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Raised serum IgG and IgA antibodies to mycobacterial antigens in rheumatoid arthritis

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SUMMARY Autoantigens cross reactive with mycobacteria are implicated in the pathogenesis of adjuvant arthritis in the rat, and there are reports of changes in the immune response to mycobacteria in human rheumatoid arthritis (RA). We have therefore examined the IgM, IgG, and IgA antibody levels to crude mycobacterial antigens and to two recombinant mycobacterial heat shock/stress proteins (65 kD and 71 kD) in sera from patients with RA, systemic lupus erythematosus (SLE), and Crohn's disease, and from healthy controls. IgA binding to the crude mycobacterial antigens was significantly raised in RA sera, though IgG and IgM binding tended to be lower than in controls. Both IgA and IgG binding to the heat shock proteins were significantly raised in the RA sera. Smaller significant rises in both classes were seen in sera from patients with SLE, and in the IgA class only to the 65 kD protein in Crohn's disease. The rises in IgG and IgA antibodies to the 65 kD protein in RA were significantly higher than in the other diseases, however. It is interesting that this protein is the one responsible for adjuvant arthritis in the rat.

Key words: antibodies to heat shock proteins, antibodies to stress proteins.

Rheumatoid arthritis (RA) is believed to be an immunological disease, possibly autoimmune, of unknown aetiology. Adjuvant arthritis, which can be induced in rats by immunisation with mycobacteria in oil, is considered by some investigators to be a model of RA. This disease can be transferred to susceptible rats by T cell clones specific for *Mycobacterium tuberculosis*.[1-4] These arthritogenic clones were found to recognise an acetone precipitable fraction of *M tuberculosis*. Interestingly, patients with RA were also reported to have raised T cell responses to an acetone precipitable fraction.[5] This, however, has recently been challenged by the observations that the T cell responses to an *M tuberculosis* antigen in patients with RA are DR4 linked and not associated with RA.[6] More recently van Eden and colleagues have shown that the arthritogenic rat T cell clones recognise a 65 kD protein, which is a component of the acetone precipitable fraction preparation.[7] Evidence that the rat model may indeed be relevant to the human condition is accumulating. Firstly, an association was detected between skin test responsiveness to tuberculin and DR4 in patients with leprosy,[8] and more recently in RA.[9] Secondly, antibody levels to crude mycobacterial sonicates in sera from patients with RA living in Kuwait (a mycobacterium rich environment) showed significant correlations with HLA-DR haplotypes known to be relevant to susceptibility to RA.[10]

The present study examines the possible associations of antibody levels to mycobacteria, and in particular to the 65 kD antigen, implicated in the adjuvant athritis model, in patients with RA living in the UK. It has been found that although patients with RA only showed raised IgA antibodies to crude mycobacterial antigens, IgG as well as IgA antibody levels were raised to the 65 kD and 71 kD antigens.

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Patients and methods

SOURCE OF SERUM SAMPLES

This study was carried out on 85 patients with RA,

as defined by the American Rheumatism Association criteria, attending the outpatient clinic of the Bloomsbury rheumatology unit. Sixty three were female and 22 male, mean age 55 years (range 27–78), duration of disease ranged from two to 20 years, disease activity was based on C reactive protein levels (Abbot Laboratories kit). They were HLA-DR tissue typed.

Eighteen patients with systemic lupus erythematosus (SLE) attending the above clinic were studied as a control disease as SLE is also a non-organ specific autoimmune disease. Seventeen were female and one male, mean age 42 years (range 30–68).

Twenty one patients with Crohn's disease were also included in the study as a non-autoimmune disease group. Ten were female and 11 male, mean age 36 years (range 18–74).

Forty five healthy laboratory staff were studied as a control group. Twenty seven were female and 18 male, mean age 35 years (range 24–63).

SOURCE OF ANTIGENS

A water extract of *M tuberculosis* $H_{37}Ra$ (Difco) was prepared as follows. Heat killed, desiccated *M tuberculosis* were ground with a homogeniser, suspended in double distilled water at 1 mg/ml, stirred for eight hours at 4°C, centrifuged for 30 minutes at 15 000 *g*, and the supernatant was lyophilised. This antigen is referred to as WE. Sonicates of fresh bacilli from *M tuberculosis* (TB) and *M vaccae* (VAC) were also used.[11] The recombinant forms of the 65 kD protein of *M bovis* BCG[12] and the 71 kD protein of *M tuberculosis* (Mehlert and Young, in preparation) were also used. These antigens are referred to as 65 kD and 71 kD respectively.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The antigens were coated at 10 μg/ml for WE, TB, and VAC and at 1 μg/ml for 65 kD and 71 kD in carbonate buffer (0·05 M, pH 9·6) onto immunoplates (Nunc) and incubated overnight at 4°C. Excess antigen was washed off with phosphate buffered saline (0·1 M, pH 7·4) containing 0·05% Tween 20 (PBS/T). The test sera were plated in doubling dilutions from 1/50 to 1/400 in PBS/T in duplicate and incubated for two hours at room temperature. After further washes with PBS/T the affinity purified $F(ab')_2$ fragments of horseradish peroxidase conjugated human antibodies (Sigma) were added at 1/1000 dilution in PBS/T and incubated overnight at 4°C. The washing process was repeated, and 0·5 mg/ml of 2,2′-azinobis-(3-ethylbenzthiazoline sulphonic acid) (Sigma) in citrate phosphate buffer (0·1 M, pH 4·1) with 0·35 μl/ml H_2O_2 vol 20 (6% w/v) was added. After approximately 30 minutes the reaction was stopped with 96 mg/ml of sodium fluoride (Sigma) in double distilled water, and the absorbance was measured at 650 nm with a Titertek multiscan ELISA reader (Flow). Throughout the assay the volume of reagents added per well at each step was 100 μl, each wash step was repeated three times with three minutes' incubation between washes at room temperature, and for each individual assay the same positive and negative controls were used.

DATA ANALYSIS

The values of optical density (OD) ratio were calculated as OD_{650} of test/OD_{650} of positive serum control in each plate. The serum dilutions used for the calculations were those within the linear phase of the antibody binding curve and were 1/100 dilution for the IgA ELISA of 65 kD antigen and 1/200 for all the other assays. As the mean age and sex of the individuals for the four groups studied were not the same the data were analysed for evidence that the age or sex influenced the antibody levels. The data were also analysed with respect to activity and duration of the disease.

HLA-DR TYPING

The HLA typing was carried out by The London Hospital, and the frequencies of DR haplotypes in the 85 patients with RA studied were DR1:15, DR2:13, DR3:19, DR4:47, DR5:10, DR6:15, DR7:8, DR8:1, DR9:1, DR10:1, and DR11:1. The data were examined for association between HLA-DR haplotypes (for which not less than five individuals were available) and antibody levels.

STATISTICAL ANALYSIS

The Mann-Whitney U two tailed test was used to compare the antibody levels in the control and experimental groups. The same test was also used to compare antibody levels of patients with RA in association with HLA-DR haplotypes. The Spearman rank correlation coefficient was used to compare antibody levels of the same group of individuals to different antigens as well as to look for correlation between raised antibody levels and sex, age, disease activity, and duration of the disease. Statistical analysis was carried out using the STSC STATGRAPHICS software package.

Results

INCREASED IgA ANTIBODY LEVELS TO CRUDE MYCOBACTERIAL ANTIGENS IN RA

Patients with RA were found to have raised IgA serum antibody levels to the WE ($p<0·01$), TB

($p<0{\cdot}0001$), and VAC ($p<0{\cdot}0001$) antigens in comparison with healthy controls. Serum samples from 19/64, 19/64, and 29/64 patients with RA showed IgA levels more than 2SD above those of the healthy control group levels to WE, TB, and VAC respectively. In addition, the IgG and IgM antibody levels to the same antigens were lower in RA than in controls (Fig. 1). Serum samples from patients with SLE and Crohn's disease were not tested against the crude antigens.

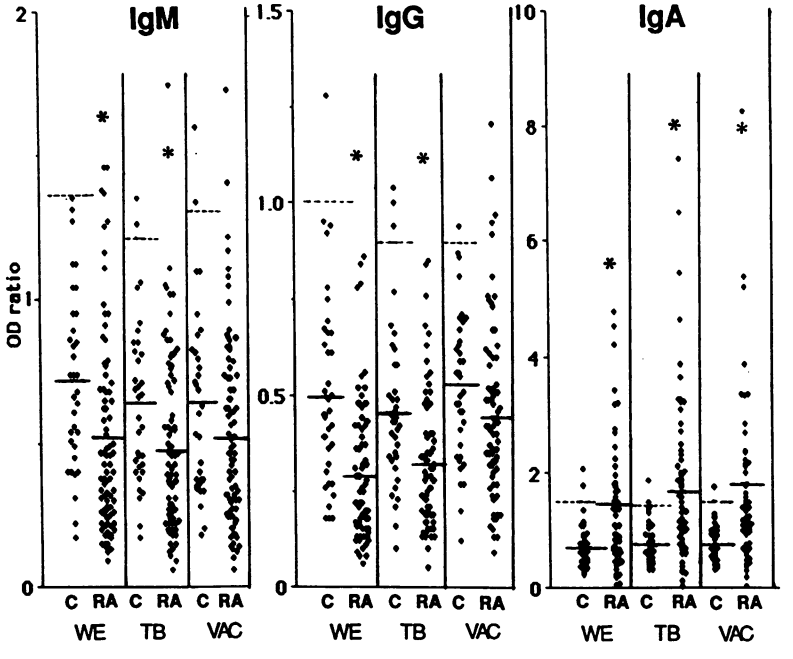

Fig. 1 *Antibody levels to WE, TB, and VAC antigens in healthy controls and patients with RA. The antibody levels are expressed as OD ratios for each group (OD ratio=OD of test/OD of control positive serum in each plate). Horizontal lines represent the mean OD ratio for each group and the dotted lines define 2SD of the control mean.* $p<0{\cdot}01$. *OD=optical density; C=control; RA=rheumatoid arthritis; WE=a water extract of M tuberculosis; TB=sonicates of M tuberculosis; VAC=sonicates of M vaccae.*

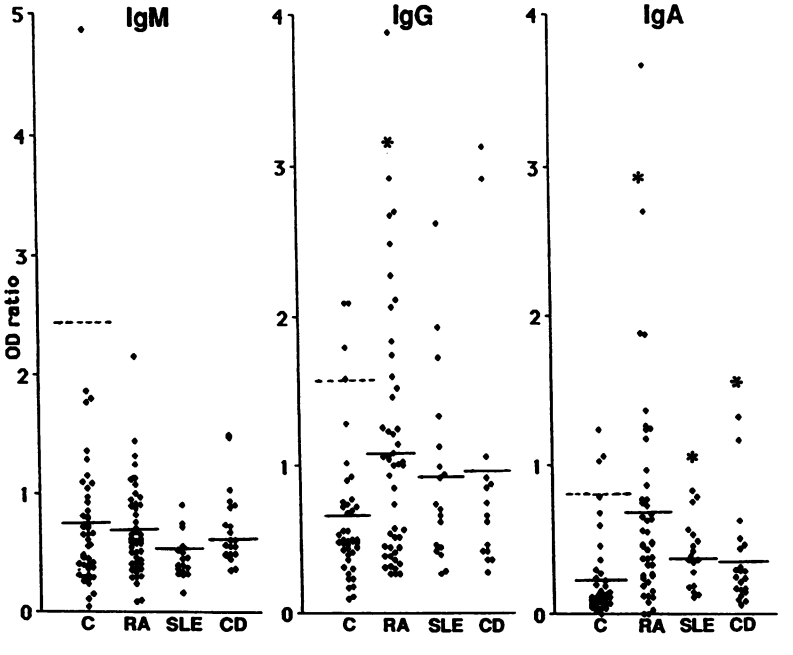

Fig. 2 *Comparison of the antibody levels to the 65 kD protein in healthy controls (C), and in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Crohn's disease (CD). Data presented as in Fig. 1.* $p<0{\cdot}01$.

INCREASED IgA AND IgG ANTIBODY LEVELS TO THE 65 kD AND 71 kD MYCOBACTERIAL ANTIGENS IN RA

When defined mycobacterial antigens were used the differences in antibody levels in RA and controls were not confined to the IgA class only ($p<0.0001$). Patients with RA also showed raised IgG antibody levels to both antigens when compared with the controls ($p<0.01$), while their IgM levels were lower (but not significantly) to both 65 kD and 71 kD antigens (Figs 2 and 3). Serum samples from 11/45 and 9/19 patients with RA showed IgA levels to the 65 kD and 71 kD antigens respectively which were more than 2SD above those of the healthy control mean. Also, 10/42 (to the 65 kD) and 7/39 (to the 71 kD) RA sera showed raised IgG levels more than 2SD above those of the control values.

ANTIBODY LEVELS TO THE 65 kD AND 71 kD MYCOBACTERIAL ANTIGENS IN PATIENTS WITH SLE AND CROHN'S DISEASE

Patients with SLE were also found to have raised IgG ($p<0.001$) and IgA ($p<0.001$) antibody levels to both antigens, but their IgM levels were not significantly different from the control levels. Although serum IgG levels were raised in SLE to the 65 kD protein, the quantity of antibodies was lower than that seen in sera from patients with RA (Figs 2 and 3). Only 3/18 sera had IgG levels more than 2SD above those of the controls to the 65 kD and 4/18 to the 71 kD antigens. With regard to IgA levels, only 1/18 sera was more than 2SD above those of the control to the 65 kD, while 10/18 sera were higher to the 71 kD protein.

Patients with Crohn's disease, on the other hand, were also found to have significantly raised IgA antibody levels to the 65 kD antigen ($p<0.001$) but again not to the same degree as the RA group (Fig. 2). Only 2/21 sera for the 65 kD antigen had a significantly higher IgA level than the control group.

LACK OF RELATION BETWEEN RAISED ANTIBODY LEVELS AND AGE, SEX, DURATION, AND DISEASE ACTIVITY

Serum samples of patients with RA with significantly higher IgG and IgA antibody levels were compared with RA sera with antibody levels equal to control levels. No correlation was found between antibody levels and age, sex, disease activity, and disease duration. Similarly, the few patients with SLE and Crohn's disease whose levels were more than 2SD above the control mean did not differ clinically from those with normal values.

ANTIMYCOBACTERIAL RESPONSES AND DR HAPLOTYPE

The data on mycobacterial responses were examined for associations between antibody levels and HLA-DR haplotypes as other investigators have suggested such associations. Our data showed no

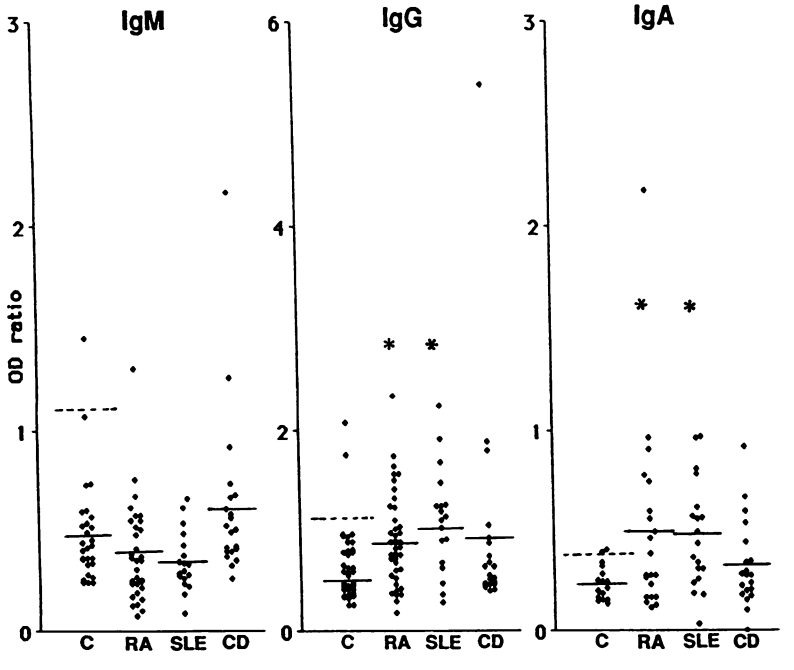

Fig. 3 *Comparison of antibody levels to the 71 kD protein in healthy controls (C), and in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Crohn's disease (CD). Data presented as in Fig. 1. *p<0·01.*

association with the DR haplotypes shown to be relevant to RA, such as DR4, DR2, and DR7. Although DR1 patients with RA showed higher IgA antibodies to the WE ($p<0.06$), TB ($p<0.05$), and VAC ($p<0.02$) antigens than non-DR1 individuals, these p values do not remain significant when corrected for the number of comparisons made.

Discussion

This study has shown that patients with RA have significantly raised antibody levels of the IgA class to the crude mycobacterial antigens WE, TB, and VAC. Our data are consistent with a recent study in Kuwait using TB, VAC, and five similar preparations from other species, where there was a tendency towards raised IgA antibody levels in RA sera, though significance was only reached with *M nonchromogenicum*. All antibody and skin test responses to mycobacteria are high in Kuwait, however, as the environment is rich in mycobacteria.[10]

To obtain more information about the specificity of antibodies to the mycobacteria in the RA sera we examined the levels of the different classes of antibodies to the recombinant 65 kD and 71 kD proteins. Both IgG and IgA levels were increased to these 'clean' antigens. The fact that these proteins show no homology with each other indicates that antibodies to at least two major mycobacterial antigens are raised in these patients with RA.

Raised levels to one recombinant protein did not correlate with the antibody levels to the other protein. In fact, analysis of 10 individual patients' sera with higher levels of IgG antibodies to the 71 kD protein than control sera (OD ratio >mean OD ratio +2SD) showed that two sera were also high for the 65 kD protein, the other eight being low for this antigen. Furthermore, some sera with high IgA levels to the 71 kD protein were lower for the 65 kD protein. These observations, together with the findings of lower (but not significant) IgM levels in patients with RA to all mycobacterial antigens tested, argue against polyclonal activation as a source of raised antibodies. Moreover, it is interesting that in Kuwait, where responsiveness to mycobacteria is very high, this tendency for IgM binding to mycobacteria to be low in RA was significantly correlated with DR7, a haplotype known to be protective in RA.[10]

No significant differences were seen in duration and activity of the disease, sex, or age of the patients with respect to antibody levels. This indicates that although raised levels of antibodies are found in these patients with RA, a relation with the disease itself is not yet clear. In this regard we analysed the relation with DR to determine whether the raised levels could be correlated with known DR haplotypes previously shown to be related to RA—for example, DR4.[13–15] No association was found between antibody levels and DR4. This result appears to be different from data on T cell responses to mycobacteria, which have been correlated with DR4 expression in RA and healthy individuals,[6] and may be important in relation to the pathogenesis in DR4 individuals. A parallel study in Kuwait with the same antigen preparations has shown comparable results with regard to raised antibody levels to the 65 kD protein in RA and lack of meaningful DR correlation (Bahr *et al*, unpublished data).

It is interesting that IgG and IgA antibody levels were raised to both the 65 kD and 71 kD proteins in patients with SLE, suggesting that the increase of antibodies in itself is not disease specific. IgG and IgA antibodies to the 65 kD protein in SLE and IgA in Crohn's disease were lower than in patients with RA, whereas antibodies in SLE sera to the 71 kD protein were raised but not significantly different from those in patients with RA ($p>0.1$). This argues in favour of the importance of the 65 kD mycobacterial protein in this disease as previously suggested from cellular studies in the rat model of RA.[7] Further recent data support this concept as IgG antibodies to the 65 kD protein from *Escherichia coli* are not raised in sera from patients with RA (paper in preparation). It is clear, however, that the mycobacterial proteins used here show high degrees of homology with similar proteins in both prokaryotic and eukaryotic organisms. Thus we cannot at this stage reliably implicate the mycobacteria as the major immunogens leading to the responses measured here.

The 65 kD and 71 kD mycobacterial proteins have recently been shown to be heat shock/stress proteins.[16] They show a great degree of homology with human stress proteins, and recent evidence has supported their role in inflammation.[17] Antibodies to a 90 kD heat shock protein have been shown to be raised in SLE sera.[18] The human 70 kD protein has been shown to be spontaneously synthesised by chondrocytes in patients with severe osteoarthritis compared with chondrocytes in healthy controls.[19] In this regard we have shown that IgG and IgA antibodies to the human 70 kD protein are also raised in the sera of these same patients with RA (paper in preparation).

In further studies we hope to evaluate the relevance of the specific antibodies to the various stress proteins (especially the 65 kD) in RA.

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