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HLA-B27 and the immune response to enterobacterial antigens in ankylosing spondylitis

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SUMMARY

Total serum immunoglobulins and class specific serum antibodies to *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa* were measured in 107 patients with ankylosing spondylitis (AS) and 110 healthy tissue typed controls by enzyme linked immunosorbent assay (ELISA). The specificity of this technique was confirmed by the use of specific bacterial murine antisera and by cross-absorption of human sera by specific bacteria. Total serum IgA in AS patients correlated with both erythrocyte sedimentation rate (ESR) ($P < 0{\cdot}001$) and C-reactive protein ($P < 0{\cdot}05$) and was significantly elevated compared to healthy individuals ($P < 0{\cdot}001$). A significant elevation of IgA antibodies to *K. pneumoniae* was detected in the serum of AS patients with active disease when compared to healthy controls ($P < 0{\cdot}01$). These studies support the involvement of an enterobacterial micro-organism in the pathogenesis of AS and further relate to the role of HLA-B27 in this disease.

Keywords HLA-B27 Klebsiella ankylosing spondylitis enterobacteriaceae HLA linked diseases

INTRODUCTION

The development of a seronegative reactive arthritis following enteric infection by certain Gram negative bacteria, in particular members of the family Enterobacteriaceae, is known to be linked with the expression of the class I MHC product HLA-B27 (Aho *et al.*, 1974).

Individuals with this phenotype inherit a susceptibility to develop arthritis as a reaction to infection by these bacteria. HLA-B27 is present in more than 90% of patients with ankylosing spondylitis (AS) (Caffrey & James, 1973; Brewerton *et al.*, 1973; Schlosstein *et al.*, 1973), but the mechanism linking the marker to the disease remains obscure. The possibility that a microbial agent is involved in the aetiopathogenesis of AS has been suggested following the independent demonstration by a number of laboratories of an elevated faecal carriage of *Klebsiella pneumoniae*, a member of the Enterobacteriaceae, in patients with active disease (Ebringer *et al.*, 1978; Kuberski *et al.*, 1981; Eastmond *et al.*, 1982).

The manifestation of chronic arthritis following *Yersinia enteritis* is known to be associated with the formation and persistence of specific serum antibodies of the IgA class (Granfors *et al.*, 1980). In AS, measurements of disease activity have been shown to correlate with both serum IgA (Cowling, Ebringer & Ebringer, 1980b) and secretory IgA concentrations (Calguneri *et al.*, 1981). It may be that patients with AS are reacting in a similar immunological fashion to some micro-organisms present in their gut, as do patients with reactive arthritis to known precipitating pathogens. To

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assess this possibility, we developed an enzyme linked immunosorbent assay (ELISA) in order to measure class specific antibodies in AS patients to Gram negative bacteria, especially the Enterobacteriaceae. The genetic component of this response, in the absence of clinical disease, was studied by comparing the antibody level to these bacteria in HLA-B27 positive and negative healthy individuals.

MATERIALS AND METHODS

Patients. Blood was obtained from 107 patients attending the Ankylosing Spondylitis Research Clinic at the Middlesex Hospital: 75 patients were seen once, 26 patients were seen twice, five patients were seen three times and one patient was bled on four separate occasions, making a total of 146 sera available for antibody studies. All sera were kept at −20°C prior to assay. The shortest interval between visits was 1 month and the New York criteria were used for the diagnosis of AS, as previously described (Ebringer *et al.*, 1978). Control sera were obtained from 110 healthy tissue typed bone marrow donors attending the Anthony Nolan Laboratories at St Mary Abbot's Hospital. The sera were selected at random from a stored collection and coded prior to analysis.

Blood samples. Serum IgA, IgG, IgM and C-reactive protein (CRP) estimations were carried out by the single radial immunodiffusion technique using commercially prepared antisera and serum standards (Seward Immunostics). The erythrocyte sedimentation rate (ESR) was measured by the Westergren Method.

Disease activity. For statistical analysis sera obtained from the patients were divided into three grades of disease activity as assessed by the serum level of the acute phase reactant: CRP. Sixty patients with a serum CRP concentration greater than or equal to 30 μg/ml were considered to have active disease; 56 patients with a CRP level between 10–30 μg/ml were considered moderately active and in 30 patients a CRP of less than 10 μg/ml was considered to indicate inactive disease.

Bacterial antigens. K. pneumoniae and *Y. enterocolitica* (Department of Rheumatology, Middlesex Hospital), *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Department of Clinical Bacteriology, Guy's Hospital) were cultured in a minimal salts medium (Clowes & Hayes, 1980) supplemented with 2 g/ℓ glucose. The bacterial cultures were centrifuged at 1,000 g (MSE-6L centrifuge) for 30 min, the supernatant decanted, millipore filtered (0·45 μm) and freeze dried before reconstitution in 0·15 M phosphate-buffered saline at pH 7·2 (PBS) to a final concentration of 50 mg/ℓ protein ($E^{1\%}_{280^{nm}}$) using purified human IgG as standard.

ELISA. The details of this method designed to estimate antibodies to bacterial antigens have been described elsewhere (Trull *et al.*, 1983).

In brief, the assay employs a double antibody technique: bacterial antigen in microtitre plate is treated first with human test or control serum, then with class specific rabbit anti-human serum (DAKO Immunoglobulins) and developed with goat anti-rabbit immunoglobulin alkaline phosphatase conjugate (Miles Yeda Ltd) and substrate. The results are expressed as OD units × 100. Comparison of ELISA results between different groups was analysed using Student's t-test.

ELISA specificity. (i) In order to assess the specificity of the ELISA in estimating serum antibody levels to Gram negative bacterial antigens, antisera were raised in mice against the four organisms selected for study. Batches of five female BALB/C mice were set up, each received 4 weekly intraperitoneal inoculations with a 200 μℓ saline suspension of bacteria at 10^8 organisms per ml. Pre- and post-immunization sera were pooled from the five mice in each batch prior to storage at −20°C. Each serum was then tested by checkerboard analysis against every bacterial antigen in the ELISA by substituting a rabbit anti-mouse immunoglobulin preparation (DAKO) for the rabbit anti-human preparation described above. Mouse antisera to whole bacteria reacted with their respective antigens (Table 1).

(ii) Absorption studies—the specificity of the ELISA for human antibodies was tested by absorbing human sera known to contain elevated titres of specific antibody to a particular bacterium with the homologous organism as well as each of the remaining bacteria selected for study. The sera were diluted 1/20 in PBS and divided into five 1 ml volumes.

To four of the aliquots was added 1 mg freeze dried, inactivated preparation of *K. pneumoniae*,

Table 1. Antibody activity after absorption

	Mouse antiserum specificity				
	Pre-immunization	*K. pneumoniae*	*S. typhimurium*	*Y. enterocolitica*	*P. aeruginosa*
K. pneumoniae	2·0	55	8	6	6
S. typhimurium	1·5	7·5	73	8·5	13·5
Y. enterocolitica	1·0	7·5	7	55·5	18·5
P. aeruginosa	1·5	6	2	9·5	102·5

Absorption of antibody to Gram negative bacteria following a single treatment with inactivated homologous and heterologous species. Results expressed as percentage absorption from pre-absorption antibody value as measured by ELISA.

Table 2. Specificity of ELISA for antibody to bacterial antigens

	Absorbing bacterium			
Test antigen	*K. pneumoniae*	*S.typhimurium*	*Y. enterocolitica*	*P. aeruginosa*
K. pneumoniae	62	12	3	0
S. typhimurium	2·9	40	0	2
Y. enterocolitica	3·0	0	40	0
P. aeruginosa	14	0	0	73

Results expressed as OD × 100.

Y. enterocolitica, *S. typhimurium* or *P. aeruginosa*. The sera were absorbed for 12 h at 4°C on a Rolamix (Luckham Ltd) and the bacteria removed by centrifugation. The absorbed sera, together with the unabsorbed sample, were diluted a further 1/5 in assay buffer and retested by ELISA. The degree of absorption of the specific antibody, as measured by ELISA, by each bacterium, could then be calculated by re-estimating the titre of antibody in the sample and expressed as a percentage of the pre-absorption value. The results of the absorption studies indicate that specific antibody was absorbed predominantly by the homologous bacteria (Table 2).

RESULTS

CRP

The mean serum CRP concentration in AS patients was 37·31 ± 3·17 mg/ℓ (mean ± s.e.) and in healthy controls it was 4·19 ± ·065 mg/ℓ. Healthy B27 positive individuals had a mean serum CRP concentration of 3·39 ± 0·75 mg/ℓ and this was comparable with the level of 4·65 ± 0·92 mg/ℓ in healthy non-B27 subjects. Thirty-seven out of 110 (34%) normals had zero values for CRP.

Serum immunoglobulins

IgA. The mean serum concentration of IgA in AS patients was 2·72 ± 0·11 g/ℓ and this was significantly higher than the level of 2·00 ± 0·09 g/ℓ in healthy controls ($t = 4·652$; $P < 0·001$). The level of serum IgA in patients with active disease (as defined above) was 3·13 ± 0·20 g/ℓ and this was significantly greater than the level of 2·00 ± 0·16 g/ℓ in inactive patients ($t = 3·704$; $P < 0·001$), or the level in healthy controls ($t = 5·783$; $P < 0·001$) (Fig. 1). The serum IgA concentration in patients with moderately active disease was 2·66 ± 0·15 g/ℓ and this was also significantly elevated when

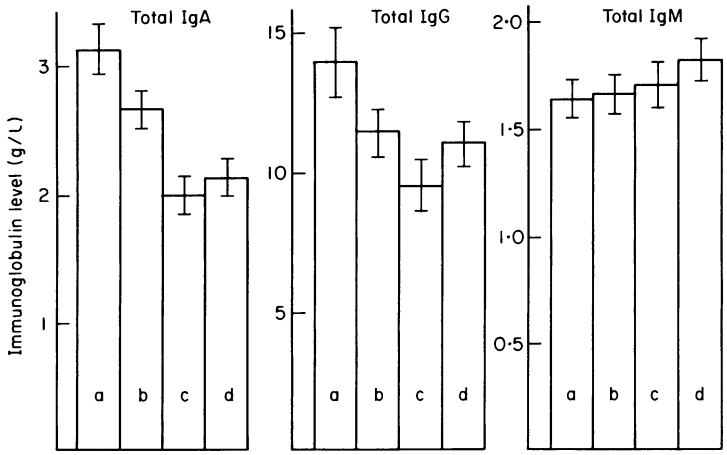

Fig. 1. Serum immunoglobulin A, G and M levels (mean ± s.e.) in active (a), moderately active (b), inactive (c) AS patients, and healthy controls (d).

compared with patients with inactive disease ($t = 2{\cdot}771$; $P < 0{\cdot}01$) and healthy controls ($t = 3.916$; $P < 0{\cdot}001$). The level of serum IgA in 40 healthy B27 positive subjects was $2{\cdot}13 \pm 0{\cdot}13$ g/ℓ and this did not differ significantly from the level of $1{\cdot}93 \pm 0{\cdot}13$ g/ℓ found in 67 healthy B27 negative subjects.

There was a statistically significant positive correlation between the serum IgA concentration and both ESR ($r = +0{\cdot}278$; $P < 0{\cdot}001$) and CRP ($r = +0{\cdot}189$; $P < 0{\cdot}05$).

IgG. The mean serum IgG in AS patients was $12{\cdot}15 \pm 0{\cdot}35$ g/ℓ and this was significantly higher than the level of $11{\cdot}01 \pm 0{\cdot}32$ g/ℓ in healthy controls ($t = 2{\cdot}216$; $P < 0{\cdot}05$). The concentration of serum IgG in active patients was $14{\cdot}01 \pm 0{\cdot}63$ g/ℓ and this was significantly greater than the level of $9{\cdot}57 \pm 0{\cdot}47$ g/ℓ in inactive patients ($t = 4{\cdot}640$; $P < 0{\cdot}001$) and the level in healthy controls ($t = 4{\cdot}565$; $P < 0{\cdot}001$).

There was again a significant positive correlation between the serum IgG concentration and both ESR ($r = +0{\cdot}370$; $P < 0{\cdot}001$) and CRP ($r = +0{\cdot}528$; $P < 0{\cdot}001$).

IgM. The mean serum IgM concentration in AS patients was $1{\cdot}67 \pm 0{\cdot}05$ g/ℓ and this was comparable with the level of $1{\cdot}70 \pm 0{\cdot}06$ g/ℓ in healthy controls. There was also no association between serum IgM levels and either ESR or CRP.

ELISA

IgA antibodies. The mean serum *K. pneumoniae* IgA in AS patients was $12{\cdot}02 \pm 0{\cdot}59$ (ELISA units ± s.e.) and this was significantly elevated when compared with the level of $9{\cdot}97 \pm 0{\cdot}57$ in healthy controls ($t = 2{\cdot}418$; $P < 0{\cdot}02$). IgA antibodies to other Gram negative bacteria were similar in patients and controls. AS patients with active disease had a mean *K. pneumoniae* IgA level of $12{\cdot}85 \pm 0{\cdot}97$ and this was significantly higher than the level of $9{\cdot}52 \pm 0{\cdot}86$ found in patients with inactive disease ($t = 2{\cdot}208$; $P < 0{\cdot}05$) and healthy controls ($t = 2{\cdot}714$; $P < 0{\cdot}01$). Moderately active patients had a mean *K. pneumoniae* IgA of $12{\cdot}47 \pm 1{\cdot}01$ and this was also significantly elevated when compared with the healthy controls ($t = 2{\cdot}318$; $P < 0{\cdot}025$), although it did not differ significantly from the *K. pneumoniae* IgA level in the inactive disease group. There was no difference between *K. pneumoniae* IgA antibody levels in inactive patients and healthy controls. IgA antibodies to *S. typhimurium*, *Y. enterocolitica* and *P. aeruginosa* were similar in patients and controls, irrespective of disease activity.

In order to further illustrate the relationship between the raised total serum IgA in AS patients and specific IgA antibodies to Gram negative bacteria, 12 patients with a total serum IgA concentration exceeding the normal mean serum IgA + 2 s.d. (equivalent to 4·6 g/ℓ) were compared with the healthy controls. AS patients with elevated serum IgA had a mean *K. pneumoniae* IgA level of $18{\cdot}65 \pm 2{\cdot}32$ and this was significantly elevated when compared with B27 positive healthy controls ($t = 3{\cdot}210$; $P < 0{\cdot}005$), healthy B22/B7 positive controls ($t = 3{\cdot}163$; $P < 0{\cdot}005$) and B7

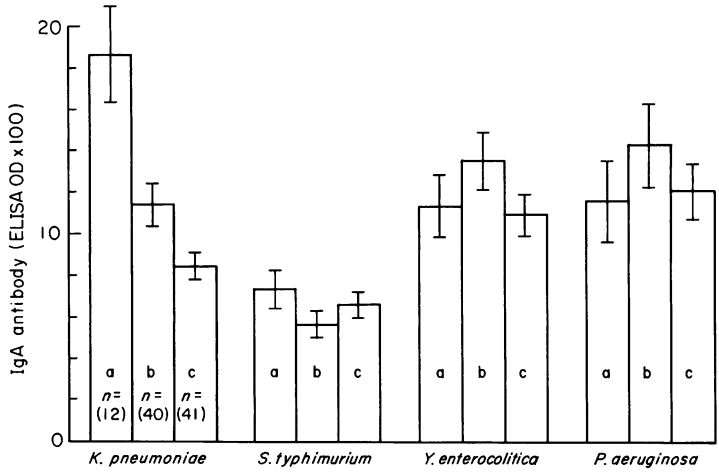

Fig. 2. Serum anti-bacterial IgA levels (mean ± s.e.) in AS patients with an elevated total serum IgA (> 4·6 g/ℓ) (a), compared to the levels found in healthy B27 (b) and non-B27 control subjects (c).

CREG negative controls (t = 6·051; $P < 0·001$). Antibodies to other Gram negative bacteria were not increased in AS patients with high total serum IgA (Fig. 2).

IgG antibodies. The mean level of IgG antibody to *S. typhimurium* antigens in AS patients was 15·88 ± 0·76 and this was significantly greater than the level of 13·15 ± 0·86 in healthy controls (t = 2·363; $P < 0·02$). When patients were divided according to disease activity, there was no difference in *S. typhimurium* antibody levels between active and inactive patients. However, there was a significant correlation between *K. pneumoniae* IgG and both ESR ($r = +0·193$; $P < 0·05$) and CRP ($r = +0·173$; $P < 0·05$). Patients with active disease had a mean serum *K. pneumoniae* IgG level of 40·92 ± 3·03 and this was significantly higher than the level of 24·50 ± 2·52 in patients with inactive disease (t = 3·535; $P < 0·001$). Patients with moderately active disease had an intermediate antibody level of 33·84 ± 3·38. However, the level of *K. pneumoniae* IgG in patients with active or moderately active disease did not differ from the level of 38·05 ± 2·12 in the healthy controls. Furthermore, AS patients with inactive disease had a significantly lower IgG antibody level when compared with the healthy control group (t = 3·084; $P < 0·005$) (and this parallels the relative levels of total serum IgG concentrations). IgG antibodies to other Gram negative bacteria did not correlate with disease activity. Healthy B27 positive controls had a mean *K. pneumoniae* IgG level of 39·09 ± 3·67 and this was similar to the level of 38·03 ± 2·79 in B27 negative healthy subjects.

IgM antibodies. There was no elevation of IgM antibodies to Gram negative bacteria in AS patients. However, the level of *K. pneumoniae* IgM in B27 positive healthy subjects was 17·50 ± 1·09 and this was significantly higher than the level of 14·63 ± 0·64 found in AS patients (t = 2·126; $P < 0·05$), although it did not differ significantly from the level of 15·10 ± 0·99 found in healthy B27 negative controls. When the patients were divided according to disease activity only, the active disease group had a significantly lower *K. pneumoniae* IgM compared with the healthy B27 positive control group (t = 2·184; $P < 0·05$).

A similar pattern was observed in the levels of *Y. enterocolitica* IgM antibody. In AS patients the mean *Y. enterocolitica* IgM level was 21·19 ± 1·02 and this was significantly lower than the level of 26·19 ± 1·50 found in all the healthy controls (t = 3·220; $P < 0·005$). Healthy B27 positive controls had a mean *Y. enterocolitica* IgM of 33·90 ± 2.83 and this was significantly elevated when compared with both AS patients (t = 5·216; $P < 0·001$) and the level of 22·35 ± 1·52 found in healthy B27 negative subjects (t = 3·833; $P < 0·001$).

The level of *Y. enterocolitica* IgM in active AS patients was found to be 17·94 ± 1·32 and this was significantly lower than the level of 26·59 ± 2·45 in the inactive disease group (t = 3·398; $P < 0·05$) and both healthy B27 positive (t = 5·666; $P < 0·001$) and B27 negative (t = 2·409; $P < 0·02$) controls. Patients with moderately active disease had a mean *Y. enterocolitica* IgM of 21·78 ± 1·70 and

although this did not differ significantly from the level in inactive patients and healthy B27 negative controls, it was significantly lower than the *Y. enterocolitica* IgM level found in B27 positive controls ($t = 3{\cdot}885$; $P < 0{\cdot}001$). The mean level of *Y. enterocolitica* IgM antibody in inactive patients was similar to that found in healthy B27 positive controls.

There was a modest but statistically significant negative correlation between the *Y. enterocolitica* IgM and ESR in patients with AS ($r = -0{\cdot}210$; $P < 0{\cdot}05$). IgM antibodies to *S. typhimurium* and *P. aeruginosa* were similar in patients and controls, irrespective of disease activity.

DISCUSSION

Elevated levels of total serum IgA and IgG in AS patients were found to correlate with both erythrocyte sedimentation rate and CRP levels, and confirm similar observations made in previous studies (Cowling *et al.*, 1980b; Calguneri *et al.*, 1981).

The possible reason for the elevation in total serum IgA in AS was investigated by measuring specific antibodies against antigens, obtained from several species of Gram negative micro-organisms, namely *K. pneumoniae*, *S. typhimurium*, *Y. enterocolitica* and *P. aeruginosa*. Increased levels of specific *K. pneumoniae* IgA antibodies were found to be present in AS patients with active disease, as assessed either by elevated CRP or total serum IgA levels.

The clear elevation in *K. pneumoniae* antibody titres, compared to the titres obtained for other Gram negative micro-organisms, suggests that exposure to *K. pneumoniae* micro-organisms, in the gastro-intestinal tract, may be associated with episodes of inflammatory activity in AS. Furthermore, clinical analysis indicates that elevated ESR and CRP levels are associated with exacerbations of both spinal and peripheral manifestations of disease in AS (Cowling *et al.*, 1980a). It is thus possible that active inflammatory enthesopathy lesions occur during periods when elevations in total and specific anti-bacterial serum IgA antibodies can be detected, suggesting that a mucosal antigenic trigger may have produced the exacerbations or relapses of the disease.

There are currently two theories attempting to explain the mechanism whereby *K. pneumoniae* could act as an aetiological factor in the pathogenesis of AS. The theories attempt to account for the high frequency of HLA-B27 in patients with AS in terms of either molecular cross-reactivity or cellular interaction between host and antigen found in bacteria.

The one gene theory, or cross-tolerance hypothesis (Ebringer, 1978), proposes that *K. pneumoniae* antigens stereochemically resemble HLA-B27 (Avakian *et al.*, 1980) and tissue damage is caused by the production of antibodies which have both an affinity for bacterial antigens and also possess anti-self or autoimmune activity. According to the cross-tolerance hypothesis, AS is produced by an infectious agent in the colon, carrying antigens partially cross-reacting with HLA-B27, which stimulate the formation of cross-reactive antibodies, reacting with tissue specific antigens found in the spine, sacro-iliac joints and uvea and a similar mechanism is thought to operate in rheumatic fever (Ebringer, 1982). The second hypothesis is that *K. pneumoniae* antigens may specifically modify HLA-B27 or a hypothetical B27 associated receptor which could then increase the susceptibility of the subject to develop the disease (Edmonds *et al.*, 1981). The adherence of pathogenic bacteria to mucosal epithelium is an important step in colonization and invasion of the intestinal tract by micro-organisms and further studies are required to assess the relevance of HLA antigens in this process. The demonstration of specific *K. pneumoniae* IgA antibodies in the study reported here is compatible with both theories; however, the mechanism by which the tissue damaging lesions are produced in a genetically susceptible population remains unclear.

Specific *K. pneumoniae* IgG antibodies were elevated in AS patients with active disease compared to patients with inactive disease, but no clear difference emerged when compared to healthy controls. Patients with active disease also had significantly lower levels of *K. pneumoniae* and *Y. enterocolitica* IgM antibodies when compared with healthy B27 positive subjects, but the significance of these results remains unclear.

In conclusion, it would appear that there is an overall gradation in the level of *K. pneumoniae* IgA antibodies from active AS patients, inactive AS patients, healthy B27 individuals to healthy

non-B27 individuals. The number of AS patients who did not possess HLA-B27 was too small for analysis. Such a gradation indicates that the development of the disease is not an all or none phenomenon, but a continuous process of repeated environmental interactions with some external antigens of commensal micro-organisms found in the bowel flora, which in a genetically susceptible individual could lead to chronic disease such as AS.

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