SHORT REPORT

ANTIBODIES TO FOUR GRAM-NEGATIVE BACTERIA IN RHEUMATOID ARTHRITIS WHICH SHARE SEQUENCES WITH THE RHEUMATOID ARTHRITIS SUSCEPTIBILITY MOTIF

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SUMMARY

The bacteria Proteus, Serratia, Escherichia and Pseudomonas possess sequences resembling the rheumatoid arthritis susceptibility sequence EQRRAA, but antibodies were elevated only to Proteus in 66 RA patients (P < 0.001) when compared to 61 active ankylosing spondylitis patients and 60 controls.

The association between rheumatoid arthritis (RA) and HLA-DR haplotypes DR1 DRB1*0101, DR4 [subtypes DRB1*0404 (Dw14), DRB1*0405 (Dw15)] and DR6 subtype DRB1*1402 (Dw16) present in Yakima Indians, has been reported [1]. A particular region of the DRβ1 chain, from position 70 to 74 (QKRAA), has been identified as the molecular sequence responsible for susceptibility to RA [2]. This sequence closely resembles one found in DRB1*0401 (DR4/Dw4) (QKRAA) where there is a conservative substitution. Glutamic acid (E) occupying position 69 is common to all DRβ1 molecules. We have identified an amino acid sequence homology between the RA susceptibility sequence EQRRAA and a biochemical/charge similarity sequence ESRRAL, spanning residues 32–37 of the surface membrane haemolysin of Proteus mirabilis (Hpm B polypeptide). The same hexamer sequence was found in the membrane haemolysin of Serratia marcescens (Shi B polypeptide) [3, 4]. Furthermore, homologous sequences were also found in Escherichia coli (QKRAA) and Pseudomonas aeruginosa (DQRRAA). Previous studies by our group and others have reported that active RA patients have increased antibodies to Proteus mirabilis [5–7]. This study was undertaken to investigate whether RA patients with active disease also have antibodies to these other microorganisms.

METHODS

Serum samples

Sera were obtained from active RA patients (ESR > 15 mm/lst h) attending the Rheumatology Department at the Lister Hospital, Stevenage, and active ankylosing spondylitis (AS) patients attending the AS Research Clinic at the Middlesex Hospital. The diagnosis of RA was according to the ARA criteria and that of AS by the New York criteria. Healthy control samples were supplied by the Blood Transfusion Service, London.

In the study, total immunoglobulin antibodies were measured in 181 individuals against S. marcescens, P. mirabilis, E. coli and P. aeruginosa. The groups examined were as follows: 66 patients with RA [21 male/45 female; mean age 48 (range 20–71) yr; mean ESR 43.4 (SE 4.3 mm/lst h)], 61 AS patients [49 male/12 female; mean age 46 (range 24–73) yr; mean ESR 42.9 (SE 3.5 mm/lst h)] and 60 healthy control subjects [30 male/30 female; mean age 34 (19–66) yr].

Preparation of bacteria

Serratia marcescens, P. mirabilis, E. coli and P. aeruginosa were clinical isolates obtained from the Department of Microbiology at King's College. Cultures were grown in 2 l flasks on an orbital shaker for 16 h at 37°C in 200 ml nutrient broth (Oxoid; 25 g/l). Flasks were inoculated with 10 ml of the corresponding starter culture left shaking at 37°C for 6 h. Batch culture cells were harvested by centrifugation at 6000 r.p.m. for 20 min at 4°C (MSE 18, 6 x 250 ml rotor). The pellets of cells was then washed three times with 0.15 m phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS. A stock solution of the suspension was prepared by diluting in 0.05 m carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 on the spectrophotometer (Corning Model 258).

Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were carried out as previously described [8]. Briefly, ELISA plates were coated with bacteria overnight at 4°C, plates washed and a dilution of patient or control serum added. The plates were incubated at 37°C for 2 h, washed and anti-human total immunoglobulin (IgG + IgA + IgM) (1:10000) (Dak) added. The plates were reincubated for 2 h, washed and substrate added. The reaction was stopped with a solution of sodium fluoride (Sigma), the plates were read at 630 nm on a microtitre plate reader (Dynatech MR 600) and results expressed as OD ± s.e.
I0.6H

Fig. 1.—Total immunoglobulin titres (mean ± s.E.) to Serratia marcescens, Proteus mirabilis, Escherichia coli and Pseudomonas aeruginosa in 60 healthy controls, 66 active RA and 61 active AS and Pseudomonas marcescens, Proteus mirabilis, Escherichia coli

was calculated to be 4, 5, 7 and 4%, was not significant. There was no significant reproducibility of the assay for each sample was tested by calculating the coefficient of variation.

CRP determination

CRP levels were determined by the single radial immunodiffusion method of Mancini et al. [9] and the results were expressed as mg/l of serum.

Statistical analysis

The mean OD units of total immunoglobulin antibodies against the various bacteria in different groups were compared with Student's t-test. The reproductibility of the assay for each sample was tested by calculating the coefficient of variation.

RESULTS

Antibodies to P. mirabilis of total (IgA + IgG + IgM) immunoglobulin were significantly elevated in the active RA patients [mean 0.869 (s.E. 0.054) OD units] compared to controls [mean 0.214 (s.E. 0.015) OD units; t = 11.22, P < 0.001] and active AS patients [mean 0.228 (s.E. 0.012) OD units; t = 11.30, P < 0.001] (Fig. 1). The difference between the OD units in AS patients and controls tested against P. mirabilis was not significant. There was no significant reactivity by RA and AS patients against P. aeruginosa, S. marcescens and E. coli in this study when compared to controls. The coefficient of variation for S. marcescens, P. mirabilis, E. coli and P. aeruginosa was calculated to be 4, 5, 7 and 4%, respectively. The mean (± s.E.) CRP (mg/l) was significantly higher in both the RA (40.4 ± 3.4; t = 7.29, P < 0.001; range 0–160.2) and AS patients (34 ± 3.3; t = 5.85, P < 0.001; range 0–108) when compared to the controls (12.0 ± 1.6; range 0–37.5).

DISCUSSION

In the present study, we found increased levels of total immunoglobulin (IgA + IgG + IgM) antibodies against P. mirabilis in active RA patients compared to active AS patients and healthy controls, but no elevations against the three other bacteria. This observation confirms our previous findings and also that of others [5-8].

In a recent study, RA patients with active disease were reported to have elevated antibodies to the 63 kDa haemolysin of P. mirabilis and to a 16mer synthetic peptide sequence containing the ESRRAL homologous residues [10]. In a related study, it was reported that Japanese patients with RA have increased autoantibodies against a 16mer synthetic peptide of DRB1*0405 (DR4/Dw15) β1 chain, which contained the EQRRAA sequence [11]. Furthermore, RA patients with early disease exhibit high antibody titres to a 15mer synthetic peptide of the dnaJ heat shock protein of E. coli which also has the EQRKAA sequence [12]. The susceptibility sequence EQKRAA is also present in the dnaJ heat shock protein of Epstein–Barr virus and a mimicking sequence is found in the isoamylase precursor of Pseudomonas (DQRRAA) with a conservative substitution of glutamic acid (E) for aspartic acid (D) (Table I). However, we were unable to find any significant elevation of antibodies against Pseudomonas or E. coli in RA patients.

The ESRRAL sequence is also present in Vibrio cholerae and Brucella ovis [Protein Information Resource data base (PIR) release 44]. However, these organisms are highly pathogenic and therefore unlikely to persist in patients following recovery. Therefore, they are unlikely to play a role in the onset of a chronic disease. One cannot assume the involvement of an organism simply because it contains a sequence which is similar to the susceptibility sequence. Other factors such as pathogenicity and persistence of the microbe are equally important, and until a particular organism can be linked to RA patients through microbiological or immunological studies, their role in the aetiology

TABLE I

Comparison of amino acid sequences of HLA-DRβ1 chain, spanning residues 69-74, and microorganisms retrieved from PIR release 44 which have similar sequences in other proteins (PIR = Protein Information Resource data base)

<table>
<thead>
<tr>
<th>Source</th>
<th>Amino acids</th>
<th>Positions</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRβ1 chain</td>
<td>EQRRAA</td>
<td>69-74</td>
<td>DR1, DR4/Dw14, Dw15, DR6/Dw16</td>
</tr>
<tr>
<td>Epstein–Barr virus</td>
<td>EQRRAA</td>
<td>60-65</td>
<td>dnaJ heat shock protein</td>
</tr>
<tr>
<td>E. coli</td>
<td>EQRRAA</td>
<td>579-584</td>
<td>dnaJ heat shock protein</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>EQRRAA</td>
<td>32-37</td>
<td>Haemolysin (Shl B)</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>ESRRAL</td>
<td>34-39</td>
<td>Haemolysin (Shl B)</td>
</tr>
<tr>
<td>V. cholerae</td>
<td>ESRRAL</td>
<td>19-24</td>
<td>Methyltransferase-adenine</td>
</tr>
<tr>
<td>B. ovis</td>
<td>ESRRAL</td>
<td>107-112</td>
<td>Isoamylase</td>
</tr>
</tbody>
</table>

E, glutamic acid; D, aspartic acid; Q, glutamine; S, serine; R, arginine; K, lysine; A, alanine; L, leucine.
remains uncertain. Gram-negative bacteria such as *Salmonella typhimurium* [13], a known pathogenic microbe, or anaerobic bowel bacteria such as *Escherichia*, *Peptostreptococcus* and *Bacteroides* [14] which do not share the RA susceptibility motif, have failed to show increased antibody responses in RA patients compared to healthy controls.

Interestingly, *S. marcescens* and *P. aeruginosa* are responsible for only 2% of urinary tract infections (UTI) in women, whilst *P. mirabilis* accounts for 15–18% of cases and *E. coli* for 80% cases. The rate of isolation of *P. mirabilis* from the urine of RA patients is increased compared to osteoarthritis patients and healthy controls [15], but some groups using different methodologies could not confirm these findings [16]. A recent study has shown a decrease in anti-*Proteus* antibody levels in RA patients during a 1 yr vegetarian diet [17]. The authors postulate that vegetarians have a greater urinary excretion of lignans and phyto-oestrogen metabolites than omnivores, and some of these substrates are known to possess antibacterial activity *in vitro* as well as inhibiting adhesion of urobacteria to uroepithelium.

Further evidence for a role of microorganisms in the aetiology of RA is illustrated by the use of antimicrobial agents such as minocycline, which have been shown to reduce joint tenderness and swelling, and decrease inflammatory parameters such as ESR [18, 19]. The role of *Proteus* in RA merits further study.

REFERENCES