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Monoclonal antibodies to different protein-related epitopes of human articular cartilage proteoglycans

Tibor T. GLANT,*† Katalin MIKECZ‡ and A. Robin POOLE

Joint Diseases Laboratory, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada; Department of Experimental Surgery, McGill University, Montreal; and †Institute of Anatomy, Histology and Embryology and ‡Department of Internal Medicine 3, University Medical School, H-4012 Debrecen, Hungary

Monoclonal antibodies produced against chondroitinase-treated human adult cartilage proteoglycans were selected for their ability to recognize epitopes on native proteoglycans. Binding analyses revealed that four of these monoclonal antibodies (BCD-4, BCD-7, EFG-4 and KPC-190) each recognized a different epitope on the same proteoglycan molecule which represents a subpopulation of a high buoyant density (D1) fraction of human articular cartilage proteoglycans (10, 30, 50 and 60% in fetal-newborn, 1.5 years old, 15 years old and 52–56 years old cartilages, respectively). Analysis of epitope specificities revealed that BCD-7 and EFG-4 monoclonal antibodies recognized epitopes on proteoglycan monomer which are associated with the protein structure in that they are sensitive to cleavage by Pronase, papain and alkali treatment and do not include keratan sulphate, chondroitin sulphate or oligosaccharides. The BCD-4 and KPC-190 epitopes also proved to be sensitive to Pronase or papain digestion or to alkali treatment, but keratanase or endo-β-galactosidase also reduced the immunoreactivity of these epitopes. These observations indicate that the BCD-4 and KPC-190 epitopes represent peptides substituted with keratan sulphate or keratan sulphate-like structures. The BCD-4 epitope is, however, absent from a keratan sulphate-rich fragment of human adult proteoglycan, while the other three epitopes were detected in this fragment. None of these four epitopes were detected in the link proteins of human cartilage, in the hyaluronic acid-binding region of human newborn cartilage proteoglycan, in Swarm rat chondrosarcoma proteoglycan, in chicken limb bud proteoglycan monomer and in the small dermatan sulphate-proteoglycan of bovine costal cartilage. EFG-4 and KPC-190 epitopes were not detected in human fetal cartilage proteoglycans, although fetal molecules contained trace amounts of epitopes reactive with BCD-4 and BCD-7 antibodies.

INTRODUCTION

The basic structural unit of the cartilage proteoglycan monomer consists of a central protein core to which glycosaminoglycans and *N*- and *O*-linked oligosaccharides are covalently attached (Hascall, 1981; Hascall & Hascall, 1981; Hascall & Kimura, 1982). A specialized region of the proteoglycan core protein, the hyaluronic acid binding region, which is present at one end of the molecule (Buckwalter *et al.*, 1982) specifically binds to hyaluronic acid to form macromolecular aggregates (Hardingham & Muir, 1972; Hascall & Heinegård, 1974). The glycosaminoglycan attachment region contains both chondroitin sulphate and keratan sulphate. In bovine nasal cartilage, the chondroitin sulphate-rich region of the proteoglycan monomer, remote from the hyaluronic acid-binding region, contains a large number of chondroitin sulphate chains interspersed with relatively less keratan sulphate chains (Heinegård & Axelsson, 1977). The core protein, between the hyaluronic acid-binding region and the chondroitin sulphate-rich region, involves a short polypeptide region containing predominantly keratan sulphate chains, and called the keratan sulphate-rich region of the molecule (Heinegård & Axelsson, 1977).

Biochemical studies of the structure of proteoglycans were greatly facilitated by the introduction of improved purification techniques (Hascall & Sajdera, 1969) which permitted the production of polyclonal antisera to purified proteoglycans and their substructures (Keiser & DeVito, 1974; Wieslander & Heinegård, 1979; Poole *et al.*, 1980*a*; Kimura *et al.*, 1981; Glant & Lèvai, 1983; Pacifici *et al.*, 1983; Ratcliffe *et al.*, 1984). The failure to produce a detectable immune response against isolated chondroitin sulphate and keratan sulphate chains (Boake & Muir, 1955; Loewi & Muir, 1965; Sandson *et al.*, 1966) led to the speculation that the glycosaminoglycans of proteoglycans are non-antigenic. Removing the chondroitin sulphate chains either with testicular hyaluronidase or with chondroitinase ABC could, however, enhance the immunoreactivity of proteoglycans and these treatments often revealed 'new' antigenic sites which suggested that the antigenic determinants are closely associated with the protein core (Buckwalter *et al.*, 1982; Wieslander & Heinegård, 1979; Poole *et al.*, 1980*a*, *b*; Glant & Lèvai, 1983). It was then discovered that polyclonal antibodies to the unsaturated oligosaccharides of chondroitin sulphate could be produced by immunization with heterologous proteoglycans treated with chondroitinase AC or ABC (Christner *et al.*, 1980). Rabbit autoantibodies

Abbreviation used: e.l.i.s.a., enzyme-linked immunosorbent assay.

* To whom correspondence and reprint requests should be addressed to: Joint Diseases Laboratory, Shriners Hospital, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada.

to degraded glycosaminoglycans, including hyaluronic acid, also occur naturally in preimmune animals (Poole *et al.*, 1985).

Introduction of monoclonal antibody technology (Köhler & Milstein, 1975), with antibodies which can recognize single epitopes (antigenic determinants), has opened a new perspective both for the immunology of proteoglycans and for the study of proteoglycan structure. Monoclonal antibodies have been produced that can recognize saturated and unsaturated oligosaccharides of chondroitin sulphate (Caterson *et al.*, 1981; Jenkis *et al.*, 1981; Couchman *et al.*, 1984) and intact keratan sulphate (Caterson *et al.*, 1983; Fudenburgh *et al.*, 1983). There are also some reports of monoclonal antibodies which react with determinants associated with the proteoglycan core protein: two reports of antibodies to the hyaluronic acid-binding region (Caterson *et al.*, 1982; Stevens *et al.*, 1985) and another paper about the partial characterization of an antibody (Dorfman *et al.*, 1980) which reacts with the core protein of proteoglycan translated from mRNA by a cell-free protein-synthesizing system (Upholt *et al.*, 1979).

Here we describe monoclonal antibodies which can recognize epitopes associated with intact protein core in the chondroitin sulphate- and keratan sulphate-rich regions of human articular proteoglycans of high buoyant density.

MATERIALS AND METHODS

Materials and chemicals

Chemicals of analytical grade or the best commercially available grade were obtained from the following: clostripain (clostridiopeptidase B; EC 3.4.22.8), neuraminidase (type VI; EC 3.2.18), α-mannosidase (EC 3.2.1.24), pepsin (crystallized, EC 3.4.23.1) papain (2 × crystallized, type IV, EC 3.4.22.2), chymotrypsin [tosyl-lysine chloromethane ('TLCK')-treated, EC 3.4.21.1], horseradish peroxidase (type VI, EC 1.11.1.7), alkaline phosphatase (type VII-S, EC 3.1.3.1), β-*N*-acetylglucosaminidase B (EC 3.2.1.30), sulphatases (EC 3.1.6.1) from limpets (*Patella vulgata*, type V) and from *Abalone entrailes* (type VIII), trypsin inhibitor (type I-S), human γ-globulins, pepstatin A, bovine serum albumin, ovalbumin, α-methyl D-mannoside, guanidinium chloride and phenylmethanesulphonyl fluoride from Sigma Chemical Co.; chondroitinase ABC (EC 4.2.2.5), keratanase (lot No. 83101), endo-β-galactosidase from *Escherichia freundii*, endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus*, keratan sulphate, chondroitin, chondroitin sulphates (C-4-S, C-6-S, dermatan) from Miles Laboratories; Sephacryl S-200 superfine, Sepharose CL-2B and CL-6B, concanavalin A–Sepharose from Pharmacia Fine Chemicals; culture media, HAT and HT containing 50 × stock solutions and fetal calf serum from Flow Laboratories; tissue culture plates, dishes and immunoplates for e.l.i.s.a. from Flow and Nunc InterMed Laboratories (Kamstrup, Denmark); di-isopropyl fluorophosphate from Aldrich Chemical Co.; iodoacetamide and chloramine T from BDH; CNBr from American Chemicals Ltd.; CsCl from Accurate Chemical & Scientific Corp.; tosylphenylalanine chloromethane ('TPCK')-treated trypsin (EC 3.4.21.4) from Millipore Corp.; poly(ethylene glycol) 1000 from Hüls Chemical Corp.; Freund's complete and incomplete adjuvants from DIFCO; bovine fibronectin and Pronase (*Streptomyces griseus*) from Calbiochem–Behring; diethylaminoethyl-cellulose (DE-52), microgranular) from Whatman Chemical Separation Ltd; ^{125}I as sodium iodide in aqueous solution from New England Nuclear.

Isolation of cartilage proteoglycans

Human fetal (23–38 weeks gestation), newborn, maturing and adult articular cartilages (1.5–90 years), fetal calf articular and nasal cartilage, bovine articular and nasal cartilage, chicken, canine and rabbit articular cartilage, chicken limb bud cartilage and Swarm rat chondrosarcoma high buoyant density A1D1 and D1 proteoglycan fractions (Heinegård, 1972) were prepared as previously described (Tang *et al.*, 1979; Glant & Lèvai, 1983). Proteoglycans isolated from combined samples of eight human articular cartilages (age range, 52–56 years) were used both for immunization and as test materials in present experiment.

Cartilage slices and tissue homogenate of rat chondrosarcoma were extracted with 4 M-guanidinium chloride containing enzyme inhibitors (Roughley & White, 1980). Proteoglycan aggregate, fraction A1, was prepared by density gradient centrifugation under associative conditions in the presence of CsCl (Hascall & Sajdera, 1969). Aggregate was dissociated with 4 M-guanidinium chloride and recentrifuged in 4 M-guanidinium chloride to purify link protein from fractions A1D5-A1D6 (Tang *et al.*, 1979) and proteoglycan monomers of high buoyant density from fraction A1D1 (Glant, 1982*a*). High buoyant density proteoglycan monomers (fraction D1) were also obtained from 4 M-guanidinium chloride extract of cartilage by using dissociative density gradient centrifugation in 4 M-guanidinium chloride/3 M-CsCl for 60 h at 100000*g*.

Keratan sulphate-enriched fragments of proteoglycan core protein

These were prepared from bovine nasal cartilage (pooled A1D1 and A1D2 fractions of two 4-year-old steers) and human articular cartilage (pooled A1D2 fractions of three 66-year-old individuals) proteoglycans by the method described by Heinegård & Axelsson (1977). Bovine nasal cartilage proteoglycan (50 mg) and human proteoglycan (40 mg) digested with chondroitinase ABC were used for trypsin digestion prior to separation on Sepharose CL-6B. Keratan sulphate-containing fragments were recovered in the first peak of bovine nasal and in the second peak of human articular cartilage proteoglycans, which later were rechromatographed on DEAE-cellulose. They represented 0.9% and 2.3% of the total glucosamine content of bovine and humam proteoglycan monomer, respectively. The glucosamine/galactosamine ratios were 9.19 and 5.71 in the keratan sulphate-enriched fragments of proteoglycans of bovine and human cartilage, respectively: they did not contain detectable amounts of uronic acid. The protein/glucosamine ratios were 5 for bovine and 2 for human, increasing to 12 and 10.8 after keratanase digestion, respectively.

Hyaluronic acid-binding region

This was a generous gift from Dr. P. J. Roughley, Joint Diseases Laboratory. It was prepared from newborn human proteoglycan aggregate by using clostripain digestion (Roughley *et al.*, 1982).

Other proteoglycans and link protein

Keratan sulphate proteoglycan was isolated from bovine corneas and purified by DEAE-cellulose chromatography, both before and after chondroitinase ABC digestion (Nakazawa *et al.*, 1983). Keratan sulphate proteoglycans were eluted from concanavalin A–Sepharose with 1 M-α-methyl D-mannoside dissolved in 50 mM-Tris/HCl, pH 7.0, containing 1 M-NaCl. The concanavalin A-bound fraction did not contain any detectable galactosamine and the protein/glucosamine ratio was 8.25. Dermatan sulphate proteoglycan (DS-PG-II) of bovine articular cartilage and human articular cartilage link protein were generously supplied by Dr. L. C. Rosenberg (Rosenberg *et al.*, 1985) and Dr. P. J. Roughley (Roughley *et al.*, 1982) respectively, having been prepared as described by these authors.

Digestions of proteoglycan

Chondroitinase ABC was used as 0.1 unit/mg of proteoglycan in 0.1 M-sodium acetate/Tris/HCl buffer, pH 7.3 (Hascall & Heinegård, 1974) containing 1 mM-iodoacetamide, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-EDTA and 5 μg of pepstatin/ml to inhibit cysteine, serine, metallo- and aspartate-proteinases, respectively. Keratanase was used at 0.1 unit/mg of proteoglycan in 50 mM-Tris/HCl, pH 7.2, containing 80 mM-NaCl with the proteinase inhibitors listed above (Conrad *et al.*, 1982). Testicular hyaluronidase (at 10 μg or 240 turbidity units/mg of proteoglycan) was used in 0.1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl, 0.05 M-Na_2SO_4 and the above proteinase inhibitors (Glant, 1982*b*). Neuraminidase at 0.2 unit/mg of proteoglycan and sulphatases at 1 unit/mg of proteoglycan were used in 0.15 M-sodium acetate buffer, pH 5.1, containing 0.25 M-NaCl, 10 mM-$CaCl_2$ and proteinase inhibitors (Friedler, 1976). Endo-β-galactosaidase (0.05 unit/mg of proteoglycan) in 0.1 M-sodium acetate, pH 5.8 (Fukunda & Matsumura, 1975) and endo-β-*N*-acetylglycosaminidase H (0.05 unit/mg of proteoglycan) in 0.1 M-citrate/phosphate buffer, pH 5.3 (Hughes & Jeanloz, 1964) were used with proteinase inhibitors listed above. Effects of glycosidic enzymes were determined by analytical methods and the protein/hexosamine ratio was expressed (e.g. Table 5). Alkaline phosphatase (1 unit/mg of proteoglycan) was used in 1 M-diethanolamine/HCl buffer, pH 9.8, containing 0.5 mM-$MgCl_2$. Tosylphenylalanine chloromethane-treated trypsin at 27.5 μg or 6.5 units/mg of proteoglycan, tosyl-lysine chloromethane-treated chymotrypsin (100 μg or 4.5 units/mg of proteoglycan) and Pronase (5 μg/mg of proteoglycan) were in 50 mM-Tris/HCl, pH 7.5 (Keiser & DeVito, 1974). Pepsin (5 μg/mg of proteoglycan) in 0.2 M-sodium acetate, pH 5.0, and papain (10 μg/mg of proteoglycan) were in 0.2 M-sodium acetate, pH 5.0, containing 5 mM-EDTA and 5 mM-cysteine (Roughley & White, 1980). After incubation, proteinase contaminants in glycosidases were inhibited with fresh proteinase inhibitors described above and the enzymes were inactivated by 3 min of boiling (Table 5). Papain was inhibited by the addition of iodoacetamide to a final concentration of 10 mM, pepsin by the addition of pepstatin dissolved in 2 M-Tris to a final concentration of 10 μg/ml, trypsin by the addition of soya bean trypsin inhibitor at 10 μg/mg of proteoglycan and chymotrypsin by the addition of diisopropyl-fluorophosphate to a final concentration of 1 mM. Pronase, which is a non-specific proteinase isolated from *Streptomyces griseus*, was inhibited with iodoacetamide, phenylmethanesulphonyl fluoride, diisopropyl-fluorophosphate (5 mM each), EDTA (10 mM) and pepstatin (10 μ/ml).

Treatments of proteoglycan monomers and glycosaminoglycans to obtain substructures

D1 proteoglycan monomer (2 mg) or each glycosaminoglycan (2 mg) were diluted in 850 μl of appropriate buffer (as indicated above) and 50 μl of enzyme solution (shown above and in the text) was added and incubated for 0, 1, 4, 12, 24 and 48 h at 37 °C. At the end of the digestions, enzyme inhibitors (as above) in 50 μl were added to each vial, boiled for 3 min and serial dilutions of enzyme-treated proteoglycan monomer were prepared immediately for inhibition assays.

Alkaline borohydride

This was used to release *O*-linked oligosaccharides and *O*-linked glycosaminoglycans (Carlson, 1968) from the core protein of human articular and bovine nasal proteoglycans. Proteoglycan monomer (2–10 mg) was dissolved in 800 μl of 50 mM-NaOH and 1 M-sodium borohydride and incubated for 48 h at 45 °C. The solution was then neutralized by addition of glacial acetic acid and 0.1 M-sodium acetate, pH 6.0, to a final volume of 2 ml. Hydrolysis of proteoglycan monomers were also performed with 50 mM-, 0.1 M-, 0.25 M- and 0.5 M-NaOH as indicated. NaOH was neutralized with HCl and the volume adjusted to 2 ml with 0.1 M-sodium acetate, pH 6.0.

Reduction and alkylation

Reduction of cysteine and a subsequent alkylation was performed by Heinegård's (1977) method. Proteoglycan monomer (D1) (2 mg) was dissolved in 50 mM-Tris/HCl, pH 7.35, containing 4 M-guanidinium chloride and 5 mM-dithiothreitol. The mixture was incubated at 40 °C for 4 h. Iodoacetic acid was added to a concentration of 15 mM and the mixture was incubated at 25 °C for a further 20 h. Sample was dialysed against potassium acetate and then exhaustively against water.

Column chromatography

Proteoglycan monomer (D1 fraction) (2 mg) was dissolved in 1.0 ml of 0.2 M-sodium acetate, pH 5.5, and chromatographed on Sepharose CL-2B or CL-6B (1 cm × 120 cm) by downward elution at 6 ml/h, collecting 2 ml fractions. Fragments of 2–10 mg of proteoglycan monomer in 2 ml produced by alkaline or enzymic digestion were prepared on Sephacryl S-200 superfine (1 cm × 120 cm) equilibrated with 0.5 M-sodium acetate, pH 5.5, as described above.

Analytical methods

Uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962) and sialic acid by the periodate/resorcinol method (Jourdian *et al.*, 1971). Samples for hexosamine analysis were hydrolysed in sealed tubes under N_2 in 4 M-HCl at 100 °C for 8 h (Roughley & White, 1980). The hydrolysates were dried under vacuum and redissolved on 0.2 M-citrate buffer, pH 3.2. Samples were analysed with a Durrum amino acid analyser, using a single-buffer system (0.2 M-citrate, pH 5.25, for hexosamines and 0.2 M-citrate/0.1 M-borate, pH 5.28, for ga-

lactosaminitol) and hexosamine and galactosaminitol contents were determined by comparison with standards. Protein content was estimated by Lowry's method using bovine serum albumin as standard (Lowry *et al.*, 1951).

Monoclonal antibodies

Female BALB/c mice (4–6 weeks) were immunized intraperitoneally with 150 μg of A1D1 fraction of human articular proteoglycans digested with chondroitinase ABC in 150 μl of Freund's complete adjuvant. Mice were reinjected (two to four times total) on every third week with chondroitinase-ABC digested human proteoglycans until the blood samples (obtained from the retrobulbar venous plexus) gave positive titre at 1:1000–1:10000 serum dilution using the e.l.i.s.a. described below. Mice were given an intravenous injection of 100 μg of chondroitinase ABC-digested human articular proteoglycans 3 days before killing. Spleens were removed and the cells were fused with Sp2/O-Ag14 murine myeloma cells by using poly(ethylene glycol) (Köhler & Milstein, 1975; Oi & Herzenberg, 1980; Zola & Brooks, 1982; Glant *et al.*, 1985). Hybridomas were selected in HAT-medium (hypoxanthine-, aminopterin- and thymidine-containing RPMI 1640 medium with 15% fetal calf serum). Antibody production of hybridomas was screened using native (non-digested) A1D1 fraction of human adult articular cartilage for the coating of 96-well microtitration plates (Nunclon; Immunoplate I, F). Positive cell lines were cloned by limiting dilution in hypoxanthine-, thymidine-containing medium in the presence of syngeneic peritoneal macrophages (Fazekas de St. Groth & Scheidegger, 1980). Cloned hybridoma cells were injected intraperitoneally into BALB/c mice to produce ascites fluids. Monoclonal antibodies were purified on Sepharose 4B and AcA44 columns followed by binding a staphyloccoccal protein A (Watanabe *et al.*, 1981).

Affinity chromatography

Sepharose CL-4B was activated with CNBr (March *et al.*, 1974) and antibodies were bound (2.4–2.6 mg/ml of wet gel) as described elsewhere (Glant, 1982*a*). Unlabelled proteoglycan monomer as carrier (2 mg) and/or 2.6×10^6 c.p.m. of ^{125}I-labelled proteoglycan monomer D1 were loaded on each column (1.4 cm × 7.0 cm) and recirculated overnight at room temperature. Unbound proteoglycans were removed by exhaustive washing with radioimmunoassay buffer (see below), and the bound fraction was eluted with 3 M-potassium thiocyanate in phosphate-buffered saline containing azide. Unbound and bound fractions of proteoglycan monomers were used for inhibition assays. By this procedure, about 90% of radiolabelled antigens was recovered.

Enzyme-linked immunosorbent assay

Supernatants of hybridomas, immunoglobulin class- and subclass-specificity of monoclonal antibodies, specificity of monoclonal antibodies to variable epitopes and the cross-reactivity of monoclonal antibodies to proteoglycans obtained from other species were determined by e.l.i.s.a. as described previously (Poole *et al.*, 1984; Glant *et al.*, 1977, 1985). Mouse immunoglobulin class- and subclass-specificity of antibodies were determined by heavy- and light-chain-specific rabbit anti-mouse immunoglobulins (Miles Laboratories).

Three monoclonal antibodies (BCD-4, BCD-7 and EFG-4) were identified as IgG1 and the KPC-190 as IgG2a. All the four monoclonal antibodies used in this experiment contained κ-light chains.

Radioimmunoassay

Proteoglycan monomers were labelled with ^{125}I by using chloramine-T (Sonada & Schlamowitz, 1970). Direct and competitive binding radioimmunoassays were used. The conditions were as described recently (Caterson *et al.*, 1983). Radioimmunoassay (RIA) buffer at pH 8.1 was used to facilitate the binding of monoclonal antibodies to protein A of *Staphylococcus aureus*. Binding curves were determined as follows. Monoclonal antibodies were serially diluted, and 100 μl of each dilution was incubated with 50 μl of ^{125}I-proteoglycan for 2 h at 37 °C. A 10% (w/v) suspension of protein A-bearing *Staphylococcus aureus* (Zysorbin) (25 μl) was added and the mixture was incubated for 30 min at 37 °C. Pellets were washed twice with radioimmunoassay buffer. Radioactivity was determined with an LKB gamma counter (model 1270, Rackgamma II) and the percentage of the total proteoglycan bound was recorded.

For competitive binding (inhibition) radioimmunoassay, monoclonal antibodies were diluted so that they would precipitate 40–50% of the total radiolabelled proteoglycan. Unlabelled proteoglycan solutions (standards, unknown samples, column fractions and proteoglycan enzyme/alkali digests) in 50 μl of radioimmunoassay buffer were mixed with 100 μl of antibody dilution and incubated overnight at 37 °C. ^{125}I-proteoglycan (50 μl) was then added, incubated for 2 h at 37 °C and the assay carried out as described for the binding assay. The inhibition of binding (%) was calculated relative to the amount of ^{125}I-labelled proteoglycan which was bound in the absence of added, unlabelled competing antigen, i.e. in the absence of inhibitor. Inhibition was expressed by the following formula:

$$\text{Inhibition (\%)} = 100 - \left(\frac{\text{c.p.m. bound in the presence of inhibitor}}{\text{c.p.m. bound in the absence of inhibitor}}\right) \times 100$$

RESULTS

Monoclonality of antibodies

Four hybridomas producing monoclonal antibodies (BDC-4, BCD-7, EFG-4 and KPC-190) against native proteoglycan monomers were selected from more than 100 antibody-producing cell lines raised to chondriotinase ABC-digested proteoglycans of human adult cartilage. In competitive e.l.i.s.a. binding assays using peroxidase-labelled and unlabelled antibodies, none of these four monoclonal antibodies inhibited the binding of the three others to proteoglycan antigens, indicating that they recognize different epitopes on these proteoglycan molecules (Fig. 1).

Proteoglycan monomer (D1 fraction) of human cartilages was separated into two subpopulations (bound and unbound) by any of these four monoclonal antibodies by using an immunosorbent procedure (Table 1). The antibody-bound subpopulation of proteoglycans reacts completely with the other three monoclonal

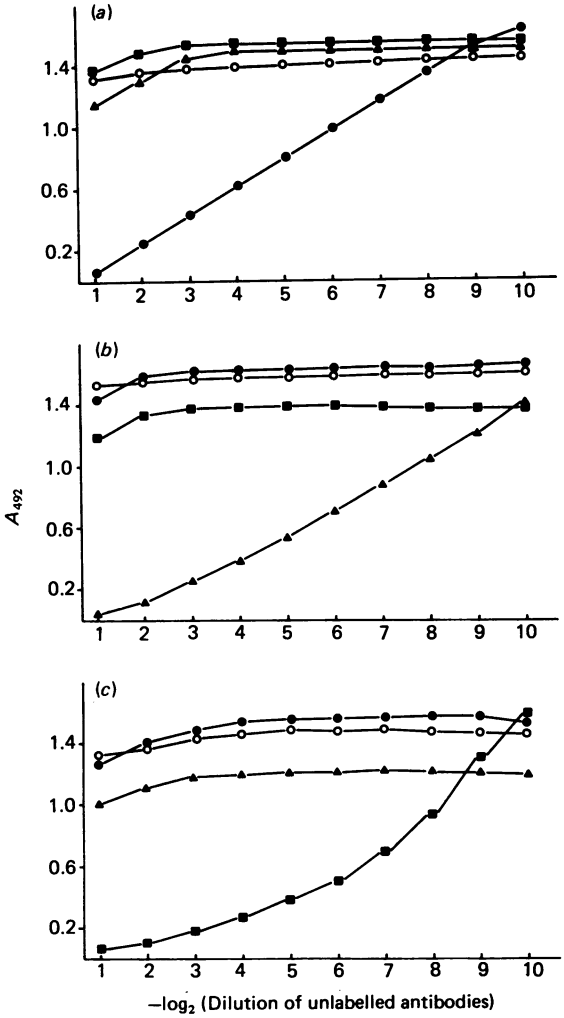

Fig. 1. Competitive binding of monoclonal antibodies to different epitopes of proteoglycan monomer of human adult cartilage

Monoclonal antibodies BCD-4 (*a*), BCD-7 (*b*) and EFG-4 (*c*) were labelled with horseradish peroxidase and used with unlabelled monoclonal antibodies [BCD- 4 (●), BCD-7 (▲) EFG-4 (■) and KPC-190 (○)] in competitive e.l.i.s.a. Microplates were coated with native proteoglycan monomer (D1) of human adult articular cartilage (5 μg/well). Two-fold dilution (50 μl volumes) of unlabelled antibodies (starting at 20 mg/ml) were added and incubated with 50 μl of peroxidase-labelled BCD-4 (2 μg/ml), BCD-7 (2.1 μg/ml) and EFG-4 (1.8 μg/ml) monoclonal antibodies, respectively.

antibodies, which means that BCD-4, BCD-7, EFG-4 and KPC-190 epitopes are present in the same proteoglycan molecule of human articular cartilage. This subpopulation of proteoglycan molecules is hardly detectable (maximum 10% of the total radiolabelled monomer fraction) in human fetal premature and neonatal cartilage (Table 1). The amount of this bound subpopulation within the total proteoglycan content increases after birth, up to 52–56 years of age (Table 1).

Cross-reactivity of monoclonal antibodies

Using radioimmunossay, all four monoclonal antibodies were shown to react with native proteoglycan monomers of adult human articular cartilage and they cross-reacted with native and chondroitinase ABC-digested proteoglycan monomers of adult bovine cartilages (Table 2). BCD-4 and BCD-7 also reacted with adult dog and rabbit proteoglycans. EFG-4 also reacted with rabbit and chicken proteoglycans but not with dog. KPC-190 did not significantly react with proteoglycans of species other than human and bovine. Both KPC-190 and EFG-4 failed to react with fetal proteoglycans. Proteoglycan monomers isolated either from chicken limb bud cartilage or transplantable rat chondrosarcoma (up to 25 μg) showed no reaction with any of these monoclonal antibodies (Table 2). Epitopes reacting with BCD-4, BCD-7, EFG-4 and KPC-190 monoclonal antibodies were not detected in the small dermatan sulphate-proteoglycan of bovine costal cartilage nor in human link protein (Table 3). Keratan sulphate-proteoglycan of bovine cornea lacked BCD-7 and EFG-4 epitopes, but the KPC-190, and a trace reaction of the BCD-4, epitopes were detected in this molecule at higher concentrations (Table 3).

Effects of alkali and sodium borohydride on epitopes

Binding of monoclonal antibodies to epitopes could be inhibited when proteoglycan monomers of human

Table 1. Age-related changes of BCD-4, BCD-7, EFG-4 and KPC-190 defined epitopes of proteoglycans in human articular cartilage

Proteoglycan monomer fractions of human articular cartilage (D1) were iodinated and separated on BCD-4, BCD-7, EFG-4 and KPC-190 monoclonal antibody–Sepharose CL-4B columns. αHFN 11F6 monoclonal antibody reacting with human fibronectins and polyclonal antibodies against human adult cartilage proteoglycan monomers were used as negative and positive controls, respectively. The subpopulation of monoclonal antibody-bound proteoglycans could be reabsorbed on columns, or bound in radioimmunoassay by any of the four monoclonal antibodies against cartilage proteoglycans. Polyclonal antibodies precipitated both the monoclonal-bound and -unbound fractions of human cartilage proteoglycans. Abbreviation: n.d., not determined.

	Bound ^{125}I-proteoglycan monomer (%)					
Antibodies...	BCD-4	BCD-7	EFG-4	KPC-190	α-HFN 11F6	Polyclonal antibody
Fetal (38 weeks)	12.4	12.1	7.0	6.8	0.05	n.d.
1.5 year old	30.9	28.6	28.9	30.0	0.00	82
15 year old	51.0	52.2	50.8	n.d.	0.00	96
52–56 year old	60.6	62.4	61.6	60.8	0.00	96

Table 2. Effect of proteoglycans of different cartilages on the binding of monoclonal antibodies to ^{125}I-labelled human adult proteoglycan monomer

Bindings of monoclonal antibodies (BCD-4, BCD-7, EFG-4 and KPC-190) to ^{125}I-labelled D1 fraction of human adult cartilage proteoglycan were inhibited with variable amounts of native cartilage proteoglycans isolated from different cartilage of different species. Results of binding curves are summarized showing the inhibition produced by different amounts of proteoglycan monomers (dry weight). Proteoglycan monomers obtained from dissociative (D1) or from associative followed by dissociative gradient centrifugation (A1D1) are indicated.

Proteoglycan	Dry weight (μg)	Inhibition (%)			
		BCD-4	BCD-7	EFG-4	KPC-190
Human adult articular D1	0.1	70	82	67	49
Human fetal D1 (28 weeks)	20.1	100	100	9	6
Bovine nasal cartilage A1D1	2.5	84	60	41	41
Bovine adult articular cartilage A1D1	2.5	61	76	49	48
Fetal calf articular A1D1 (210 days)	312.0	52	30	14	14
Dog adult articular A1D1	0.128	63	65	19	13
Rabbit articular A1D1	0.312	32	40	51	20
Chicken articular A1D1	10.0	50	20	56	2
Chicken sternal A1D1	4.0	64	22	67	14
Chicken limb bud A1D1*	39.0	2	0	0	0
Swarm rat chondrosarcoma A1D1	25.0	0	0	0	0

* Chicken limb bud cartilage was isolated from Hamburger–Hamilton stage 23–24 (Hamburger & Hamilton, 1951)

Table 3. Effect of proteoglycans and their substructures on the binding of monoconal antibodies to ^{125}I-labelled human adult proteoglycan monomer

Bindings of monoclonal antibodies (BCD-4, BCD-7, EFG-4 and KPC-190) to ^{125}I-labelled D1 fraction of human adult cartilage proteoglycan were inhibited at concentrations indicated. Abbreviations used: C-4-S, chondroitin-4-sulphate; C-6-S, chondroitin-6-sulphate; DS, dermatan sulphate; KS, keratan sulphate; PG, proteoglycan.

Proteoglycan or subcomponent	Dry weight (μg)	Inhibition (%)			
		BCD-4	BCD-7	EFG-4	KPC-190
Human D1	0.10	70	82	67	49
C-4-S, C-6-S and DS	12.50	0	0	0	0
C-4-S, C-6-S and DS digested with chondroitinase ABC	12.50	0	0	0	0
KS	12.50	58	0	0	5
KS digested with keratanase	12.50	7	0	0	0
KS-PG (bovine cornea)	4.00*	16	4	2	62
DS-PG II	10.24	0	0	0	0
Link protein (human)	12.50	0	0	0	0

* Protein content.

articular cartilage were treated with sufficient NaOH or alkaline borohydrine. The BCD-4 and KPC-190 reactive epitopes seem to be the most sensitive to alkaline treatments (Table 4). When proteoglycans treated with 50 mM-alkali and 1 M-borohydride were examined at a higher concentration (20 μg), some immunoreactivity was detected (Table 4). Proteoglycan thus treated (20 mg) was chromatographed on Sephacryl S-200 and analysed for protein, hexuronic acid, glucosamine, sialic acid and galactosaminitol. Fig. 2 shows that BCD-7, EFG-4 and KPC-190 monoclonal antibodies react with higher M_r component(s) in a region before the chondroitin sulphate peak. However, there is also immunoinhibition of all antibodies where chondroitin sulphate is mainly concentrated, particularly for BCD-4, and where both chondroitin sulphate and keratan sulphate, are present. The trailing shoulders of KPC-190 and BCD-4 correspond to the keratan sulphate peak. The protein elution profile indicates that the alkaline borohydride procedure extensively degraded the core protein and that the majority of peptide fragments co-eluted with or after the smaller oligosaccharides, as described by Lohmander *et al.* (1980). There was no evidence of reactions of monoclonal antibodies with either *O*-linked or *N*-linked oligosaccharides.

Role of glycosaminoglycans and oligosaccharides in determining epitope structure; reactions with glycosaminoglycans

None of the antibodies reacted with chondroitin or dermatan sulphates either before or after digestion with chondroitinase ABC (Table 3). Only BCD-4 clearly reacted with isolated keratan sulphate and this reaction was almost completely abolished by prior digestion of this

Table 4. Effects of alkali and alkaline borohydride on the epitopes of proteoglycan monomer

Human adult cartilage proteoglycan (D1) was incubated as indicated. Samples were neutralized and diluted to contain 0.2 μg or 20 μg of proteoglycan monomer calculated on dry weight of untreated material. Alkaline borohydride treatment was at 45 °C for 48 h.

Treatments	Amount (μg)	Antibody binding to epitopes (%)			
		BCD-4	BCD-7	EFG-4	KPC-190
Untreated	0.2	100	100	100	100
Treated	0.2				
50 mM-NaOH, 45 °C, 4 h		68	75	76	62
50 mM-NaOH, 45 °C, 48 h		0	20	14	0
50 mM-NaOH/1 M-$NaBH_4$		0	0	4	0
250 mM-NaOH, 45 °C, 4 h		0	0	0	0
Treated	20				
50 mM-NaOH/1 M-$NaBH_4$		28	48	58	24

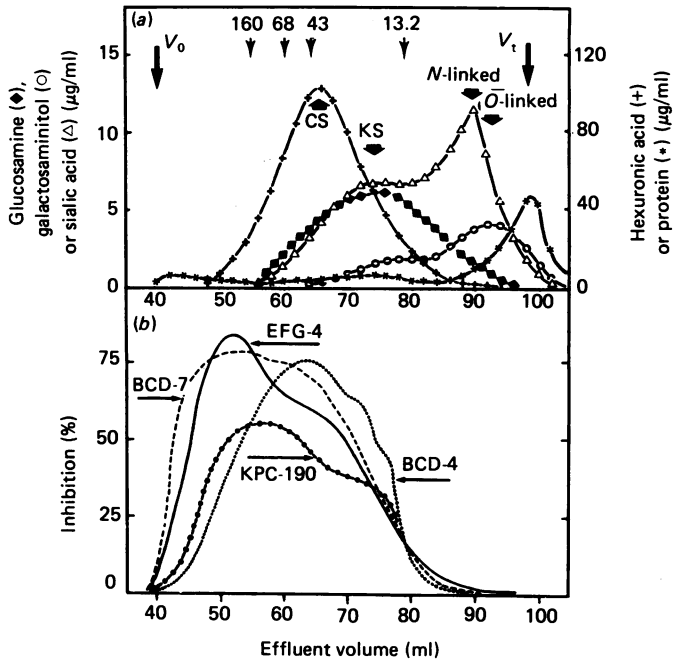

Fig. 2. Sephacryl S-200 column chromatography (117 cm × 1 cm) of alkaline-borohydride treated cartilage proteoglycans

Proteoglycan monomer (D1) of human adult cartilage (20 mg) was treated with 50 mM-NaOH and 1 M sodium borohydride for 48 h at 45 °C. Hexuronic acid (+), sialic acid (△), glucosamine (◆), galactosaminitol (○) and protein (*) contents were determined. Peaks of chondroitin sulphate (CS, hexuronic acid), keratan sulphate (KS), *N*-linked and *O*-linked oligosaccharides (glucosamine, sialic acid and galactosaminitol) are indicated in (*a*). Inhibitions by column fractions of specific binding of antibodies BCD-4, BCD-7, EFG-4 and KPC-190 to ^{125}I-labelled human adult proteoglycan monomer are shown in (*b*). Arrows indicate the void volume (V_0) and the total volume (V_t) as well as the positions of M_r standards [human γ-globulin (160000), bovine serum albumin (68000), ovalbumin (43000) and cytochrome *c* (13200)].

glycosaminoglycan with keratanase (Table 3). Radioimmunoassay of proteoglycans treated with chondroitinase ABC or keratanase revealed that immunoreaction was unaffected, indicating that chondroitin sulphate and keratan sulphate side chains do not mask and are not part of the epitopes recognized by BCD-7 and EFG-4 (Table 5). Keratanase or endo-β-galactosidase digestion did, however, reduce the binding of BCD-4 and KPC-190, and chondroitinase ABC treatment enhanced their binding (Table 5). This indicates that keratan sulphate constitutes in part at least the epitopes recognized by BCD-4 and KPC-190 antibodies and that these epitopes can be masked by chondroitin sulphate. Further, more detailed analyses of antibody BCD-4 are shown on Fig. 3. Digestions of proteoglycan monomer with endo-β-galactosidases, however, never abolished completely the immunoreactivity of antigen to the monoclonal antibodies used in this experiment. Sensitivity of epitope KPC-190 to keratanase, endo-β-galactosidase and proteinases was similar to that observed with BCD-4. Effects of other enzyme treatments on the expression of determinants are also shown in Table 5. Sulphatases, alkaline phosphatase and neuraminidase alone had no effect on antibody binding. Complete immunoinhibition profiles for BCD-4 are shown in Fig. 3 where details of the effects of chondroitinase ABC, keratanase, mixed glycosidases and endo-β-galactosidase are given.

Effect of proteolytic cleavage

Pronase and papain digestions essentially abolished the binding of 200 ng proteoglycan monomer to all four monoclonal antibodies (Table 5). A full immunoinhibition curve for BCD-4 is shown in Fig. 3. Pepsin, chymotrypsin and trypsin reduced reactivity to a lesser degree. In each case the epitope recognized by BCD-4 was more susceptible to proteinase treatments. Column fractions of pepsin, chymotrypsin and trypsin-treated proteoglycan monomer, however, showed a broad range of inhibition from the void (V_0) volume of a Sephacryl S-200 column to 30–40 kDa fragments (results not shown).

Localization of epitopes in core protein of proteoglycan monomers

Epitopes BCD-4, BCD-7, EFG-4 and KPC-190 were not detectable in the hyaluronic acid binding region of

Table 5. Effects of glycosidic and proteolytic cleavage on ability of non-iodinated adult proteoglycan to inhibit binding of monoclonal antibodies to ^{125}I-proteoglycans

The effect of each enzyme treatment on the expression of the determinant was studied by comparing the antibody binding to epitopes using 0.2 μg of proteoglycan monomer (high buoyant density proteoglycan D1 fraction of human adult articular cartilage) and by comparing the amounts of proteoglycan monomer in control (zero-time) and in treated samples (time 4 h) required to achieve 50% inhibition in radioimmunoassay. Control samples were treated exactly as the test samples in each procedure (4 h at 37 °C), except that the enzymes were inactivated at the beginning of the incubation period (zero-time). Protein: glucosamine ratios were 1.96, 4.20 and 4.18 in native (undigested), keratanase- and endo-β-galactosidase-treated samples, respectively.

Treatments	Antibody binding to epitopes (%)				Adult D1 required for 50% inhibition (ng)			
	BCD-4	BCD-7	EFG-4	KPC-190	BCD-4	BCD-7	EFG-4	KPC-190
Untreated control	100	100	100	100	60	42	38	170
Control (100 °C, 3 min)	100	99	100	100	60	42	38	170
Chondroitinase ABC	118	103	100	138	42	40	38	68
Keratanase	66	98	100	58	200	42	38	680
Sulphatases	100	100	100	100	60	42	38	170
Alkaline phosphatase	100	100	100	100	60	42	38	170
Neuraminidase	100	100	100	100	60	42	38	170
Endo-β-galactosidase	72	100	100	60	120	42	38	560
Pronase	0	0	0	0	2000	16400	22200	18800
Papain	0	8	2	2	14900	6500	5700	9600
Pepsin	35	52	58	66	460	370	400	300
Chymotrypsin	44	67	59	67	420	240	280	320
Trypsin	65	82	88	66	340	220	160	300

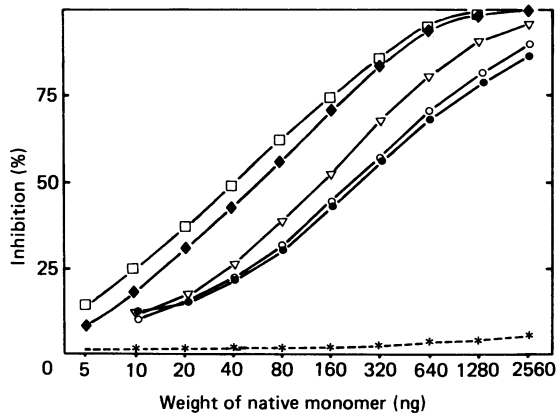

Fig. 3. Immunoinhibition assays of monoclonal antibody BCD-4

Binding of monoclonal antibody BCD-4 to ^{125}I-labelled proteoglycan monomer of 52–56-year-old human cartilage in the presence of unlabelled proteoglycan monomer (D1) (◆), or by unlabelled proteoglycan monomer digested with pronase (*), endo-β-galactosidase (○), chondroitinase ABC (□), keratanase (●) or chondroitinase ABC plus keratanase (▽). Effects of keratanase and endo-β-galactosidase are shown in Table 5. In radioimmunoassay, monoclonal antibody BCD-4 (2.5 μg/100 μl) precipitated 50% of ^{125}I-labelled proteoglycan (8200 c.p.m./50 μl) in the absence of unlabelled antigen. Inhibition (%) was calculated relative to the amount of ^{125}I-labelled antigen bound to the antibody in the absence of added unlabelled antigen.

proteoglycan monomer of human newborn articular cartilage (Table 6). Reduction and alkylation of proteoglycan monomer did not influence the binding of monoclonal antibodies. A keratan sulphate-rich fragment of human adult cartilage proteoglycan digested with trypsin and isolated by Sepharose CL-6B and DEAE-cellulose chromatography proved to be free of the BCD-4 epitope (Table 6 and Fig. 4) although this epitope was found to contain a keratanase-sensitive component (Table 5 and Fig. 3). All the other three monoclonal antibodies reacted with the keratan sulphate-rich fragment of human adult cartilage, but much more protein was required to produce similar inhibition compared with native cartilage proteoglycan (Table 6). Full immunoinhibition curves for BCD-4, KPC-190 and EFG-4 are shown in Fig. 4. Keratanase treatment significantly reduced the immunoreactivity of the KPC-190 epitope located in this fragment (Table 6). A mild alkaline treatment slightly reduced the inhibitory effect of this fragment on the binding of EFG-4 and BCD-7 monoclonal antibodies and abolished it on the binding of KPC-190 antibody (Table 6). Pronase digestion of the keratan sulphate-rich fragment destroyed the immunoreactivity to all the antibodies (Table 6).

DISCUSSION

Monoclonal antibodies characterized in this paper react with different epitopes of the same native adult human proteoglycan monomer which are all sensitive to enzymic and non-enzymic degradation. Column profiles of cleaved proteoglycan monomers suggested that the antigenic determinants reside in a polypeptide region of minimum M_r 30000–40000 (results not shown).

Epitopes revealed by BCD-7 and EFG-4 monoclonal antibodies proved to be sensitive to proteinase digestions and alkaline cleavages yet resistant to all the glycosidases, sulphatases and phosphatases studied, suggesting that these epitopes of proteoglycan molecule are highly associated with the protein structure and do not include

Table 6. Binding of proteoglycan monomers and their substructures to monoclonal antibodies

Inhibition of binding of monoclonal antibodies to ^{125}I-labelled human adult proteoglycan monomer (D1) is shown. Abbreviations used: R/A, reduced and alkylated proteoglycan monomer; HABR, hyaluronic acid-binding region of human newborn cartilage proteoglycan; KS-D1, keratan sulphate-rich fragment of adult monomer (D1, 66 years old); Kase-KS-D1, keratanase treated KS-D1; Pronase-KS-D1, Pronase-treated KS-D1; NaOH-KS-D1, alkaline treatment of KS-D1 in 50 mM-NaOH for 4 h at 45 °C was performed; n.d., not determined.

Proteoglycan or subcomponent	Protein (μg)	Inhibition (%)			
		BCD-4	BCD-7	EFG-4	KPC-190
Native adult D1 (52–56 year)	0.01	69	80	66	50
R/A adult D1 (52–56 year)	0.01	67	81	66	48
HABR	1.80	0	0	0	0
KS-D1	0.50	2	58	71	33
Kase-KS-D1	0.50	0	58	70	18
Pronase-KS-D1	0.50	n.d.	0	0	0
NaOH-KS-D1	0.50	n.d.	48	61	0

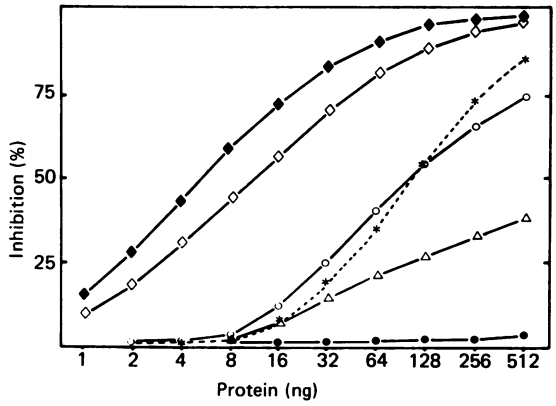

Fig. 4. Immunoinhibition of monoclonal antibodies BCD-4, EFG-4, and KPC-190

Binding of monoclonal antibodies to ^{125}I-proteoglycan monomer of human articular cartilage in the presence of unlabelled proteoglycan monomer (◇, ◆), keratan sulphate-attachment region of proteoglycan monomer of human articular cartilage before (○, ●) or after (△) keratanase. The protein/glucosamine ratio was increased from 2 to 10.8 by keratanase treatment. Open symbols indicated the binding curve of KPC-190, closed symbols the binding of BCD-4 monoclonal antibodies. The broken line shows the binding of EFG-4 monoclonal antibody to the keratan sulphate attachment region of human articular proteoglycans.

keratan sulphate, chondroitin sulphate or oligosaccharides. Since the hyaluronic acid-binding region of newborn cartilage could not bind either of these two monoclonals, and the reduction and alkylation of proteoglycan monomers did not reduce the antibody binding [disulphide bridges of hyaluronic acid-binding region can preserve the original immunoreactivity of this region (Ratcliffe *et al.*, 1984)], we believe that these epitopes are present in the protein core outside the hyaluronic acid-binding region.

Digestion of proteoglycans with keratanase or endo-β-galactosidase reduced the binding of monoclonal antibodies BCD-4 and KPC-190 to native proteoglycans. Since binding was also inhibited by treatment with Pronase or papain or alkali, especially with borohydride, these observations indicate that both these epitopes represent peptides substituted with a keratan sulphate or with a keratan sulphate-like structure. The role of keratan sulphate in the structure of BCD-4 and KPC-190 epitopes is not the same. When isolated keratan sulphate was used for inhibition there was an inhibitory effect on the binding of BCD-4 monoclonal antibody to native proteoglycan monomer but no effect on KPC-190. This inhibition was seen at relatively high concentrations: inhibition required 100–150-fold more keratan sulphate (on the basis of dry weight) than native cartilage proteoglycan monomer. Also KPC-190 reacted much more strongly with corneal keratan sulphate proteoglycan and with keratan sulphate-rich fragments of human adult proteoglycan monomer. Together these results indicate that the KPC-190 epitope has an obligate requirement for protein core for recognition of keratan sulphate by antibodies. Also the keratan sulphate binding would appear to be weaker than that observed for BCD-4. BCD-4 and KPC-190 are clearly present in different parts of the molecule, in view of the analysis of fragments isolated from adult monomer. The production of antibodies to keratan sulphate is presumably selected to the absence of this molecule from mice (Venn & Mason, 1985).

Since both the hyaluronic acid-binding region and the keratan sulphate-enriched fragment of human cartilage proteoglycan proved to be free of BCD-4 epitope, we believe that this epitope is located only in the chondroitin sulphate-rich part of the core protein. The BCD-7, EFG-4 and KPC-190 epitopes were detected both in keratan sulphate- and chondroitin sulphate-bearing fragments of the core protein of proteoglycan monomer. The putative locations of all four epitopes are shown diagrammatically in Fig. 5.

This study also presents some new information on the heterogeneity of human proteoglycans. The monoclonal antibodies BCD-4, BCD-7, EFG-4 and KPC-190 recognized protein related epitopes on the same proteoglycan molecule, but this was only a subpopulation in the D1 fraction of human adult articular cartilage proteoglycans. Thus, there may exist in cartilage different molecular species with genetically distinct core proteins

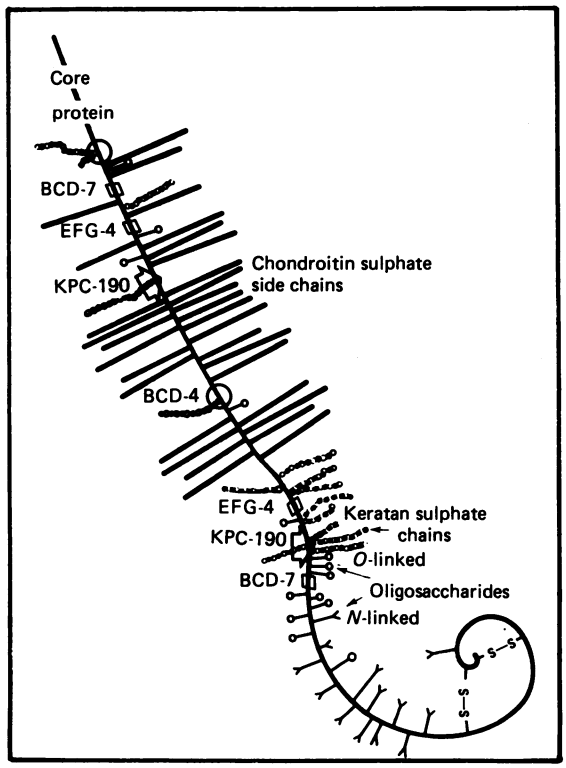

Fig. 5. Schematic presentation of epitopes located on the core protein of proteoglycan monomer of human adult cartilage

BCD-7 and EFG-4 epitopes (peptides) are present in both keratan sulphate- and chondroitin sulphate-attachment region. BCD-4 epitope (protein associated with a keratanase-sensitive component) is located only in the chondroitin sulphate-attachment region. KPC-190 epitope (protein associated with keratan sulphate) was found mainly in the keratan sulphate-rich fragment of proteoglycan monomer, but it is also present in chondroitin sulphate-attachment region of the molecule.

(Hopwood & Robinson, 1975; Stanescu *et al.*, 1977; Roughley & White, 1980; Heinegård *et al.*, 1981; Champion *et al.*, 1982; Stanescu & Stanescu, 1983). The absence of these epitopes in fetal proteoglycans also suggests the absence of distinct proteoglycan subpopulations in fetal cartilage containing these epitopes. After birth all four epitopes appear in human articular cartilage and increase in amount with age, indicating the appearance of distinct proteoglycan molecules characteristic of more mature chondrocytic expression, as suggested earlier (Champion *et al.*, 1982). Their presence in proteoglycans of some other cartilages of different species indicate that these antigenic determinants are associated with structural features which are partly conserved during phylogenesis rather than during ontogenetic development (Glant *et al.*, 1975). Further analysis of proteoglycans isolated by monoclonal antibodies can probably help to understand the structural organization of proteoglycans during cartilage development and the biosynthetic abnormalities in arthritic cartilage.

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