Immune complexes in ankylosing spondylitis

I L MacLean, J R Archer, M I D Cawley, B L Kidd, B P O'Hara, F S Pegley, P W Thompson

Abstract
Immune complexes have been reported in ankylosing spondylitis (AS) and may implicate infectious agents. Serum samples from 49 patients with AS were assayed for immune complexes by polyethylene glycol precipitation, followed by radial immunodiffusion and pepsinogen binding immunoassay. Both methods showed increases in IgA containing immune complexes, which correlated with serum IgA and with IgA rheumatoid factor concentrations, but did not show increases in other immune complex components. Increased immune complexes were associated with peripheral joint synovitis, but showed no correlation with other clinical or laboratory indices of disease activity. Immune complexes from nine AS serum samples and one AS synovial fluid were electrophoretically separated then probed with anti-Klebsiella pneumoniae, but AS specific antigens were not identified. This study did not suggest a major role for immune complexes in AS without peripheral disease, nor provide serological evidence for the involvement of klebsiella antigens.

There is epidemiological evidence for environmental factors in ankylosing spondylitis (AS), and the clinical overlap between AS and reactive arthritis suggests an infectious agent. Increases in the serum IgA concentration in AS, and additional circumstantial evidence includes increased bowel permeability and subclinical distal ileitis in a proportion of patients with AS.

Ebringer’s group reported an immunological cross reaction between HLA-B27 and Klebsiella pneumoniae, with increased antibodies to klebsiella and faecal carriage of klebsiella in patients with active AS, whereas Geczy investigated a klebsiella product (‘modifying factor’) which specifically interacts with B27. Unfortunately, others have had difficulty repeating these observations, and an alternative approach to studying the involvement of klebsiella antigens in AS seems appropriate.

Although the classical clinical manifestations of immune complex disease are not prominent in AS, there are anecdotal reports of IgA deposition glomerulonephritis in AS, and a Leiden group has investigated IgA containing immune complexes in patients with AS. A number of studies have reported increased concentrations of circulating immune complexes in AS (reviewed by Mcguigan et al). The prevalence of increased immune complexes varies markedly between studies, and seems to be associated with active disease and, in particular, with peripheral joint synovitis. One group reported disease specific immune complexes in AS, but the antigen was not characterised and this finding has not been confirmed. Yersinia antigens have been detected in circulating immune complexes from patients with post-yersinial reactive arthritis, and we suggested that immune complexes from patients with AS may similarly implicate particular bacterial antigens in AS.

In this study immune complexes were measured in serum samples from patients with AS by two different methods and related to indices of disease activity, and immune complexes from selected subjects were probed for the presence of klebsiella antigens.

Materials and methods

CLINICAL MATERIAL
The 49 patients with AS and 23 control subjects were part of a group described previously. Ten patients with AS had peripheral joint synovitis. Seven were taking sulphasalazine and the remainder non-steroidal anti-inflammatory drugs, simple analgesics, or no drugs. Blood was collected into prewarmed (37°C) 20 ml glass universals and maintained at 37°C throughout clotting, transport to the laboratory, and serum separation. Serum samples were frozen in multiple aliquots at −70°C, and thawed once only. Synovial fluid from the knee joint of one patient with AS was processed similarly.

IMMUNE COMPLEX ASSAYS
Immune complexes were precipitated by 2% polyethylene glycol (PEG), molecular weight 6000, and the concentrations of C1q, C3c, IgG, IgM, and IgA in the PEG insoluble fractions were determined by single radial immunodiffusion using a commercial kit (Merrid M202; Mercia Diagnostics). In an enzyme linked immunosorbent assay (ELISA), based on the affinity of immune complexes for proteolytic enzymes, ELISA plate wells were coated for 24 hours at 4°C with 100 ml freshly prepared 25 μg/ml porcine pepsinogen (EC 3.4.23.1; Sigma; marketed as ‘Pepsin’) in bicarbonate buffer, pH 8.3. The wells were washed twice with 0.05% Tween 20 in phosphate buffered saline (PBST), blocked with 200 μl 1% bovine serum albumin in PBST (PBST–BSA), then washed a further four times. Triplicate 100 μl aliquots of patient serum diluted (1:10 for IgG and IgM, 1:5 for IgA) in PBST–BSA were
added for three hours at 37°C. Pepsinogen binding immune complexes were detected with peroxidase conjugated rabbit antibody to human IgG, IgM, or IgA (Dako) in PBST–BSA, followed by H2O2/o-phenylenediamine substrate solution and measurement of absorbance at 492 nm. The means of the triplicate absorbance values were normalised relative to that of a reference serum from a healthy subject (arbitrarily designated as 100%) included on each plate, to allow comparison between assays.

**IgA Rheumatoid Factor**

IgA rheumatoid factor was measured by a modification of the method of Haskard et al. Briefly, ELISA plate wells were coated with 2.5 μg human IgG in PBS, then washed and treated with PBST–BSA. Serum (100 μl) diluted 1:100 in PBST–BSA was added, in triplicate, for three hours at 37°C, followed by washing, then addition of 1:500 peroxidase–anti–IgA and substrate as above. The optimal serum dilution was determined by preliminary titration. The secondary antibody was absorbed with human IgG before use.

**Probing of Immune Complexes for Klebsiella Antigen**

Immune complexes for qualitative analysis were prepared from serum samples of nine patients with AS, selected according to various arbitrary criteria of disease activity (raised PEG precipitated or pepsinogen binding immune complexes (8/9), peripheral joint synovitis (6/9), (6/9), early morning stiffness >30 min (6/9), erythrocyte sedimentation rate >30 mm in 1st hour (6/9), and/or increased serum IgA (3/9)), and from the synovial fluid from the knee joint of one patient with AS, using 3% PEG. Controls were two healthy male laboratory volunteers, two healthy HLA–B27 positive subjects, and two male patients with active rheumatoid arthritis. The *Klebsiella pneumoniae* serotype K43 isolate ‘BTS1’ was propagated in nutrient broth, inactivated in formalin, and disrupted by vortexing with glass beads followed by ultrasonication. Rabbit antiserum No 85 was supplied by Dr Geczy, and in his hands reacts with cells bearing modifying factor from patients with AS, and antiserum No 157 was produced by hyperimmunisation with *K1 pneumoniae* serotype F77. In a preliminary ELISA both antisera bound strongly to the klebsiella sonicate, though the titre of the No 85 was about twice that of No 157. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) was performed under reducing, denaturing conditions with a vertical slab ‘minigel’ system (Atto), using immune complexes diluted 1:2 to 1:15 relative to the original serum concentrations. The separated immune complex components were electroblotted to a nitrocellulose membrane (Biorad) then probed with anti-klebsiella (1/25–1/250) in PBST–BSA, followed by peroxidase conjugated swine-anti-rabbit immunoglobulin, then H2O2/diaminobenzidine substrate. Immunoblot bands were compared with protein bands noted after 0-1% Coomassie blue staining of parallel unblotted gels.

**Results**

**Measurement of Immune Complexes**

Figure 1 shows the results of assays for PEG precipitated immune complexes, pepsinogen binding immune complexes, and IgA rheumatoid factor. Almost all of the AS C1q, C3c, IgG, and IgM PEG precipitated immune complexes concentrations were within the range spanned by the control serum samples. Twenty six of 49 (53%) of AS IgA results exceeded the mean value for the control group plus two standard deviations, however, with the AS group as a whole significantly higher (p<0.02, Mann–Whitney U test). Sixteen per cent of AS serum samples had increased IgA pepsinogen binding immune complexes, with the AS group again significantly higher (p<0.05).

Of the 10 AS subjects with peripheral joint synovitis, eight had increased immune complex concentrations by one or both assays. The serum total IgA concentration correlated moderately with the IgA pepsinogen binding immune complex concentration (R=0.33, p<0.05), but not with the IgA PEG precipitated immune complex concentration. No other immune complex parameter correlated with serum immunoglobulin concentrations, or with erythrocyte sedimentation rate, serum cytokine deaminase concentration, morning stiffness, or a subjective visual analogue pain scale (detailed results not included). Ankylosing spondylitis IgA rheumatoid factor concentrations were higher than those of healthy subjects (p<0.001) and correlated both with the IgA pepsinogen binding immune complexes level (R=0.50, p<0.001) and with serum IgA concentration (R=0.30, p<0.05).
Discussion
This study investigated immune complexes in the serum samples of patients with AS and correlated the results with peripheral joint synovitis and disease activity, as an alternative approach to studying the involvement of klebsiella antigens. Polyethylene glycol (2%) precipitation and pepsinogen binding assays showed increased IgA immune complexes in AS; other results did not differ significantly from those for healthy controls. In agreement with previous reports, which used a variety of different assay systems, increases were noted more often in subjects with peripheral joint synovitis, but the correlation with other clinical and laboratory indicators of disease activity (apart from serum IgA concentration) was poor. Correlation between the two assays was also disappointing. The World Health Organisation’s 1977 collaborative study on 18 different methods for immune complex detection similarly noted poor agreement between different methods.

There has been little previous investigation of IgA rheumatoid factor in AS. It may impair solubilisation of serum immune complexes and hence raise the apparent serum immune complex concentrations. Increases in total serum immunoglobulin concentration may also lead to the precipitation of non-complexed immunoglobulin by PEG. The correlation found between IgA containing immune complexes and both serum IgA and IgA rheumatoid factor concentrations in patients with AS may in part be due to these phenomena, but their precise contribution is hard to ascertain. Two studies have previously reported IgA rheumatoid factor in AS, and in the latter this was associated with the presence of immune complexes. By contrast, an earlier study using rabbit IgG Fc as the target antigen did not detect IgA rheumatoid factor in AS serum samples. Immune complex detection methods do not discriminate between true specific antibody–specific antigen’ immune complexes and less specific immunoglobulin aggregates formed in vivo or in vitro, and existing methods cannot entirely eliminate this as a source of artefact.

We are not aware of previously published attempts to detect specific bacterial antigens in AS immune complexes. Bands noted on western blotting of immune complexes with anti-klebsiella in this study were weak and not disease specific, and may represent non-specific binding by the many non-immune complex proteins precipitated by PEG. Given the difficulties in identifying microbial antigens in immune complexes, and without comparable parallel experiments with immune complexes from patients with unequivocal postinfectious reactive arthritis, we cannot exclude a failure to detect klebsiella antigens on technical grounds. Another group also obtained negative results using similar methods (Espinoza, L, personal communication). The involvement of klebsiella in AS remains open to speculation.

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