Humoral immunity to link protein in patients with inflammatory joint disease, osteoarthritis, and in non-arthritic controls

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SUMMARY Cartilage link protein of high purity was prepared and used in an enzyme linked immunosorbent assay (ELISA). Antibodies to link protein were sought in the sera of 98 patients with rheumatic disorders; 38 with rheumatoid arthritis (RA), 29 with osteoarthritis (OA), 13 with psoriatic arthritis (PA), nine with ankylosing spondylitis (AS), nine with systemic lupus erythematosus (SLE), and in 83 healthy controls. Antibodies were detected in all groups with the following prevalences: 21/83 normals, 9/38 RA, 7/29 OA, 7/13 PA, 3/9 AS, and 4/9 SLE. No statistically significant differences existed between the groups with regard to either prevalence or mean titre of anti-link antibodies. Serum antibodies to proteoglycan link protein appear to be no more common in patients with rheumatic disorders than in healthy controls.

Key words: autoimmunity, rheumatoid arthritis.

Autoimmunity to the components of articular cartilage—proteoglycan (PG), link protein, and collagens II, IX, and XI—may help to trigger or maintain, or both, the abnormal immune reactions seen in the synovium of patients with inflammatory arthritides. Injection of types II^{1 2} and XI³ collagen can induce a polyarthritis in genetically defined strains of rats and mice. Immunity to injected cartilage proteoglycan can lead to development of inflammatory arthritis in rabbits,⁴ dogs,⁵ and BALB/c mice.⁶ Adjuvant arthritis in mice may be triggered by structural mimicry between the immunising antigens on *Mycobacterium tuberculosis* and PG core protein.⁷ Conversely, as cartilage is broken down in mechanically induced experimental arthritis, autoimmunity to homologous cartilage proteoglycan and collagens develops.⁸

Both humoral and cell mediated immune mechanisms can play a part in the arthritogenic responses to cartilage components. After the

transfer of either anticollagen II antibodies⁹ or lymphoid cells¹⁰ or cloned T lymphocytes responsive to PG core protein¹¹ from arthritic to naive animals the recipient animals develop arthritis, suggesting that both B cell and T cell immunity are important in the development of clinical disease. Genetic factors are also important, however, as not all animals with natural or acquired immunity to cartilage components become arthritic.^{6 8}

Our knowledge of the degree of autoimmunity to cartilage matrix in human inflammatory arthropathy is much more fragmentary. Only a small proportion (about 3%) of patients with rheumatoid arthritis possess autoantibodies that react immunocytochemically with cartilage.¹² In patients with RA, however, the estimated incidence of antibodies to native collagen types II, IX, and XI is 10%, 2%, and 2.8% respectively,¹³ though the incidence of antibodies to denatured collagens is higher.^{13 14}

Both cell mediated and humoral immunity to cartilage PG antigens have been demonstrated in patients with rheumatoid arthritis and ankylosing spondylitis.^{15 16} The high incidence of anti-PG antibodies reported in patients with RA by Glant et

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al (38%; 11/29 patients) was obtained from experiments in which proteoglycan-rich extracts of cartilage, rather than purified proteins, were used.¹⁵

The present study describes the preparation of link protein from human articular cartilage and its use in ELISA assays to detect autoantibodies in the sera of patients with inflammatory and degenerative arthritis.

Materials and methods

MATERIALS

AH-Sepharose 4B and Sephadex G-150 Superfine (SF) were obtained from Pharmacia, Milton Keynes, UK; and nitrocellulose paper from Schleicher and Schuell, Dassel, West Germany. Other reagents were obtained from the following sources: avidin D-horseradish peroxidase conjugate (avidin-HRP), Vector Laboratories, Peterborough; peroxidase conjugated, affinity purified goat anti-rabbit IgG (GARS-HRP), Bio-Rad Laboratories, Watford, Herts; biotinylated protein A, Sera Labs Ltd, Crawley Down, West Sussex; sodium dodecyl sulphate (specially pure), acrylamide (grade 1), *N,N'*-methylenebisacrylamide (specially pure), silver nitrate, glycine, hydrogen peroxide (all Analar), casein (Hammarsten grade), caesium chloride, and thiomersal (both lab reagent grade) were all purchased from BDH Ltd, Poole, Dorset. Unless specified, other reagents were from Sigma, Poole, Dorset.

PROTEOGLYCAN EXTRACTION AND FRACTIONATION

Macroscopically normal articular cartilage was sliced from the crown region of 50 human osteoarthritic femoral heads obtained at total hip replacement. The minced cartilage was extracted with 4 M guanidinium hydrochloride (GuHCl) containing enzyme inhibitors.¹⁷ The proteoglycans were then fractionated by associative and dissociative density gradient centrifugation by the method of Roughley *et al.*¹⁸ with modification of the initial density of the associative gradient to 1.45 g/ml (M Bayliss, personal communication).

HYALURONIC ACID AFFINITY CHROMATOGRAPHY

The link protein-rich fraction from the dissociative gradient (A1A1D6) was fractionated by chromatography on a column (12.5 ml) of hyaluronic acid coupled to Sepharose 4B prepared and developed as described by Tengblad.¹⁹ Link protein eluted in 4 M GuHCl was further purified by Sephadex-G150 Superfine column chromatography.

SEPHADEX G-150 SUPERFINE CHROMATOGRAPHY

Link protein in 4 M GuHCl (2–3 mg in 2 ml) was dialysed for 24 hours at 4°C against 1 M NaCl, 10 mM 3-(*N*-morpholino) propanesulphonic acid (MOPS) buffer, pH 7.0. Link protein is soluble in this buffer and forms oligomers (8–10S),^{20, 21} which are excluded by G-150 Superfine. The dialysed solution was applied to the column (2.5×20.5 cm) equilibrated with 1 M NaCl, 10 mM MOPS, pH 7.0, and eluted in the same solution at a flow of 22.5 ml/h. The absorbance of the eluate was monitored at 280 nm.

The concentration of link protein in the void peak was calculated from the absorbance at 280 nm using the published absorbance coefficient of 1.4.²⁰

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Samples were analysed electrophoretically by the method of King and Laemmli²² on 7.5–10.0% (total acrylamide) slab gels. The gels were stained serially with Coomassie brilliant blue R250 and silver nitrate using the method of De Moreno *et al.*²³

IMMUNOBLOTTING

Conditions used in the electrophoretic transfer of proteins to nitrocellulose were as described by Burnette.²⁴ Subsequently, the nitrocellulose was washed briefly in water and blocked with phosphate buffered saline (PBS)/0.5% Tween 20 before incubation (4°C overnight) with primary antibody (1:1000 dilution in PBS/0.05% Tween 20). Unbound proteins were removed by rinsing (3×5 min) in PBS/0.05% Tween 20. Bound primary antibody was detected after a two hour incubation with GARS-HRP (1:3000 dilution), washing, and visualisation of HRP activity in the chromogen solution (160 µg/ml 3-amino-9-ethylcarbazole, 0.2 M sodium acetate, 0.1 mM H₂O₂, pH 5.2).

ENZYME LINKED IMMUNOSORBENT ASSAY

Ninety six well polystyrene ELISA plates (Immuno-plate IF, Nunc, Denmark) were coated with 50 µl of link protein (10 µg/ml) in 1 M NaCl, 10 mM MOPS, pH 7.0 buffer. After one hour's incubation at room temperature the plates were washed three times with distilled water and exposed to 0.5% Tween/PBS buffer (250 µl) for one hour to remove weakly adsorbed protein.

Non-specific binding sites were blocked by treatment for two hours with 0.1% w/v casein in 10 mM trometamol (TRIS)-HCl pH 7.6 buffer containing 154 mM NaCl and 0.02% thiomersal (casein buffer). Serum samples were diluted 1:20 in casein buffer, and 50 µl aliquots were added to the wells in

quadruplicate. After overnight incubation at 4°C the wells were emptied and washed five times (250 µl/wash) with casein buffer. Bound IgG antibodies were detected using biotinylated protein A and avidin-HRP conjugate. Equal volumes of biotinylated protein A (0.8 µg/ml) and avidin-HRP (6.4 µg/ml) in casein buffer were mixed rapidly and 50 µl aliquots added to each well for four hours at room temperature. Wells were then washed five times with casein buffer. Chromogen solution (1.8 mM 2,2'-azinobis(3-ethylbenzthiazoline sulphonic acid), 0.1 mM H₂O₂ in PBS, 200 µl/well) was added and the colour developed at room temperature on a plate shaker. Absorbance at 414 nm was measured with a Bio-Rad model 2550 plate reader.

Standards of human IgG (0–100 ng/well) immobilised on the wells and blocked as described above were incorporated into each assay to relate colour development to the amount of bound IgG. Sera were classed as negative if absorbance was less than three SDs above the mean absorbance of the zero IgG standard. The Wilcoxon rank sum test and χ^2 test were used to analyse the differences in titres between disease groups and prevalence of positive samples in the different groups respectively.

SERUM SAMPLES

Serum samples were obtained from outpatients attending the rheumatology clinic. Patients with RA had definite or classical disease,²⁵ those with SLE satisfied the revised American Rheumatism Association criteria,²⁶ and those with AS the New York criteria.²⁷ Osteoarthritis was diagnosed on the basis of clinical and radiological findings. Patients with psoriatic arthritis had a deforming peripheral arthritis; two also had psoriatic spondylitis.

Results

DENSITY GRADIENT CENTRIFUGATION

The generation of crude proteoglycan fractions by this method has become a standard procedure since its development by Hascall and Sajdera.²⁸ Analysis of the top fraction from the dissociative gradient (A1A1D6) by SDS-PAGE showed appreciable amounts of link protein present, though several minor contaminant bands were also seen. The main contaminant (M_{av} approximately 65 kilodaltons) was probably human serum albumin. A more disperse band seen in the 65–75 kilodalton region might have resulted from variable cleavage of the hyaluronic acid binding region of core protein (HABR).

AFFINITY CHROMATOGRAPHY ON HYALURONIC ACID-SEPHAROSE 4B

Fig. 1a shows the eluting solutions used and a typical

Fig. 1A

Fig. 1B

Fig. 1 (A) Sequential elution of protein from a hyaluronic acid-Sepharose 4B column. The A1A1D6 fraction in 0.4 M GuHCl, 0.05 M sodium acetate, pH 5.8, was applied and the column washed with (a) 0.4 M GuHCl; (b) 1 M NaCl; (c) 1–3 M NaCl gradient; (d) 4 M GuHCl; (e) 0.2 M trometamol HCl, pH 8.0; (f) 0.2 M trometamol HCl, pH 8.0 plus pronase; (g) 0.5 M NaCl; (h) 4 M GuHCl. (B) Hyaluronic acid affinity column fractions a-h, a sample of bovine nasal cartilage link protein (s), and a sample of pronase (p) run on a 10% total acrylamide sodium dodecyl sulphate gel. Samples (10 µg and 5 µg) of fraction (d) eluted with 4 M GuHCl were applied. Three bands (48, 45, and 41 kilodaltons) corresponding to bovine nasal cartilage link protein standard and a trace of a high molecular weight contaminant are indicated (◄).

Fig. 2 Elution of affinity purified link protein applied in 1 M NaCl, 10 mM MOPS, pH 7.0, to a Sephadex G-150 SF column and eluted with the same buffer. Electrophoretic separation of fraction 1 on SDS-10% polyacrylamide gels shows bands at 48 and 45 kilodaltons and resolution of the 41 kilodalton band into three species.

elution profile. Link protein was eluted by the 4 M GuHCl step. The disperse HABR band, however, although reduced in level relative to link protein, was still present (Fig. 1b).

SEPHADEX G-150 SUPERFINE CHROMATOGRAPHY

Separation of the hyaluronic acid affinity purified link protein by chromatography on Sephadex G-150 SF (Fig. 2) yielded a large excluded peak (fraction 1) with a broad shoulder (fraction 2). Analysis by SDS-PAGE (Fig. 2) showed fraction 1 to contain only link protein, whereas in fraction 2 HABR was present in increased proportion relative to link protein. Also of interest was the resolution of the lower molecular weight band at 40 kilodaltons into several distinct components. Whether this is a product of the oligomerisation process has yet to be ascertained. All the bands interacted with a specific rabbit antiporcine link protein antibody (kindly provided by Dr T Hardingham, Kennedy Institute, London), previously shown to cross react with human link protein. Link protein from fraction 1 was used in the assay.

ANTI-LINK PROTEIN ANTIBODY ASSAY

During the development of the ELISA it was found that the binding of IgG to control wells was higher than to the link protein coated wells in a minority (<20%) of the sera. To overcome this a competitive binding assay was introduced. Soluble link protein (0–9 µg/ml diluted serum) was preincubated with the serum samples before their addition to the link protein coated plates. Subsequent steps in the assay were carried out as described in the 'Materials and

methods' section. Fig. 3 demonstrates the inhibitory action of added soluble link protein on IgG binding. Maximal inhibition was achieved at a soluble link

Fig. 3 Inhibition of antibody binding by added soluble link protein in the ELISA. A rheumatoid serum (■) and a normal serum (●) are shown.

protein concentration of 4.5 µg/ml. In subsequent assays the specific anti-link protein IgG binding was determined by subtraction of the mean binding in the presence of 4.5 µg link protein/ml from the mean binding in the absence of competing link protein. Intra- and interassay variability were assessed by (a) incorporation of a standard serum onto each plate within an assay and (b) relating values obtained for individual serum samples in several assays to a standard serum. Table 1 shows the mean and standard deviation (SD) of the standard serum incorporated on each plate in four assays. The coefficient of intra-assay variation ranged from 4 to 12% with a mean of 7.51%. Table 2 shows mean and SD values obtained for individual serum samples related to a standard serum in a number of assays. The interassay coefficient of variation ranged from 6 to 19% with a mean of 11.4%.

The variation in antibody levels with time was examined in four normal individuals selected on the basis of their absorbance readings in preliminary assays. The results showed that within an individual the serum level of anti-link protein antibody remains essentially constant over periods of up to five weeks (Fig. 4).

Antibodies reactive against link protein were detected in all groups (Fig. 5). The geometric mean titre of antibodies to link protein in the control group (8.61 ng) was very close to those in the RA (8.54 ng) and OA (10.03 ng) groups, and no significant differences were observed between the control population and any disease group. Only four sera (one control, one RA, and two OA) had antibody levels more than two SDs above the

positive control mean value. None exceeded this mean by more than three SDs.

Analysis of the prevalence of antibodies to link protein showed that there were no significant

Table 1 Coefficient of intra-assay variation (CV) calculated from the mean absorbance and standard deviation (SD) of a standard serum incorporated on all plates in four assays

Assay No	No of plates	Mean	SD	Coefficient of variation (CV) (%)
1	6	0.191	0.016	8.16
2	2	0.038	0.001	3.72
3	3	0.187	0.011	6.02
4	5	0.042	0.005	12.14

Mean coefficient of intra-assay variation (CV) = 7.51%.

Table 2 Coefficient of interassay variation (CV) calculated from the mean absorbance and standard deviation (SD) of individual sera measured in separate assays

Serum No	No of assays	Mean	SD	Coefficient of variation (CV) (%)
1	4	0.489	0.044	9.09
2	4	1.320	0.257	19.44
3	2	0.772	0.049	6.32
4	3	0.763	0.063	8.22
5	3	1.358	0.189	13.95

Mean coefficient of interassay variation (CV) = 11.40%.

Fig. 4 Anti-link protein antibody levels in serum samples from four normal individuals taken at intervals over a period of up to five weeks.

Fig. 5 Anti-link protein antibody bound in the ELISA. Normal, rheumatoid arthritis (RA), osteoarthritis (OA), psoriatic arthritis (PA), ankylosing spondylitis (AS), and systemic lupus erythematosus (SLE) sera are shown. Horizontal bars indicate the geometric mean values. $\square$ = Male, $\bullet$ = female subjects.

Table 3 Prevalence of anti-link protein antibodies in arthritic and non-arthritic groups

Condition	Proportion of subjects with anti-link antibodies		
	All	Men	Women
Control	21/83	7/34	14/49
RA	9/38	4/18	5/20
OA	7/29	2/10	5/19
PA	7/13	4/6	3/7
AS	3/9	2/7	1/2
SLE	4/9	—	4/9

differences between the control and arthritic groups, or between men and women (Table 3). The main patient groups were closely age matched (mean ages: RA 55.2 years; OA 55.0 years; control 48.4 years), and regression analysis showed no significant correlation of anti-link protein antibody levels with age in any group or overall.

Discussion

Articular cartilage contains large aggregates of proteoglycans bound to hyaluronic acid, which are

stabilised by link protein and entrapped within a collagen network. The human link protein prepared from osteoarthritic femoral heads was substantially pure and gave the electrophoretic pattern expected of normal link protein preparations.¹⁸ Although autoantibodies to cartilage collagens and proteoglycans have been described previously,^{1 6 8 13-16 29} the present study is the first to present evidence of circulating autoantibodies to link protein. The present ELISA assay was shown to be reproducible, and the specificity of antibody binding to immobilised link protein was demonstrated by the inhibitory effect of the addition of small amounts of competing soluble antigen. Serum anti-link protein antibody levels were stable over a period of up to five weeks in the four normal individuals studied.

The fact that ELISA assays present absorbance results as a continuous variable can present problems with regard to the definition of a normal range. Antibody titres significantly above zero were found in 25% of the normal control, 24% of the RA, 24% of the OA, 54% of the PA, 33% of the AS, and 44% of the SLE groups. There were no statistically significant differences between the groups either in the prevalence, the positive response, or the mean antibody titre. On another basis of defining the normal range, only one RA, two OA, and one control sera showed antibody titres greater than two SDs above the mean positive control titre. As antibody titres greater than three SDs above the mean are absent in all patients of the RA (n=38) and OA (n=29) groups, by applying χ^2 goodness of fit analysis it can be shown that the incidence of high titre antibodies in all patients with RA or OA is likely ($p < 0.05$) to be less than 8% and 10% respectively. The possibility that in arthritic patients excess circulating link protein arising from damaged articular cartilage causes erroneously low titres of antibody is being investigated.

Autoantibodies to a variety of antigens have been detected in normal individuals and may have a clearance role for the disposal of the products of catabolism.³⁰ It is possible that the antibodies detected in this study and autoantibodies to degraded glycosaminoglycans and hyaluronic acid detected in preimmune rabbits²⁹ fall into this category. An alternative possibility that structural mimicry leads to cross reaction between link protein and antibodies to foreign antigens cannot be excluded and requires further study.

In conclusion, we describe a reproducible assay for link protein antibodies. Such antibodies appear to be present in similar proportions and titre in both healthy persons and patients with different forms of arthritis. It thus appears that antibodies to link protein are unlikely to play a major part in the

pathogenesis of cartilage damage. Nevertheless it may be that, as with anticollagen antibodies,¹³ in small subgroups of patients anti-link protein antibodies are of significance.

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