antibodies were added to the wells. After 2 h at room temperature, the plates were washed five times with PBS-azide and 100 μl of goat anti-mouse immunoglobulin (GAG, hexosaminoglycan).

**Fig. 1.** Schematic representation of the procedures used to generate the bovine nasal cartilage proteoglycan substructures that were used as antigens in competitive inhibitory radioimmunoassays to determine the specificity of the 1/20/5-D-4 monoclonal immunoglobulin. GAG, glycosaminoglycan.
culture supernatants (100–200 μl/well) were added and incubated for 90 min at 37 °C. The plates were washed three times with PBS-azide. Enzyme-linked second antibody (alkaline phosphatase-conjugated goat anti-mouse κ and λ light chains) was added (200 μl/well) and similarly incubated. The plates were washed three times with PBS-azide and then 200 μl of alkaline phosphatase substrate (p-nitrophenyl phosphate, 1 mg/ml, in 0.25 mM MgCl₂, 1 M diethanolamine, pH 9.8) was added to each well and the plates were incubated 30–90 min at 37 °C until optimal color development occurred. The reaction was stopped by the addition of 5 M NaOH (50 μl/well). Clonal antibody-positive wells were identified by the presence of yellow color resulting from the conversion of the p-nitrophenyl phosphate to p-nitrophenol by the enzyme-linked second antibody. The p-nitrophenol was quantitated by measuring the absorption at 405 nm of the solution from each well (EIA Multiskan, Flow Laboratories).

Mouse immunoglobulin subclass and heavy and light chain specificities were determined by a modification of the above EIA procedure. HAC-PG-Core(ABC) was coated on the EIA plate, unreacted sites were blocked with EIA buffer, and the supernatants from the wells of positive clones were incubated as described above. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin subclass, heavy and light chain-specific second antibodies (a generous gift from Dr. John F. Kearney, University of Alabama in Birmingham) were used to characterize the hybridoma monoclonal antibody. The 1/20/5-D-4 monoclonal antibody was identified as producing immunoglobulin containing κ heavy chains and λ light chains. The identification of 1/20/5-D-4 as an IgG1-producing monoclonal facilitated its use in radioimmunooassay procedures utilizing S. aureus protein A (42).

Radioimmunoassay for Detecting the Antigen Specificity of the 1/20/5-D-4 Monoclonal Immunoglobulin.—The EIA procedure used was a modification of one described in previous publications (21, 24, 41). A higher pH was used in the buffers to facilitate the binding of mouse immunoglobulin to the heat- and formalin-treated S. aureus cells, which allows all subclasses of immunoglobulin except IgM to bind to the S. aureus (42). Incubations were performed in a solution of 1% bovine serum albumin, 0.5% deoxycholate. 0.25% Nonidet P-40, 0.02% NaN₃ in 0.15 M sodium phosphate, pH 8.1. The presence of detergents in the incubation and wash buffers ensured the complete solubilization of antigens. Antigen-antibody complexes bound to S. aureus pellets were washed with 0.15 M sodium phosphate, pH 8.1.

Antigens (HAC-PG-Core(ABC), BNC-PG-Core(ABC), and RC-PG-Core(ABC)) were iodinated with ¹²⁵I using chloramine-T (43). Competitive binding radioimmunoassays used the conditions described in previous publications (21, 24, 41), except for the buffer changes indicated above, and measured the ability of unlabeled BNC proteoglycan antigens to compete with ¹²⁵I-BNC-PG-Core(ABC) for a known dilution of the 1/20/5-D-4 culture medium. The per cent inhibition given by each unlabeled antigen is expressed as 100 – (100 × cpm bound in the presence of unlabeled antigen/cpm bound in the absence of unlabeled antigen).

RESULTS

Preliminary examination of the specificity of the 1/20/5-D-4 monoclonal supernatants in an EIA indicated that the immunoglobulin recognized an antigenic determinant common to proteoglycan preparations from a wide variety of animal species and tissues but not to Swarm rat chondrosarcoma proteoglycan (Fig. 2). Analyses in the EIA indicated that the antibody recognized antigens from articular cartilage to a greater degree than preparations from nasal septum. As keratan sulfate is absent from rat chondrosarcoma proteoglycan and enriched in articular antigen proteoglycans, these results suggested that 1/20/5-D-4 may be recognizing some part of keratan sulfate.

EIA analyses of the 1/20/5-D-4 immunoglobulin subclass were performed and indicated that the hybridoma was synthesizing immunoglobulins of the IgG1 subclass containing κ light chains (as mentioned above). The results of RIA analyses using serial dilutions of the 1/20/5-D-4 monoclonal media versus ¹²⁵I-labeled HAC-PG-Core(ABC), BNC-PG-Core(ABC), and RC-PG-Core(ABC) are shown in Fig. 3. Greater than 80% of the ¹²⁵I-labeled HAC-PG-Core(ABC) and BNC-PG-Core(ABC) proteoglycans were bound by the

![Fig. 2. Enzyme immunoassay of 1/20/5-D-4 hybridoma media against proteoglycan preparations from several different animal species and tissues. BNC, bovine nasal cartilage; BAC, bovine articular cartilage; HAC, human articular cartilage; RC, Swarm rat chondrosarcoma; SAC, sheep nasal cartilage; SAC, sheep articular cartilage; CSt, chicken sternal cartilage; CEp, chicken epiphyseal cartilage; MNC, monkey nasal cartilage; MAC, monkey articular cartilage; ShB, shark bursal cartilage; ShC, shark cranial cartilage. Proteoglycan fractions A1 and A1D follow the notation suggested by Heinegård (34) for proteoglycan aggregate and monomer, respectively.](http://www.jbc.org/)

The 1/20/5-D-4 monoclonal IgG1, ¹²⁵I-RC-PG-Core(ABC)³ was not recognized by the 1/20/5-D-4 IgG1, showing similar results to the EIA analyses which used intact proteoglycan preparations as antigens.

Amino acid and hexosamine analyses of the antigen used for immunization and the BNC proteoglycan substructures used as competitive inhibitors in RIA analyses are given in Table I. The analyses (Table 1) are consistent with the identifications and descriptions of the fractions. BNC proteoglycan substructures were used as antigens in competitive binding RIA to determine the specificity of the 1/20/5-D-4 monoclonal IgG1 (Fig. 4). Inhibition analyses are compared relative to the amount of total amino acids present in each antigen. Proteoglycan substructures showing the greatest inhibition were those derived from the keratan sulfate attachment region of bovine nasal cartilage proteoglycan. The most inhibitory antigens were those derived from sequential chondroitinase ABC, trypsin, and chymotrypsin digestion of BNC-A1D1 and the 0.5 M NaCl glycosaminoglycans eluted from DEAE-cellulose (antigens G and D, respectively; Table I and Fig. 1). BNC chondroitin sulfate-oligosaccharide peptides were poor competitive inhibitors in the RIA. A summary of the relative inhibition of each of the BNC proteoglycan sub-

³ The 1/20/5-D-4 monoclonal antibody did recognize antigenic determinants present on ²⁰¹I-labeled or ³⁵S-labeled proteoglycan isolated from rat articular cartilage (B. Caterson, unpublished observation).
A Monoclonal Antibody to Keratan Sulfate

TABLE I

Analyses of proteoglycan fragments used as competing antigens in radioimmunoassays

| Amino acid | Antigen | A | B | C | D | E | F | G | residues/1000 amino acids |
|------------|---------|---|---|---|---|---|---|---|--------------------------|
| Aspartic acid |         | 71 | 75 | 52 | 49 | 37 | 63 | 40 |                         |
| Threonine |         | 38 | 59 | 40 | 42 | 43 | 31 | 45 |                         |
| Serine |         | 114 | 90 | 163 | 151 | 142 | 174 | 121 |                         |
| Glucosamine |         | 150 | 101 | 155 | 163 | 180 | 149 | 195 |                         |
| Proline |         | 85 | 133 | 116 | 134 | 164 | 59 | 149 |                         |
| Glycine |         | 146 | 93 | 152 | 138 | 131 | 186 | 118 |                         |
| Alanine |         | 56 | 83 | 60 | 60 | 42 | 49 | 42 |                         |
| Valine |         | 77 | 60 | 69 | 66 | 61 | 80 | 55 |                         |
| Isoleucine |         | 37 | 29 | 34 | 32 | 28 | 40 | 24 |                         |
| Leucine |         | 82 | 67 | 85 | 75 | 60 | 98 | 57 |                         |
| Tyrosine |         | 25 | 23 | 10 | 12 | 7 | 4 | 8 |                         |
| Phenylalanine |         | 36 | 50 | 28 | 38 | 56 | 15 | 60 |                         |
| Histidine |         | 14 | 10 | 6 | 6 | 8 | 15 | 16 |                         |
| Lysine |         | 27 | 22 | 11 | 15 | 29 | 13 | 51 |                         |
| Arginine |         | 44 | 10 | 18 | 18 | 14 | 24 | 20 |                         |
| Glucosamine |         | 45 | 460 | 205 | 462 | 140 | 79 | 356 |                         |
| Galactosamine |         | 29 | 1044 | 2414 | 4173 | 43 | 50 | 96 |                         |
| HexN/amino acid |         | 0.074 | 1.340 | 4.419 | 11.933 | 0.184 | 0.129 | 0.452 |                         |
| GaN/GlcN |         | 0.64 | 8.1 | 20.60 | 9.03 | 0.21 | 0.04 | 0.27 |                         |

* See Fig. 1 and "Experimental Procedures" for description of antigen preparation.

* Molar ratios.

structures in the RIA is shown on Table II. In general, inhibition is greater for keratan sulfate (glucosamine-rich) glycosaminoglycan peptide fragments. However, the 0.3 M NaCl DEAE-cellulose glycosaminoglycan fraction which is rich in glucosamine (antigen B, galactosamine/glucosamine ratio, 8.1; Tables I and II) was found to be a relatively poor inhibitor in the RIA.

Competitive binding RIA analyses were performed using bovine corneal keratan sulfate preparations and compared with skeletal keratan sulfate samples, to determine whether the antigenic determinant recognized by the 1/20/5-D-4 immunoglobulin occurred in the polysaccharide chain or the peptide portion of the proteoglycan fragments (Fig. 5). Inhibition analyses were compared relative to the amount of glucosamine present in each antigen. The results indicate that

Fig. 4. Competitive inhibition radioimmunoassay analyses depicting the ability of unlabeled antigens (proteoglycan substructures, antigens A-G; Fig. 1 and Table I) to compete with 125I-BNC-PG-Core(ABC) for the 1/20/5-D-4 immunoglobulin. Corneal keratan sulfate, which has a distinctly different carbohydrate linkage to peptide from skeletal keratan sulfate, gave an inhibition curve similar to that of skeletal keratan sulfate in RIA analyses (50% inhibition at 17 and 55 pmol of glucosamine, respectively). Furthermore, chemical desulfation of the corneal keratan sulfate (where 75% of the ester sulfate was removed from the polysaccharide) caused a significant reduction in the antigenicity of the glycosaminoglycan (Fig. 5, Table III). These results indicate that the antigenic determinant recognized by the 1/20/5-D-4 monoclonal antibody resides in the polysaccharide structure common to both corneal and skeletal keratan sulfate. In addition, the presence of ester sulfate on the polysaccharide significantly enhances the antigenicity of the keratan sulfate determinant recognized by the 1/20/5-D-4 IgG1. During the chemical desulfation process, some methylation of the glycosaminoglycan sugars may have occurred but this was subsequently reversed by alkali treatment.4 In addition, some cleavage occurs during the desulfation procedure.4 However, it is unlikely that the small amount of depolymerization would significantly influence the antigenic determinant.

4 J. Baker, unpublished analyses.
These fucolipids contain one N-acetyllactosamine moiety similar to that found in keratan sulfate. In addition, pig gastric mucin which contains a structure similar to that found in the repeating disaccharide portion of keratan sulfate (e.g. 6-sulfated N-acetylgalactosaminyl residue of (1→3) to galactose; see Ref. 45). Many of these fucolipids did not show inhibition in the RIA. These results further indicate the specificity of the 1/20/5-D-4 immunoglobulin for a determinant which is characteristic of the keratan sulfate glycosaminoglycan chain.

**DISCUSSION**

Many oligosaccharides of known structure have been shown to be antigenic (45). There is such a diversity of oligosaccharide structures on mammalian glycoproteins that many are likely to be antigenic. The glycosaminoglycan chains of proteoglycans from all sources tend to have the same linear disaccharide repeat sequences and are therefore less likely to be antigenic, although antibodies specific for some feature of chondroitin 4-sulfate chains have been reported (27). Recently, antibodies to oligosaccharide moieties of chondroitinase-digested proteoglycans have been raised (24). The determinants are the protein-linked oligosaccharide "remnants" of chondroitin sulfate chains.

In this paper, the characterization of a monoclonal antibody raised against the core proteins of human articular cartilage proteoglycan is described. It is evident that the only glycosaminoglycan which it recognizes is keratan sulfate, although keratan sulfates from cornea and cartilage bind the antibody. Desulfation of keratan sulfate decreases binding. These characteristics are also common to a rabbit antisemur to bovine corneal proteoglycan as recently described by Conrad et al. (46). The rabbit antisemur appears to possess antibody activities to the proteoglycan core protein. In contrast, the monoclonal antibody must recognize one specific structural feature of the keratan sulfate chain. To identify this structural feature will be difficult as little is known of the arrangement of differently substituted galactosyl and N-acetylgalactosaminyl residues along the keratan sulfate chains. (Comparatively, much more is known of other glycosaminoglycans (30).) Thus, either or both sugar residues may be 6-sulfated and the antibody may recognize a specific pattern of sulfation on two to four consecutive sugar residues. Keratanase, an endo-β-galactosidase, will be useful for generating fragments from keratan sulfate which can be characterized and then tested for competitive binding to the antigen binding site of monoclonal antibody 1/20/5-D-4.

Monoclonal antibody 1/20/5-D-4 can recognize and bind keratan sulfate chains in their native form attached to proteoglycan. Therefore, it is likely to be a useful tool for studying localization and distribution of keratan sulfate proteoglycans in tissue sections employing immunohistological methods. Indeed, this antibody has been successfully employed by Vertel to localize keratan sulfate in the Golgi of chondrocytes.

**Acknowledgments**—The expert technical assistance of Irene Par- rish is greatly appreciated. We express appreciation to Vicki McLeod, Sherri Nielson, and Sally Bierer for typing of the manuscript and to Dr. John F. Kearney for his helpful discussions on the intricacies of monoclonal antibody procedures. Human articular cartilage was pro- vided through the Tissue Procurement Service of the Comprehensive Cancer Center at University of Alabama, Birmingham (Grant CA13148).

**REFERENCES**

1. Kempson, G. E., and Muir, H., Swanson, S. A. V., and Freeman, M. A. R. (1970) Biochim. Biophys. Acta 215, 70–77

B. M. Vertel, unpublished findings.
A Monoclonal Antibody to Keratan Sulfate

2. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4232–4241
3. Hardingham, T. E., and Muir, H. (1974) Biochim. Biophys. Acta 344, 565–581
4. Oegema, T. R., Jr., Hascall, V. C., and Dziewiatkowski, D. D. (1975) J. Biol. Chem. 250, 6151–6159
5. Kimura, T. H., Hardingham, T. E., Hascall, V. C., and Solursh, M. (1979) J. Biol. Chem. 254, 2600–2609
6. Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H., and Heinegård, D. (1980) J. Biol. Chem. 255, 6084–6091
7. Mitchell, D., and Hardingham, T. E. (1982) Biochem. J. 202, 387–395
8. Faltz, L. L., Reddi, A. H., Hascall, V. C., Ford, J. D., and Baker, J. R. (1978) J. Biol. Chem. 253, 2399–2407
9. Heinegård, D., and Axelsson, J. (1977) Biochem. J. 165, 1487–1493
10. Gregory, J. D. (1979) Cancer Res. 39, 25–41
11. Oegema, T. R., Jr., Brown, M., and Dziewiatkowski, D. D. (1977) J. Biol. Chem. 252, 4250–4256
12. Rosenberg, L., Hellmann, W., and Kleinenschmidt, A. K. (1975) J. Biol. Chem. 250, 1877–1883
13. Thonar, E. J., and Sweet, M. B. E. (1979) Hybridomas (Hammerling, G., ed) pp. 259–267, Elsevier, New York
14. Oegema, T. R., Jr., Brown, M., and Dziewiatkowski, D. D. (1974) J. Biol. Chem. 249, 4250–4256
15. Rosenberg, L., Hellmann, W., and Kleinenschmidt, A. K. (1975) J. Biol. Chem. 250, 1877–1883
16. Thonar, E. J., and Sweet, M. B. E. (1979) J. Biol. Chem. 254, 2387–2393
17. Caterson, B., and Baker, J. R. (1979) J. Biol. Chem. 254, 2393–2399
18. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4250–4256
19. Hascall, V. C., and Heinegård, D. (1977) J. Biol. Chem. 252, 4670–4677
20. Wieslander, J., and Heinegård, D. (1979) Biochem. J. 179, 35–45
21. Caterson, B., Baker, J. R., Levitt, D., and Paslay, J. W. (1979) J. Biol. Chem. 254, 9369–9372
22. Wieslander, J., and Heinegård, D. (1980) Biochem. Biophys. Res. Commun. 80, 496–503
23. Oegema, T. R., Jr., Brown, M., and Dziewiatkowski, D. D. (1977) J. Biol. Chem. 252, 1971–1979
24. Christner, J. E., Caterson, B., and Baker, J. R. (1980) J. Biol. Chem. 255, 7102–7105
25. Köhler, G., and Milstein, C. (1975) Nature (Lond.) 256, 495–497
26. Upholt, W. B., Vertel, B. M., and Dorfman, A. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4847–4851
27. Jenkins, R. B., Hall, T., and Dorfman, A. (1981) J. Biol. Chem. 256, 8279–8282
28. Caterson, B., Baker, J. R., Christner, J. E., Kearney, J. F., and Stohrer, R. C. (1981) in Monoclonal Antibodies and T-cell Hybridomas (Hammerling, G. J., Hammerling, U., and Kearney, J. F., eds) pp. 259–267, Elsevier, New York
29. Cullen, S. E., and Schwartz, B. D. (1978) J. Immunol. 117, 136–142
30. Rodén, L., Baker, J. R., Cifonelli, J. A., and Mathews, M. B. (1972) Methods Enzymol. 28, 73–140
31. McKibbon, J. M. (1978) J. Lipid Res. 19, 131–147
32. Bitter, T., and Muir, H. (1962) Anal. Biochem. 4, 330–334
33. Ford, J. D., and Baker, J. R. (1978) Anal. Biochem. 84, 559–550
34. Silvestri, L. J., Hurst, R. E., Simpson, L., and Settine, J. M. (1982) Anal. Biochem. 123, 303–309
35. Baker, J. R., and Caterson, B. (1979) J. Biol. Chem. 254, 2387–2393
36. Hascall, D. (1972) Biochim. Biophys. Acta 285, 193–207
37. Kearney, J. F., Radbruch, A., Liesegard, B., and Rajewsky, K. (1979) J. Immunol. 123, 1548–1550
38. Kearney, J. F., Barletta, R., Quan, Z. S., and Quintans, J. (1981) Eur. J. Immunol. 11, 877–883
39. Littlefield, J. W. (1964) Science (Wash. D. C.) 145, 709–710
40. McCarthy, M. M. U., and Baker, J. R. (1979) Carbohydr. Res. 69, 151–154
41. Baker, J. R., Caterson, B., and Christner, J. E. (1982) Methods Enzymol. 83, 216–234
42. Ey, P. L., Prowse, S. J., and Jenkins, C. R. (1978) Immunchemistry 15, 429–436
43. Sonada, S., and Schlamowitz, M. (1970) Immunochemistry 7, 885–898
44. Clamp, D. A., Tsai, C-M., and Ginsburg, V. (1979) in Carbohydrate-Protein Interaction (Goldstein, I., ed) pp. 990–101, American Chemical Society, Washington, D. C.
45. Conrad, G. W., Ager-Johnson, P., and Woo, M-L (1982) J. Biol. Chem. 257, 464–471
Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. Monoclonal antibodies to cartilage proteoglycan.

B Caterson, J E Christner and J R Baker

J. Biol. Chem. 1983, 258:8848-8854.