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

A K Austin, R N Hobbs, J C Anderson, R C Butler, and B A Ashton

From the Department of Rheumatology, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry; and the Department of Biochemistry and Molecular Biology, University of Manchester Medical School, Manchester

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, and XI 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, dogs, and BALB/c mice. Adjuvant arthritis in mice may be triggered by structural mimicry between the immunising antigens on Mycobacterium tuberculosis and PG core protein. Conversely, as cartilage is broken down in mechanically induced experimental arthritis, autoimmunity to homologous cartilage proteoglycan and collagens develops.

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 antibodies or lymphoid cells or cloned T lymphocytes responsive to PG core protein 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.

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 immunologically with cartilage. 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, though the incidence of antibodies to denatured collagens is higher. Both cell mediated and humoral immunity to cartilage PG antigens have been demonstrated in patients with rheumatoid arthritis and ankylosing spondylitis. The high incidence of anti-PG antibodies reported in patients with RA by Grant...
al (38%; 11/29 patients) was obtained from experiments in which proteoglycan-rich extracts of cartilage, rather than purified proteins, were used.\textsuperscript{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\textsuperscript{-methylenebisacrylamide} (spectrally pure), silver nitrate, glycine, hydrogen peroxide (all Analytical), 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.\textsuperscript{17} The proteoglycans were then fractionated by associative and dissociative density gradient centrifugation by the method of Roughley et al.\textsuperscript{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.\textsuperscript{19} Link protein eluted in 4 M GuHCl was further purified by Sephaxex-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),\textsuperscript{20,21} which are excluded by G-150 Superfine. The dialysed solution was applied to the column (2.5×20 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.\textsuperscript{20}

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Samples were analysed electrophoretically by the method of King and Laemmli\textsuperscript{22} on 7.5-10% (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.\textsuperscript{23}

**Immunoblotting**

Conditions used in the electrophoretic transfer of proteins to nitrocellulose were as described by Burnette.\textsuperscript{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 (Immunoplate 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 tris(metamol) (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’-azino-bis(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 χ² 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, those with SLE satisfied the revised American Rheumatism Association criteria, and those with AS the New York criteria. 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. 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 (Mₓ 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...
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).

SPHADEX 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
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

<table>
<thead>
<tr>
<th>Assay No</th>
<th>No of plates</th>
<th>Mean</th>
<th>SD</th>
<th>Coefficient of variation (CV) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>0.191</td>
<td>0.016</td>
<td>8.16</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.038</td>
<td>0.001</td>
<td>3.72</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.187</td>
<td>0.011</td>
<td>6.02</td>
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<tr>
<td>4</td>
<td>5</td>
<td>0.042</td>
<td>0.005</td>
<td>12.14</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Serum No</th>
<th>No of assays</th>
<th>Mean</th>
<th>SD</th>
<th>Coefficient of variation (CV) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>0.489</td>
<td>0.044</td>
<td>9.09</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1.320</td>
<td>0.257</td>
<td>19.44</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.772</td>
<td>0.049</td>
<td>6.32</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0.763</td>
<td>0.063</td>
<td>8.22</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1.358</td>
<td>0.189</td>
<td>13.95</td>
</tr>
</tbody>
</table>

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.
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. Although autoantibodies to cartilage collagens and proteoglycans have been described previously, 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 χ² 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. It is possible that the antibodies detected in this study and autoantibodies to degraded glycosaminoglycans and hyaluronic acid detected in preimmune rabbits 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

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Proportion of subjects with anti-link antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>All</strong></td>
</tr>
<tr>
<td>Control</td>
<td>21/83</td>
</tr>
<tr>
<td>RA</td>
<td>9/38</td>
</tr>
<tr>
<td>OA</td>
<td>7/29</td>
</tr>
<tr>
<td>PA</td>
<td>7/13</td>
</tr>
<tr>
<td>AS</td>
<td>3/9</td>
</tr>
<tr>
<td>SLE</td>
<td>4/9</td>
</tr>
</tbody>
</table>

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**Humoral immunity to link protein**

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. □=Male, ○=female subjects.
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References


