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Studies of a monoclonal antibody to skeletal keratan sulphate

Importance of antibody valency

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A mouse monoclonal antibody (AN9P1) to keratan sulphate is described. In a competitive-inhibition solution-phase radioimmunoassay employing ^{125}I-labelled intact proteoglycan, it reacts preferentially with keratan sulphate bound to the core protein of adult human articular-cartilage proteoglycan and to a much lesser degree with keratan sulphate purified from this proteoglycan. Proteolytic cleavage of the proteoglycan by pepsin and trypsin has little effect on antibody binding, but treatment with papain decreases binding considerably and more than does treatment with keratanase. An even greater decrease in binding is observed after treatment with alkaline borohydride. A comparison of binding of antibody AN9P1 with that of another previously described monoclonal antibody, 1/20/5-D-4, to keratan sulphate [Caterson, Christner & Baker (1983) J. Biol. Chem. **258**, 8848–8854] revealed similar binding characteristics, both showing much diminished binding after papain digestion of proteoglycan and even less with purified skeletal keratan sulphate. Removal of the Fc piece of antibody AN9P1 had no significant effect on the differential binding of divalent F(ab')$_2$ fragment to proteoglycan, to papain-digested proteoglycan and to keratan sulphate, although there was a small decrease in binding to papain-digested proteoglycan. Conversion of the antibody into univalent Fab fragment with removal of the Fc piece resulted in diminished binding to proteoglycan, compared with that observed with IgG, and in enhanced binding to free keratan sulphate and to papain-digested proteoglycan. These results suggest that close proximity of keratan sulphate chains on the core protein of proteoglycans favours preferential reactivity of bivalent antibody with these species through cross-bridging of chains by antibody. Conversely, much decreased binding to keratan sulphate on proteoglycan core-protein fragments and to free keratan sulphate results from a lack of close proximity of keratan sulphate. By using univalent Fab fragment in these assays these differences in binding are minimized by preventing cross-bridging and thereby enhancing detection of smaller fragments without sacrificing too much sensitivity of detection of larger proteoglycan species. The persistent preferential binding of Fab fragment to proteoglycan is probably in part the result of the increased epitope density in the intact molecule compared with keratan sulphate in a more disperse form.

INTRODUCTION

The high-buoyant-density proteoglycans of cartilage matrix display a complex structural organization that changes with age (Roughley & Mort, 1986; Poole, 1986; Webber *et al.*, 1987). The advent of the use of polyclonal and monoclonal antibodies for the study of these molecules has enabled us to gain further insight into their chemical structures when these are used in conjunction with more traditional biochemical approaches. The use of antibodies now permits us to detect very small amounts of these molecules, to assay them in body fluids and to study biosynthesis, differences in their structural organization, their interaction with other molecules and their distribution and organization in tissues (Wieslander & Heinegård, 1979; Poole *et al.*, 1980*a*,*b*, 1982; Kimura *et al.*, 1981; Buckwalter *et al.*, 1982; Thonar *et al.*, 1982; Ratcliffe *et al.*, 1984; Glant *et al.*, 1986*b*; Witter *et al.*, 1987; Webber *et al.*, 1987).

Antibodies have been prepared that react with the hyaluronic acid-binding region (Thonar *et al.*, 1982; Ratcliffe & Hardingham, 1983; Stevens *et al.*, 1985; Witter *et al.*, 1987), with epitopes on the protein core where the glycosaminoglycans chondroitin sulphate and keratan sulphate are attached (Glant *et al.*, 1986*a*) and with chrondroitin sulphate (Christner *et al.*, 1980; Jenkins *et al.*, 1981; Couchman *et al.*, 1984) and hyaluronic acid (Poole *et al.*, 1985) once these glycosaminoglycans have been cleaved by eliminases, exposing immunoreactive 4,5-unsaturated glucuronosyl residues at the non-reducing ends. Antibodies have also been prepared that react with keratan sulphate attached to core proteins of both cartilage proteoglycans (Caterson *et al.*, 1983; Zanetti *et al.*, 1985; Glant *et al.*, 1986*a*) and corneal proteoglycans (Conrad *et al.*, 1982; Fudenburgh *et al.*, 1983; SundarRaj *et al.*, 1985). Of those that react with the cartilage molecule, it has been reported that these also recognize keratan sulphate, or oligosaccharides thereof, isolated from the corneal keratan sulphate proteoglycan (Caterson *et al.*, 1983; SundarRaj *et al.*, 1985; Zanetti *et al.*, 1985). There has been one published study demonstrating that these monoclonal antibodies react with purified keratan sulphate that has been isolated from cartilage proteoglycan (Mehmet *et al.*, 1986). In this case reactivity of antibody 1/20/5-D-4 with skeletal keratan sulphate was shown to be much less than that with

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corneal keratan sulphate, whereas antibodies 1-B-4 and MZ15 showed more similar reactivities. But no studies have been made of the importance of antibody valency in binding to keratan sulphate and whether the Fc piece influences binding in any way. Here we describe a monoclonal antibody AN9P1 that reacts with keratan sulphate of high-buoyant-density cartilage proteoglycans. It resembles another monoclonal antibody to keratan sulphate, 1/20/5-D-4 (Caterson *et al.*, 1983), in that it reacts well with skeletal keratan sulphate only when it is bound to core protein, in contrast with earlier indications (Thonar *et al.*, 1985). Also, we show that binding of antibody AN9P1 to keratan sulphate is not enhanced when the Fc part of the immunoglobulin is removed but binding to the keratan sulphate of cartilage proteoglycans is dependent upon the bivalency of the antibody.

MATERIALS AND METHODS

Preparation of human cartilage proteoglycans

Source of tissue. Human articular-cartilage proteoglycans were obtained from the distal femoral condyles of fetuses and adults at autopsy, within 12 h of death, from individuals in whom there was no macroscopic evidence of joint trauma, connective-tissue abnormality or arthritic diseases (Roughley *et al.*, 1982).

Extraction and purification. Proteoglycan monomers were prepared from an A1 preparation from which an A1D1 preparation of buoyant density greater than 1.54 g/ml was isolated by the methodology described by Roughley *et al.* (1982). Adult dog articular-cartilage and rabbit chondrocostal-cartilage proteoglycan were also prepared as A1D1 preparations. Swarm rat chondrosarcoma proteoglycan monomers were isolated as a D1 preparation (Roughley & White, 1980). Proteoglycan monomer preparations were dialysed at 4 °C, twice against 100 vol. of water, once against 100 vol. of 100 mM-sodium acetate or -potassium acetate, pH 6.0, then exhaustively against water, before being freeze-dried. The dried materials were stored desiccated at 4 °C. Bovine nasal-cartilage proteoglycan monomer (A1D1) was generously donated by Dr. L. C. Rosenberg (Montefiore Hospital and Medical Center, Bronx, NY, U.S.A.). Keratan sulphate proteoglycan isolated from bovine cornea (Axelsson & Heinegård, 1978) was generously donated by Dr. T. Glant (Glant *et al.*, 1986*a*).

Preparation of proteoglycan fragments and keratan sulphate

Chondroitin ABC lyase digestion. Proteoglycan monomer at 20 mg/ml in 0.1 M-sodium acetate/0.1 M-Tris/HCl buffer, pH 7.3, containing 1 mM-EDTA, 1 mM-iodoacetic acid, 1 mM-phenylmethanesulphonyl fluoride and 5 μg of pepstatin/ml, to inhibit contaminating proteinases in the enzyme preparation, was digested with chondroitin ABC lyase (Miles Scientific, Naperville, IL, U.S.A.) at 0.01 unit/mg of proteoglycan at 37 °C for 24 h.

Keratanase digestion. Proteoglycan monomer at 10 mg/ml in 80 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-EDTA, 1 mM-iodoacetic acid, 1 mM-phenylmethanesulphonyl fluoride and 5 μg of pepstatin/ml, was digested with endo-β-D-galactosidase (keratanase, from *Pseudomonas* sp.; Miles Scientific) at a concentration of 1 unit/ml at 37 °C for 24 h.

Testicular-hyaluronidase digestion. Proteoglycan monomer dissolved at 20 mg/ml in 150 mM-NaCl/0.1 M-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA, 1 mM-iodoacetic acid, 1 mM-phenylmethanesulphonyl fluoride and 5 μg of pepstatin/ml, was digested with testicular hyaluronidase (bovine type VI; Miles Scientific) at 240 units/mg of proteoglycan at 37 °C for 24 h.

Endoglycosidase F digestion. Adult human proteoglycan monomer was dissolved at 1 mg/ml in 100 mM-sodium phosphate buffer, pH 6.1, containing 50 mM-EDTA, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Endoglycosidase F (New England Nuclear, Boston, MA, U.S.A.) was added in the same buffer at 6 units/mg of proteoglycan. After incubation at 37 °C for 24 h, the solution was boiled for 3 min to inactivate the enzyme.

Pepsin, papain and trypsin digestion. Proteoglycan monomer was dissolved at 2 mg/ml in 0.2 M-sodium acetate buffer, pH 5.0 (for pepsin digestion), in the same buffer containing 5 mM-EDTA and 5 mM-cysteine (for papain digestion), or in 50 mM-Tris/HCl buffer, pH 7.5 (for trypsin digestion). Papain (2× crystallized; ICN Pharmaceuticals, Irvine, CA, U.S.A.), pepsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) or trypsin [L-tosylphenylalanylchloromethane-('TPCK'-)treated; Worthington Biochemical Corp.] in the appropriate buffer was added at 10 μg/mg of proteoglycan, at the start of incubation and again after 4 h. After incubation for a total of 24 h at 37 °C, papain was inhibited by the addition of iodoacetamide to a final concentration of 10 mM, pepsin was inhibited by the addition of pepstatin to a final concentration of 10 μg/ml, and trypsin was inhibited by the addition of soya-bean trypsin inhibitor (type II-S; Sigma Chemical Co., St. Louis, MO, U.S.A.) at 10 μg/mg of proteoglycan. In all these studies enzyme-free controls were also incubated under the conditions described.

Alkaline-borohydride digestion. Alkaline-borohydride digestion (Carlson, 1976) was used to release intact *O*-linked glycosaminoglycan chains and *O*-linked oligosaccharides from the core protein. Proteoglycan monomer (2 mg) was dissolved in 800 μl of 50 mM-NaOH/1 M-$NaBH_4$, and incubated for 48 h at 45 °C. The solution was then neutralized by addition of 100% acetic acid.

Purification of keratan sulphate

Preparation of keratan sulphate. Adult (73-year-old) articular-cartilage proteoglycan (116 mg) was dissolved in 0.2 M-Tris/HCl buffer, pH 7.35, at 8 mg/ml. It was digested for 20 h at 37 °C with chondroitin ABC lyase at 1 unit/20 mg of proteoglycan. After dialysis against water to remove oligosaccharides, the solution was concentrated to a total volume of 9 ml with a YM5 membrane (Amicon), and adjusted to 50 mM-NaOH. $NaBH_4$ was added to 1 M and the solution was incubated at 45 °C for 48 h. The solution was neutralized with acetic acid, and 5 ml was chromatographed on a column (97 cm × 2.5 cm) of Sephacryl S-200 (Pharmacia, Montreal, Quebec, Canada) at a flow rate of 25 ml/h with 400 mM-NaCl/0.1 M-Tris/HCl buffer, pH 7.5. Frac-

tions (5 ml) were monitored for uronic acid, galactose and A_{214}. The initial galactose-containing peak was concentrated to 20 ml with a YCO5 membrane, and 5 ml was desalted on a column (26 cm × 1 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) at a flow rate of 5 ml/h. The column was monitored for A_{214}. A yield of 23 mg of keratan sulphate was obtained, representing approx. 20% keratan sulphate by weight of proteoglycan. Further analysis revealed a sulphate/galactose molar ratio of 0.92:1.

Assays for uronic acid, galactose and sulphate. Uronic acid was determined by the carbazole assay (Bitter & Muir, 1962). Galactose was determined as neutral sugar by the anthrone assay (Dische, 1962). Sulphate was measured as described by Terho & Hartiala (1971).

Chromatographic analysis on Sepharose CL-4B of purified keratan sulphate and proteolytic digests of adult human cartilage proteoglycan

Native human adult proteoglycan (1 mg), a pepsin digest (3 mg), a papain digest (4 mg) and purified keratan sulphate (3 mg) were chromatographed on Sepharose CL-4B columns (115 cm × 1 cm) in 4 M-guanidinium chloride with 50 M-Tris/HCl buffer, pH 7.3, at a flow rate of 6 ml/h. Fractions were dialysed against 0.2 M-sodium acetate buffer, pH 5.5, and analysed by radioimmunoassay with antibody AN9P1.

Production, purification and characterization of monoclonal antibody AN9P1

Immunization. Adult human articular cartilage proteoglycan (A1D1) was dissolved at 20 mg/ml in 0.1 M-sodium acetate/0.1 M-Tris/HCl buffer, pH 7.3. Eight-week-old female BALB/c mice were injected on day 0 with a total of 120 μg of proteoglycan, emulsified in 300 μl of 50% (v/v) Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) in water, and on day 4 with the same amount emulsified in 300 μl of 50% (v/v) Freund's incomplete adjuvant (Difco) in water. Subsequent injections of 120 μg in 300 μl of 145 mM-NaCl were given on days 7, 29 and 33. A final injection of 200 μg in 500 μl of 145 mM-NaCl was given 8 weeks later. All injections were administered subcutaneously at multiple sites on the back and limbs, except for the final booster, which was given intraperitoneally.

Hybridoma generation. The BALB/c-mouse-derived plasmacytoma line SP2/0.Ag 14 (Shulman *et al.*, 1978) was used. It was kindly supplied by Dr. A. Fuks of the McGill University Cancer Centre. The culture of these cells and the fusion procedure have been described previously (Rosenberg *et al.*, 1985). Cells were cloned by limiting dilution. A monoclonal antibody AN9P1 was selected for study. The hybridoma cells were injected into primed BALB/c mice, where they grew as ascites tumours (Rosenberg *et al.*, 1985). Control ascitic fluid (containing no proteoglycan-specific antibodies) was produced in the same manner by injecting cells of the parent plasmacytoma line. Ascitic fluids were harvested 1–3 weeks after injection, cells were separated by centrifugation at 600 *g* for 10 min, and fluids were stored at −20 °C.

Immunoglobulin purification. Immunoglobulins were concentrated and partially purified from ascitic fluid by precipitation with an equal volume of saturated $(NH_4)_2SO_4$ (Tang *et al.*, 1979). Protein A–Sepharose affinity chromatography, based on the method of Ey *et al.* (1978), modified by Watanabe *et al.* (1981), was used to isolate monoclonal mouse IgG and to provide an initial indication of immunoglobulin class and subclass. Protein A–Sepharose was obtained from Pharmacia. Briefly, immunoglobulin was dialysed into 0.14 M-phosphate buffer, pH 8.0, and a 500 μl sample was applied to a Protein A–Sepharose column of 4 ml bed volume equilibrated with this buffer. Unbound material was eluted. Bound immunoglobulin was eluted with 0.1 M-sodium citrate buffer, pH 3.0, followed by rapid neutralization with NaOH.

Determination of monoclonal IgG subclass. The subclass of the purified AN9P1 IgG was determined as IgG_{2a} by Ouchterlony immunodiffusion in 0.7% agarose in phosphate-buffered saline (Poole *et al.*, 1980*b*), against subclass-specific antisera (Miles Laboratories, Napierville, IL, U.S.A.).

Antibody 1/20/5-D-4. This antibody, which is an IgG_1, has been described in earlier work by Caterson *et al.* (1983). It was purchased from ICN (Montreal, Quebec, Canada).

Preparation of $F(ab')_2$ and Fab antibody subunits. $F(ab')_2$ fragment was prepared from antibody AN9P1, which is an IgG_{2a}, by digestion with pepsin as described by Parham (1983). Fab fragment was prepared from antibody AN9P1 by digestion of immunoglobulin with papain as described by Porter (1959). $F(ab')_2$ and Fab fragments were separated from undigested IgG and Fc fragments by chromatography of the digest on an affinity column of Protein A–Sepharose as described above.

Immunoassays

E.l.i.s.a. This was used for identifying antibody in hybridoma cultures. It was performed with adult human articular-cartilage proteoglycan digested with chondroitin ABC lyase in 0.1 M-Tris/acetate buffer, pH 7.3 (Poole *et al.*, 1985), diluted in 0.1 M-sodium carbonate buffer, pH 9.0, and bound to 96-well Immulon plates (Dynatech, South Windham, ME, U.S.A.) at a concentration of 2.5 μg/well. The binding and assay were conducted as previously described (Poole *et al.*, 1985) with the use of hyperimmune IgG (from a pig immunized with mouse IgG) coupled to alkaline phosphatase.

Radioimmunoassay. Human proteoglycan monomer was iodinated by using the chloramine-T method (Sonada & Schlamowitz, 1970). A 100 μl portion of a 1 mg/ml proteoglycan solution in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.5, was combined in a glass test tube with 0.25 mCi of $Na^{125}I$ (New England Nuclear) in 5 μl. Then 10 μl of chloramine-T (600 μg/ml) was added, the tube was capped, and the solution was gently vortex-mixed for exactly 2 min. Then 100 μl of sodium metabisulphite (1.2 mg/ml) and 200 μl of NaI (10 mg/ml) were added. Iodinated proteoglycan was separated from free I_2 by chromatography on a Sephadex G-25 (Pharmacia) column (10 ml bed volume) equilibrated with iodination buffer. Void-volume fractions containing greater than 40000 c.p.m./10 μl were pooled for use in the radioimmunoassay.

For all radioimmunoassays, other than those dealing

with comparative binding of antibody IgG, $F(ab')_2$ fragment and Fab fragment to keratan sulphate, which are described in Fig. 3 and Table 4, an assay with Protein A was employed. The buffer was 0.15 M-sodium phosphate buffer, pH 8.1, containing 1% (w/v) bovine serum albumin, 0.5% (w/v) sodium deoxycholate, 0.25% (v/v) Nonidet P-40 and 0.02 (w/v) NaN_3. Since the affinity of Protein A for each mouse IgG subclass varies with pH (Ey *et al.*, 1978), a buffer pH of 8.1, at which all mouse IgG subclasses are bound, was used. Antibodies were serially diluted in radioimmunoassay buffer to give approx. 50% of the total binding of radiolabelled proteoglycan, and 100 μl of each dilution (in duplicate) was then combined with 100 μl of ^{125}I-labelled proteoglycan antigen (10000 c.p.m.) and incubated at room temperature for 90 min. Then 50 μl of a 10% (w/v) suspension of heat- and formalin-killed Protein A-bearing *Staphylococcus aureus* (Zymed; Cedarlane Laboratories, Hornby, Ont., Canada) was added, and the mixture was incubated at room temperature for 20 min. Then 1 ml of buffer was added and the suspension was centrifuged at 4000 *g* for 20 min at 4 °C. The supernatant was discarded and the radioactivity of the pellet was counted to determine bound radioactivity.

The radioimmunoassay procedure used in the studies reported in Fig. 3 and Table 4, where binding of IgG_{2a}, $F(ab')_2$ fragment and Fab fragment were compared, employed a triple-antibody method. To the radiolabelled proteoglycan (approx. 10000 c.p.m. per tube) in 150 μl of radioimmunoassay buffer (10 mM-sodium/potassium phosphate buffer, pH 7.2, containing 147 mM-NaCl, 0.05% NaN_3 and 0.2% radioimmunoassay-grade bovine serum albumin) is added 50 μl of the sample to be assayed (standard or unknown) in radioimmunoassay buffer and 50 μl of antibody to keratan sulphate as IgG or $F(ab')_2$ fragment or Fab fragment diluted in radioimmunoassay buffer to give approx. 50% of maximal binding of ^{125}I-labelled proteoglycan. The mixture is incubated for 1 h at 37 °C. Then 25 μl of a hyperimmune rabbit antiserum to mouse IgG (Cappel) diluted 1/16 in radioimmunoassay buffer is added and the incubation at 37 °C is continued for 1 h. Then 25 μl of non-immune rabbit serum (diluted 1/16 in radioimmunoassay buffer) is added followed by 100 μl of an $(NH_4)_2SO_4$-concentrated preparation of pig immunoglobulin to rabbit F(ab') fragment (prepared from a hyperimmune serum as described by Poole *et al.*, 1985). After overnight incubation for 18 h at 4 °C, 1 ml of radioimmunoassay buffer was added, the assay tubes were centrifuged and the radioactivities of the pellets were counted. Assays were performed in tripicate.

The percentage inhibition of binding was calculated relative to the amount of ^{125}I-labelled proteoglycan bound in the absence of unlabelled competing antigen according to the formula:

$$\text{Percentage inhibition} = 100 - 100 \times \frac{\text{c.p.m. bound in presence of inhibitor}}{\text{c.p.m. bound in absence of inhibitor}}$$

By reference to a standard curve prepared with native adult proteoglycan monomer, μg equivalents of native proteoglycan were determined where indicated. For further details, reference should be made to Poole *et al.* (1985), where a similar assay was employed.

Table 1. Binding of monoclonal antibody AN9P1 to cartilage proteoglycans

Amounts of proteoglycan per total assay volume required to achieve 50% inhibition of binding of monoclonal antibody AN9P1 to ^{125}I-labelled native adult human proteoglycan are shown.

Proteoglycan	Amount required for 50% inhibition (μg)
Adult human articular-cartilage A1D1	0.32
Fetal human articular-cartilage A1D1	50
Adult rabbit articular-cartilage A1D1	0.20
Adult dog articular-cartilage A1D1	0.85
Adult bovine nasal-cartilage A1D1	10
Rat chondrosarcoma D1	30*
Bovine corneal keratan sulphate proteoglycan	0.15

* Rat chondrosarcoma D1 produced no inhibition up to 30 μg and is hence considered to contain no immunoreactive epitopes.

RESULTS AND DISCUSSION

Binding of antibody AN9P1 to cartilage proteoglycans

Effects of enzymic and chemical treatments of proteoglycan on immunoreactivity and comparison with antibody 5D4. A comparison of binding of antibody AN9P1 to different proteoglycans was made by determining the amount of proteoglycan required to achieve 50% inhibition of binding to radiolabelled adult human cartilage proteoglycan (Table 1). Most binding was seen with adult human, rabbit and dog articular-cartilage proteoglycans and with bovine corneal keratan sulphate. Less binding was observed with bovine nasal-cartilage proteoglycan and even less with fetal human cartilage proteoglycan. No binding was seen with rat chondrosarcoma proteoglycan, since there was no inhibition with up to 30 μg of proteoglycan/ml. These observations indicated that the epitope recognized by this antibody was most common in proteoglycans of adult articular cartilages of different species, deficient in the fetus and absent from the rat chondrosarcoma. However, the epitope was present on the keratan sulphate-containing proteoglycan isolated from the bovine cornea. This observation, combined with the fact that keratan sulphate is deficient in or absent from cartilage proteoglycans isolated from fetal human cartilage and most commonly found in adult human cartilage (Roughley & White, 1980), and is absent from the rat chondrosarcoma (Choi *et al.*, 1971; Oegema *et al.*, 1975), suggested that this antibody reacted with keratan sulphate and resembled antibody 1/20/5-D-4 described by Caterson *et al.* (1983).

Adult human cartilage proteoglycan was subjected to various enzymic and chemical treatments to characterize antibody binding further. The molecular sizes of some of these degradation products were analysed by chromatography on Sepharose CL-4B (Fig. 1). Intact proteoglycan was eluted at the void volume. Pepsin-digest fragments were intermediate in size between native proteoglycan and papain-digest fragments. Free keratan sulphate chains were eluted after the papain-digest fragments, indicating that the latter are composed of small clusters of two or more keratan sulphate chains attached to core protein. Radioimmunoassays of adult human cartilage

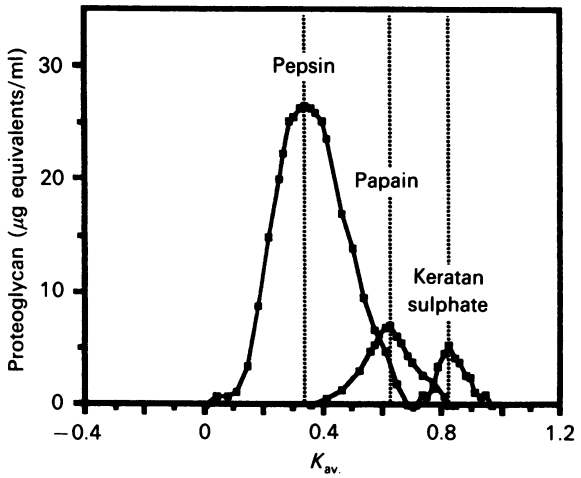

Fig. 1. Chromatography on Sepharose CL-4B of native adult human proteoglycan (eluted at V_0; results not shown), pepsin and papain digests of this molecule and purified keratan sulphate

Fractions were analysed by radioimmunoassay with antibody AN9P1.

proteoglycan treated with these and other degradative agents are shown in Table 2. They reveal that removal of chondroitin sulphate by chondroitin ABC lyase and treatment with endoglycosidase F, which cleaves high-mannose-type and complex-type *N*-linked oligosaccharides (Elder & Alexander, 1982), have no effect on binding. They also show that limited cleavages of core protein by pepsin and trypsin produce little decrease in binding. But, when the molecule is cleaved by papain, which is thought to produce single chondroitin sulphate chains (Roughley, 1978), there is a considerable decrease in binding. The greatest decrease was seen after alkaline-borohydride treatment, which removes *O*-linked oligosaccharides, keratan sulphate and chondroitin sulphate from core protein and cleaves core protein (Lohmander *et al.*, 1980). The observation that keratanase produced a significant but lesser decrease in binding indicated that the epitope may reside on keratan sulphate. The limited decrease in reactivity after keratanase digestion may reflect the continued reactivity of keratan sulphate 'stubs' left attached to core protein.

Table 2. Effect of various treatments on binding of monoclonal antibody AN9P1 to human adult cartilage proteoglycan

Amounts of adult human proteoglycan per assay volume required to achieve 50% inhibition of binding to native adult human proteoglycan are shown. Control samples for each treatment were treated exactly as the test samples, except that the enzyme was inactivated or the alkaline borohydride was neutralized before incubation with the proteoglycan.

Treatment	Amount required for 50% inhibition (μg)
None	0.25
Chondroitin ABC lyase	0.25
Endoglycosidase F	0.25
Keratanase	1.2
Pepsin	0.35
Trypsin	0.35
Papain	7.5
Alkaline borohydride	20.0

The decrease in binding observed with papain and alkaline-borohydride treatments also indicated that antibody binding to cartilage proteoglycan may be dependent upon the close proximity of keratan sulphate chains that results when they are bound to the core protein of the proteoglycan. More detailed studies were therefore made of antibody binding to adult human articular-cartilage proteoglycan after some of these treatments; binding to purified keratan sulphate was also studied. Earlier work on monoclonal antibody 1/20/5-D-4, which reacts with keratan sulphate of skeletal and non-skeletal origin, indicates that this antibody reacts equally well with free skeletal keratan sulphate as with keratan sulphate bound to cartilage proteoglycan, although only corneal keratan sulphate and trypsin-digest fragments of cartilage proteoglycans were studied (Caterson *et al.*, 1983). For this reason, and to establish the similarity of antibody AN9P1 to antibody 1/20/5-D-4, we decided to compare the binding of antibody 1/20/5-D-4 with that of AN9P1. These results are shown in Fig. 2. It can be seen that both antibodies displayed very similar inhibition profiles with all proteoglycan preparations, and a much diminished reaction after papain cleavage. Moreover, both antibodies reacted with purified keratan sulphate but to a much lesser degree than with intact proteoglycan. The decreased binding was similar to that observed after treatment of proteoglycan with alkaline borohydride, which frees keratan sulphate from core protein. Table 3 shows the amounts required to achieve 50% inhibition of binding for each antibody.

Our results clearly demonstrate in this radioimmunoassay that both antibody AN9P1 and antibody 1/20/5-D-4 react with skeletal keratan sulphate but that binding is considerably enhanced when keratan sulphate is part of a cluster of chains attached to core protein.

Dependence of antibody binding on bivalency. The indications of antibody binding being dependent upon binding of keratan sulphate to core protein raised the question whether binding was also antibody-valency-dependent and modified by removal of the Fc piece. In Fig. 3 binding of AN9P1 antibody IgG and $F(ab')_2$ and Fab subunits to adult human articular-cartilage proteoglycan, to papain-digested proteoglycan and to purified keratan sulphate are compared. In Table 4 are shown the amounts of proteoglycan, papain-digested proteoglycan and keratan sulphate required to achieve 50% inhibition of binding. The relative amounts are also indicated to permit a comparison of binding for each set of assays with IgG, $F(ab')_2$ fragment or Fab fragment. Removal of the Fc piece alone leaving bivalent $F(ab')_2$ fragment results in relatively little change in absolute and relative binding to proteoglycan and to keratan sulphate, indicating that the Fc piece does not influence binding of the Fab arms to epitopes. The binding to papain-digested fragments of proteoglycan does, however, show a small decrease. Conversion of antibody into univalent Fab with papain also results in removal of the Fc piece, but now binding to proteoglycan is decreased whereas binding to papain-digested proteoglycan and particularly to free keratan sulphate is increased compared with that of IgG.

These observations indicate that, when intact radio-

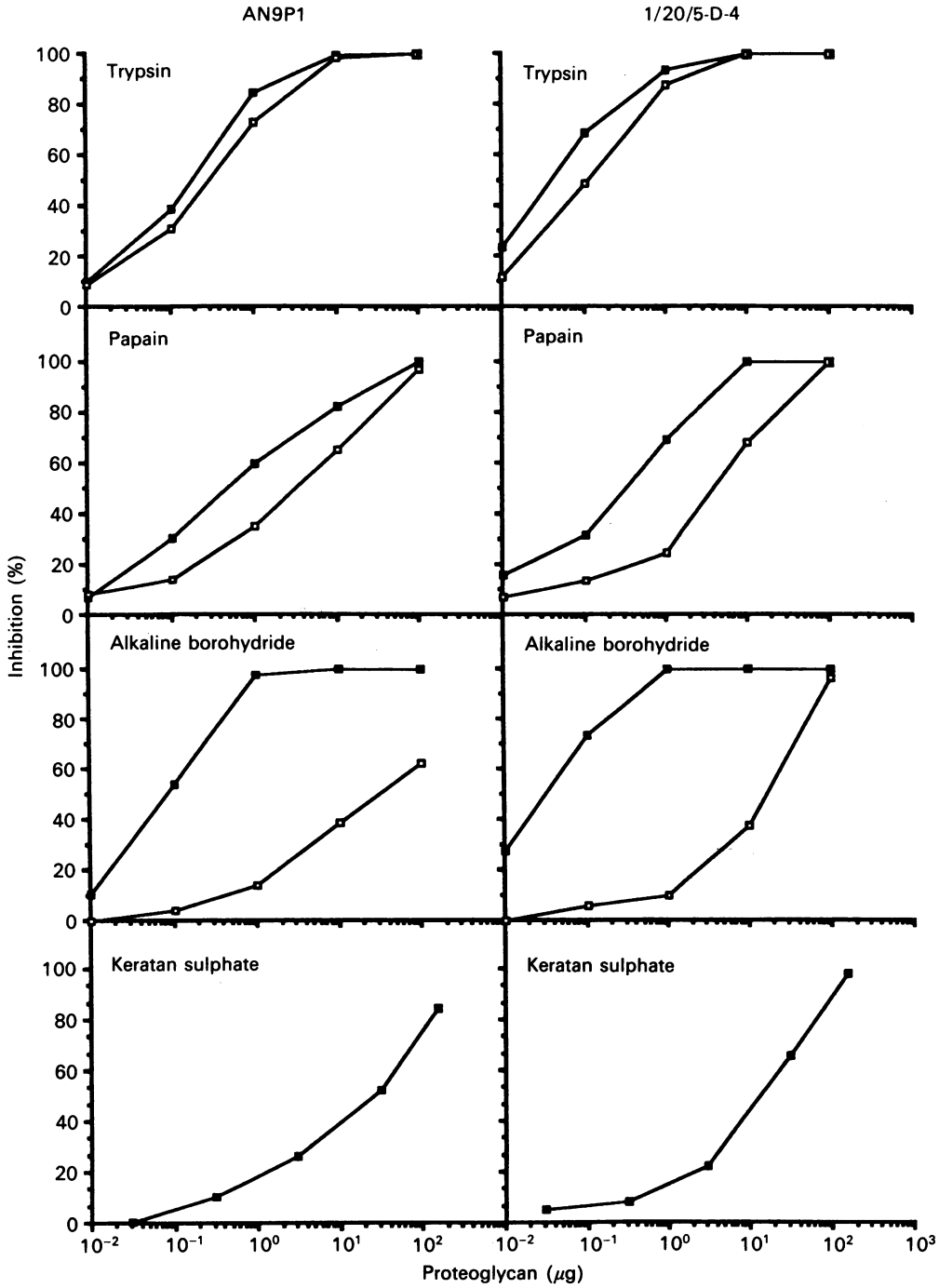

Fig. 2. Effects of enzymic and chemical treatments of adult human articular-cartilage proteoglycan on its capacity to inhibit binding of monoclonal antibodies AN9P1 and 1/20/5-D-4 to radiolabelled native adult human cartilage proteoglycan

Inhibition of binding by keratan sulphate is also shown. The amounts (μg) of proteoglycan per total assay volume in sample are shown. ■, Control; □, digest. Keratan sulphate concentration is recorded as the equivalent concentration of intact cartilage proteoglycan to permit a comparison of binding between these two molecular species. This conversion is based on the keratan sulphate content being 20% by weight of the total adult human proteoglycan as determined by our analyses (see the Materials and methods section).

labelled proteoglycan is used in the assay, the bivalent antibody preferentially cross-bridges adjacent keratan sulphate chains where these are present on proteoglycan core protein, leading to preferential binding to 'clusters' of keratan sulphate rather than to one or two chains of keratan sulphate attached to small fragments of core protein produced by papain digestion (Roughley, 1978) or to single keratan sulphate chains. The use of univalent Fab fragment negates the possibility of cross-bridging and ensures more uniform reactivity with keratan sulphate. The persisting preferential binding of antibody to keratan sulphate in intact proteoglycans probably in part results from the greater density of epitopes when keratan sulphate is attached to core protein than when it is in a

Table 3. Comparison of binding of antibodies AN9P1 and 1/20/5-D-4 to cartilage proteoglycan degradation products

Amounts of proteoglycan or keratan sulphate per total assay volume required to achieve 50% inhibition of binding of monoclonal antibodies to ^{125}I-labelled native adult human proteoglycan are shown. These data are derived from Fig. 2.

Treatment or product	AN9P1 antibody		1/20/5-D-4 antibody	
	Amount required for 50% inhibition (μg)	Relative amount	Amount required for 50% inhibition (μg)	Relative amount
No treatment	0.18	1.0	0.04	1.0
Trypsin	0.30	1.7	0.11	2.8
Papain	3.00	16.7	4.00	100
Alkaline borohydride	31.0	172	17.0	425
Keratan sulphate	25.0	139	13.0	325

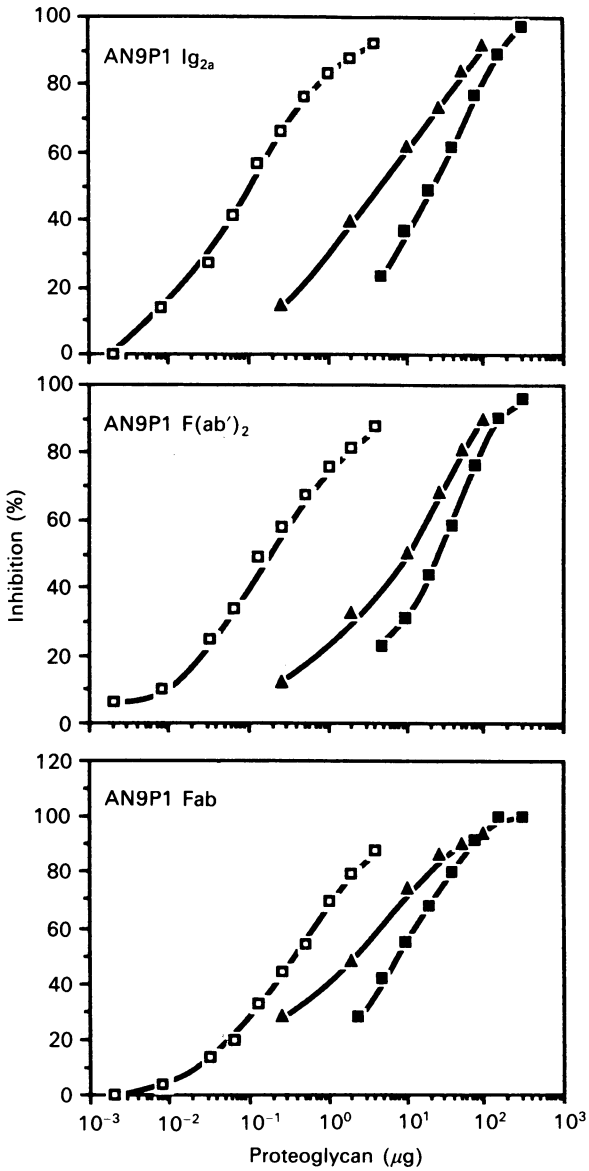

Fig. 3. Effects of removal of Fc piece and use of bivalent $F(ab')_2$ fragment and univalent Fab fragment in radioimmunoassay with antibody AN9P1 of native adult human articular-cartilage proteoglycan (□), papain-digested proteoglycan (▲) and purified skeletal keratan sulphate (■)

Other details are as described in the legend to Fig. 2.

more disperse form after core protein has been cleaved. The diminished slope of the inhibition curves for papain-digested proteoglycan (Fig. 3) compared with similar curves for keratan sulphate and intact proteoglycan also suggests a difference in the conformation of keratan sulphate on papain fragments with a decrese in reactivity with antibody. The further decrease in binding observed after alkaline-borohydride treatment to release free keratan sulphate from core protein would also be expected to result from destruction of clusters of keratan sulphate on papain fragments with a decrease in reactivity after papain cleavage, as suggested by the chromatographic data shown in Fig. 1. The possibility remains, however, that alkaline-borohydride treatment may lead to some desulphation of the keratan sulphate chain, and loss of sulphate might be expected to affect antibody reactivity. It has been shown that some of these monoclonal antibodies to keratan sulphate, including 1/20/5-D-4, bind to oversulphated keratan sulphate hexasaccharides (Mehmet *et al.*, 1986). However, alkaline hydrolysis of chondroitin sulphate under identical conditions maintains a sulphate/glucuronic acid ratio at 1:1 (results not shown).

These results clearly demonstrate that with assays of this kind there is a need to investigate antibody valency and compare antibody binding to free skeletal keratan sulphate as well as to keratan sulphate on degraded and intact cartilage proteoglycan. Previously, published studies have concentrated on analyses of antibody binding to corneal keratan sulphate, which reacts well in its free (non-protein form) for reasons not yet understood. When assaying mixtures of molecules, such as proteoglycan degradation products in body fluids or tissues, single or small clusters of keratan sulphate chains may be found that react differently with antibody. If intact immunoglobulin is employed, preferential reactivity would be observed with clusters of keratan sulphate: smaller molecular fragments of proteoglycan may go undetected. The use of univalent Fab fragment decreases the differences in reactivity between larger and small fragments bearing keratan sulphate, although differences in reactivity still exist, making accurate quantitative analyses of total keratan sulphate impossible. However, by using univalent Fab fragment, these differences would be minimized by enhancing detection of small fragments without too much sacrifice of sensitivity of detection of larger species. If sensitivity is not a limiting factor it would be desirable to convert all skeletal keratan sulphate into single chains in order to establish an accurate

Table 4. Comparison of binding of AN9P1 IgG, F(ab')$_2$ and Fab to cartilage proteoglycan degradation products

Amounts of proteoglycan or keratan sulphate per total assay volume required to achieve 50% inhibition of binding of monoclonal antibody as IgG or F(ab')$_2$ fragment or Fab fragment to ^{125}I-labelled native adult human proteoglycan are shown. These data are derived from Fig. 3.

	IgG_{2a}		F(ab')$_2$ fragment		Fab fragment	
Treatment or product	Amount (μg)	Relative amount	Amount (μg)	Relative amount	Amount (μg)	Relative amount
No treatment	0.1	1.0	0.14	1.0	0.38	1.0
Papain	4.4	44	10.0	71	2.4	6.3
Keratan sulphate	20.5	205	25.0	179	8.5	22.4

immunoassay of this glycosaminoglycan with antibodies of this kind. Since tissues and body fluids commonly contain smaller proteoglycan fragments produced by degradation (Witter *et al.*, 1987), the use of such assays employing univalent Fab fragment should represent a significant improvement on existing assays.

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