Elevated levels of IgM and IgA antibodies to *Proteus mirabilis* and IgM antibodies to *Escherichia coli* are associated with early rheumatoid factor (RF)-positive rheumatoid arthritis

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Objectives. Antibodies to *Proteus mirabilis* were previously detected in patients with established rheumatoid arthritis (RA). We examined the prevalence of antibodies to *P. mirabilis* and their associations with RA in early synovitis patients.

Methods. Two hundred and forty-six patients with inflammatory arthritis for less than 1 yr were prospectively evaluated for 1yr. Of these patients, 30% had rheumatoid factor (RF)-positive RA, 16% RF-negative RA, 17% a spondyloarthropathy and 37% undifferentiated arthritis. Serum antibodies to *P. mirabilis*, *Escherichia coli* and other potentially arthritogenic organisms (*Chlamydia*, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* and parvovirus B19) and for antibodies specific for immunoglobulin (Ig) G damaged with advanced glycation end-products (anti-IgG-AGE) were measured.

Results. IgM and IgA anti-*Proteus* antibodies were significantly higher in patients with RF-positive RA compared with all other patient groups (*P* < 0.0005 and *P* < 0.005). Anti-*P. mirabilis* IgG, and IgG, IgA, and IgM antibodies to other potentially arthritogenic pathogens did not differ in the patient groups. IgM antibodies to *E. coli* were elevated in RF-positive RA patients. Anti-*P. mirabilis* IgM and IgA results were not explained by false-positive reactions, because after absorption of RF there was no decrease in antibodies to *Proteus* in 10 of 12 patients. *Proteus* and *E. coli* antibodies were highest in patients positive for both RF and anti-IgG-AGE antibodies (*P* < 0.001). Patients with erosions tended to have higher IgA anti-*Proteus* titres, but no association with the shared HLA epitope or treatment was detected.

Conclusion. Anti-*P. mirabilis* IgM and IgA and anti-*E. coli* IgM antibody elevations are associated with early seropositive RA and the presence of anti-IgG-AGE antibodies. The role that *P. mirabilis* or *E. coli* plays in early RF-positive RA requires further investigation.

Key words: Proteus antibodies, Early synovitis, Rheumatoid arthritis, Rheumatoid factor, Spondylarthritits.

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects 0.5–1% of the general population, predominantly women, and is characterized by persistent inflammation and proliferation of the synovial tissue [1]. The resulting destruction of the inflamed joints leads to early disability and increased morbidity [2, 3]. The clinical heterogeneity of this disease is thought to be a reflection of the complex interactions of genetic and environmental factors that are involved in the susceptibility, course and outcome of this disease. Many infectious agents have been suggested to be involved in the aetiology of RA. However, to date their role in the direct pathogenic mechanisms that lead to RA has not been conclusively documented [4, 5].

Several studies of RA patients from different ethnic backgrounds have documented the presence of elevated levels of serum antibodies to *Proteus mirabilis* [6–11]. Raised levels of antibodies specific to *P. mirabilis* were observed in RA patients when compared with normal controls and patients with ankylosing spondylitis [12–14]. Furthermore, urine samples from patients with RA showed the presence of infection with *P. mirabilis* strains [15] of particular proteic types [16] more frequently than in those from healthy controls. A significant positive correlation between high anti-*Proteus* antibody levels in serum and the number of *Proteus* colony-forming units in urine specimens of RA patients supports the view that the urinary tract may be a source of the bacterial infection that contributes to the aetiology or pathogenesis of RA [17]. Although a pathogenic role for *Proteus* in the development of RA has not been defined, it has been suggested that molecular mimicry between an epitope shared either between HLA DRB1*0401* and *P. mirabilis* haemolysin or between type XI collagen and *P. mirabilis* urease could be a possible mechanism to activate autoimmune responses in the joint [18]. However, the lack of conclusive epidemiological data in the course of early RA and the lack of appropriate inflammatory controls in some of the other studies prompted us to examine the prevalence of antibodies to *P. mirabilis*, another Gram-negative bacteria associated with bacteruric, namely *Escherichia coli*, as well as to

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Submitted 16 May 2005; revised version accepted 24 June 2005.

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a number of other arthritogenic organisms in a heterogeneous cohort of early synovitis patients and to examine the potential diagnostic and prognostic value of the detection of elevated anti-Proteus antibodies. We also measured antibodies to immunoglobulin (Ig) G damaged by advanced glycation end-products (AGE) [19], and found that the RA patients with this reactivity were also positive for the anti-Proteus and anti-E. coli response. Our data suggest that recent infections with Proteus and or E. coli, as measured by IgM elevations, can be detected in patients with seropositive RA early in their disease.

Materials and methods

Patients
A cohort of 246 patients with inflammatory arthritis for less than 1 yr involving one or more swollen joints was enrolled into an early synovitis study at the National Institute of Arthritis and Musculoskeletal and Skin Diseases (protocol 94-AR-0194). Patients had persistent synovitis of at least one peripheral joint for more than 6 weeks. Patients with traumatic, septic and crystal-induced arthritis were excluded. As previously reported [19], the patients were evaluated clinically, serologically and radiographically at the initial visit, and later at 6 weeks, 6 months and 1 yr. Diagnosis was established at the end of year 1. The number of swollen joints was determined by evaluating for the presence of joint effusion or synovial thickening or both of at least 66 peripheral joints, excluding hip joints. Anteroposterior and lateral radiographs of the hands and feet were taken at the initial visit and at the 1-yr follow-up visit and were evaluated for the presence of erosions. Erosions were defined as the presence of radiographic erosions of any involved joint, detected by 1 yr on either the initial or the 1-yr follow-up radiographs. The American College of Rheumatology (ACR) criteria for RA [20], and the European Spondylarthropathy Study Group (ESSG) criteria for spondylarthropathy [21] were applied to each member of the cohort based on the clinical, radiographic and laboratory data obtained. All patients not fulfilling either set of criteria were classified as having undifferentiated arthropyathy (UA) for the purposes of this study.

This study was approved by the local medical ethics committee and patients gave their written informed consent.

Measurement of antibodies to P. mirabilis and other microbial organisms

Serum taken at the patients’ initial visit was assayed by enzyme-linked immunosorbent assay (ELISA) for antibodies to microbial organisms, including P. mirabilis, as previously described [15]. Eleven strains of P. mirabilis representing the 11 commonest O serotypes of strains associated with a P. mirabilis urinary tract infection [22]; namely serotypes O3, O6, O10, O11, O13, O23, O24, O27, O28, O29 and O30, were each cultured overnight at 37°C on plates of cystine lactose electrolyte-deficient (CLED) medium (CM 301; Oxoid, Basingstoke, UK). Equal numbers of cells of all the 11 different O serotypes were pooled together and used as the source of Proteus antigen. The cells of each serotype were harvested and suspended in phosphate-buffered saline (PBS) pH 7.2 containing sodium azide 0.04% (PBS-Az) and then washed in this solution. The washed cells were resuspended in PBS-Az to an optical density (OD) (550 nm) of 1 and stored as a standard suspension at 4°C. Equal volumes of each of the standard 11 O serotype suspensions of P. mirabilis were then pooled. Aliquots (100 μl) of a 1 in 10 dilution of the pooled standard suspension in PBS-Az were added to flat-bottomed wells of microtitration plates (Maxisorb F8; Nunc, Nottingham, UK), which were covered and left overnight at room temperature. The contents of the wells were then discarded and the wells washed three times in water. Two hundred microlitres of Tween-20 0.05% (PBST) supplemented with bovine serum albumin 0.05% (200 μl) was added to each well and incubated for 1 h at room temperature. The contents of the wells were then discarded and the wells washed three times in water. One hundred microlitres of either serum diluted in PBST (for IgG, dilution was 1 in 1000; for both IgA and IgM, dilution was 1 in 100) or of PBST alone (control) was added to each well in triplicate and the plates were incubated for 2 h at room temperature. After incubation for 1.5 h at room temperature, the contents of the wells were discarded and the wells washed three times in water. Two hundred microlitres of p-nitrophenyl phosphate 1 mg/ml in substrate buffer (100 mM sodium bicarbonate, 1 mM magnesium chloride, pH 10.2) was added to each well. After incubation at 37°C for 30 min the OD₄₀₅ of each well of the plate was immediately measured by a Dias multiaellite reader (Dynatech Medical Products, Guernsey, Channel Islands, UK). Results are expressed as the mean of three OD₄₀₅ readings. Comparisons are made across all of the patient groups, thus providing groups of appropriately matched patients who served as controls. The reliability of the assay was as previously reported [15].

Antibodies (IgG, IgA and IgM) to E. coli were measured by ELISA. In brief, E. coli (ATCC 3521) washed in PBS were made to a stock solution at an OD₅₅₀ of 1.0. ELISA plates (ICN, Montreal, QC, Canada) were coated with the E. coli diluted 1 part stock to 10 parts of a sodium carbonate/bicarbonate buffer, pH 9.4 at 4°C for 16 h. After three washes with PBS/0.1% Tween 20, sera were added at the appropriate dilution (for IgG, 1/2000; for IgA and IgM, 1/100) and incubated for 2 h at 37°C. Plates were washed three times and the appropriate horseradish peroxidase-conjugated (Fab’2) fragments of anti-human IgG, IgA or IgM (BioCan; Jackson Immunolabs, Mississauga, ON, Canada) were added. Dilutions in PBS/Tween of the antisera were used to determine positivity for E. coli for the antibody in question. Since normal individuals have antibodies to these bacteria, it is not possible to assign positivity based on the mean plus 2 s.d. of normal controls.

Antibodies to a panel of possible arthritogenic organisms (Chlamydia trachomatis, Salmonella typhi, Shigella flexneri, Campylobacter jejuni, Yersinia enterocolitica and parvovirus B19) were measured by ELISA at a clinical laboratory (Specialty Laboratories, OneQuest, Santa Monica, CA, USA). Positive tests were defined as follows: C. trachomatis IgM, >0.45 EIA units; C. trachomatis IgG, >0.18 EIA units; C. jejuni >3 s.d. above the mean of a reference group of normal subjects; Salmonella IgG, >10 U; Salmonella IgM, >20 U; Shigella IgG, >20 U; Yersinia IgG, >3 s.d. above the mean of a reference group of normal subjects; Yersinia IgM, >3 s.d. above the mean of a reference group of normal subjects; and parvovirus B19 IgG, >10 EIA units; parvovirus B19 IgM, >10 EIA units.

Detection of RF
RF was measured by nephelometry. In addition, in a subset of patients in one experiment the RF was also assayed by an
alternative method in which 50 μl of serum diluted 20-fold in glycine–saline buffer, pH 8.2, was mixed with 50 μl of RF latex reagent (IgG-coated latex particles; BioStat Diagnostics, Stockport, UK), rocked for 2 min and then examined for agglutination of the latex particles. Positive and negative RF sera were included as controls in each batch of tests. Agglutination indicated the serum had an RF content of ≥20 IU/ml. Doubling dilutions of such positive sera were retested. The RF concentration was equal to the highest dilution of serum giving visible agglutination of the latex particles [15]. RF was removed from sera by repeated adsorption with latex beads coated in human IgG, and centrifugation to deposit the latex beads until the RF level of the serum supernatant was <20 IU/ml. The supernatant was then measured for IgM antibodies to P. mirabilis by ELISA.

**Measurement of antibodies to IgG-AGE**

IgM and IgA anti-IgG-AGE antibodies were detected in serum or plasma by ELISA as previously described [19, 23, 24], the testing laboratory being blinded to the diagnosis. IgGs of all four subclasses, which were fully glycated in vitro, were used at a concentration of 2 μg/ml (100 μl/well) to coat the wells of an ELISA plate (EIA; ICN, Montreal, QC, Canada). After washing the plates, 100 μl of the sera or plasma diluted 1 in 1000 were applied in duplicate to each well, and were incubated in the AGE-modified IgG-coated wells for 2 h at 37°C. After washing the plates in PBS/0.1% Tween 20, the bound antibodies were detected with peroxidase-conjugated F(ab’)2 fragments of anti-human IgM, or IgA (Jackson Immunolabs) diluted 1 in 10 000 in PBS/0.1% Tween. To follow the reactivity over time and to maintain consistent results, a control serum from a normal control were tested each time the assay was performed. After washing the plates three times with PBS/0.1% Tween, 100 μl of 0.05 M Na2HPO4, 0.012% H2O2 was added. The reaction was stopped by the addition of 4 M H2SO4 approximately 30 min later. The OD492 (reference OD690; the reference wavelength is 0.05 M Na2HPO4, 0.012% H2O2) was added. The reaction was measured for IgM antibodies to 
P. mirabilis by ELISA.

**Results**

As previously reported, of the 246 patients with synovitis of recent onset in the cohort, 113 (46%) met the ACR criteria for RA [19]. Of the 246 patients, 43 (17%) fulfilled the ESSG criteria for a spondyloarthropathy (SpA), and 90 (37%) patients had undifferentiated arthritis (UA), respectively. The SpA group appeared atypical (Table 1), but this may reflect the fact that the ESSG criteria do not discriminate as well in an early synovitis cohort. Table 1 shows the demographic and clinical data of the patients studied. Radiographic erosions were either present at entry or developed during the year of follow-up. Of the patients with RF-positive RA, 40% had erosions that were either present at study entry or developed within the year of study follow-up, and 37% of RF-negative RA patients had erosions either at study entry or at the 1 yr follow-up evaluation. By comparison, only 12 and 15%, respectively, of patients with SpA or UA had radiographic erosions at 1 yr. As expected, a significantly larger number of patients with RA received treatment with prednisolone and/or DMARDs (P <0.005 and P <0.0001 respectively). All patient groups received similar levels of NSAID therapy.

**Table 1. Demographic, clinical and treatment characteristics of the patients (for additional details of the cohort see references 19 and 25)**

<table>
<thead>
<tr>
<th></th>
<th>All patients (n = 246)</th>
<th>RF+ RA (n = 75)</th>
<th>RF- RA (n = 38)</th>
<th>SpA (n = 43)</th>
<th>UA (n = 90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>43 ± 13</td>
<td>48 ± 12*</td>
<td>44 ± 14</td>
<td>37 ± 11</td>
<td>41 ± 14</td>
</tr>
<tr>
<td>Female (%)</td>
<td>160 (65)</td>
<td>45 (60)</td>
<td>28 (74)</td>
<td>27 (63)</td>
<td>60 (70)</td>
</tr>
<tr>
<td>Symptom duration (weeks)</td>
<td>33 ± 31</td>
<td>33 ± 17</td>
<td>30 ± 24</td>
<td>31 ± 36</td>
<td>35 ± 37</td>
</tr>
<tr>
<td>Total joint count</td>
<td>12 ± 12</td>
<td>19 ± 12*</td>
<td>22 ± 12*</td>
<td>5 ± 10</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>39 ± 30</td>
<td>45 ± 28</td>
<td>41 ± 31</td>
<td>40 ± 31</td>
<td>33 ± 30</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>1.7 ± 2.2</td>
<td>1.8 ± 1.8</td>
<td>1.8 ± 2.0</td>
<td>1.9 ± 3.2</td>
<td>1.5 ± 1.9</td>
</tr>
<tr>
<td>RF &gt;20</td>
<td>93 (38)</td>
<td>75 (100)*</td>
<td>0 ± 0</td>
<td>2 (5)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>RF titre</td>
<td>205 ± 165</td>
<td>208 ± 165</td>
<td>N/A</td>
<td>361 ± 223</td>
<td>189 ± 169</td>
</tr>
<tr>
<td>Anti-AGE IgG positivity</td>
<td>65 (26)</td>
<td>37 (49)*</td>
<td>4 (11)</td>
<td>9 (21)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>Shared epitope</td>
<td>113 (46)</td>
<td>48 (64)*</td>
<td>17 (45)</td>
<td>18 (42)</td>
<td>30 (33)</td>
</tr>
<tr>
<td>B27</td>
<td>45 (18)</td>
<td>12 (16)</td>
<td>6 (16)</td>
<td>18 (42)</td>
<td>9 (10)</td>
</tr>
<tr>
<td>Erosions (%)</td>
<td>48 (27)*</td>
<td>23 (40)**</td>
<td>13 (37)**</td>
<td>3 (12)*</td>
<td>9 (15)*</td>
</tr>
<tr>
<td>Prednisolone+DMARD (%)</td>
<td>31 (13)</td>
<td>16 (21)*</td>
<td>7 (18)*</td>
<td>4 (9)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Antibiotics (%)</td>
<td>40 (16)</td>
<td>14 (19)</td>
<td>5 (13)</td>
<td>6 (14)</td>
<td>15 (17)</td>
</tr>
</tbody>
</table>

Comparisons were made between groups.

Numbers represent either mean ± s.d. or total numbers (%).

*A complete set of radiographs was available for only 180 patients [57 with RF+ RA, 35 with RF- RA, 26 with a spondyloarthropathy (SpA) and 62 with undifferentiated arthritis (UA)].

**HLA typing**

Patients were HLA-typed for MHC class I and DR alleles by the molecular polymerase chain reaction-sequence specific primer method using sequence-specific primers, as previously described [25].

**Statistical analysis**

Statistical analyses were performed using SAS (Bethesda, MD, USA) and EpilInfo 16 statistical software for parametric and non-parametric comparisons between groups (Centers for Disease Control and Prevention, Atlanta; http://www.cdc.gov/epiinfo). Patient groups were compared using parametric and non-parametric statistics. Analysis of variance (ANOVA) and the Kruskal–Wallis test were used for continuous variables, and the χ2 test for proportions. Bonferroni correction was made for multiple comparisons, where appropriate.
Anti-IgA and IgM *P*. mirabilis-specific serum antibody titres are elevated in patients with RF-positive RA

Figure 1 shows the anti-*P*. mirabilis antibody levels in the sera of all the patients at their initial visit to the National Institutes of Health. No significant differences in the means of the sera *Proteus* IgG antibodies were detected in patients with RF-positive RA, RF-negative RA, SpA or UA. In contrast, the means of sera anti- *Proteus* IgM and IgA antibody levels were both significantly higher in patients with RF-positive RA compared with all other patient groups (OD\_M 0.74 ± 0.29 vs 0.58 ± 0.20 (P < 0.0003), and OD\_A 0.144 ± 0.057 vs 0.121 ± 0.049 (P < 0.015), respectively).

**Proteus antibodies associate with markers of disease severity only in patients with RA**

The results in Table 2 show that anti- *Proteus* IgA, IgM and IgG antibody levels correlated moderately with total serum IgA, IgM and IgG levels (r = 0.49, 0.45 and 0.28, respectively; P < 0.0001). A moderate correlation between the titre of the IgM antibodies to *P*. mirabilis and the RF titre was observed (r = 0.46; P < 0.0001). RF levels were more weakly correlated with the anti- *Proteus* IgA levels (r = 0.21; P < 0.01). In contrast, anti- *Proteus* IgG levels did not correlate with the RF. In addition to the association with RF, anti- *Proteus* IgA was weakly associated with the ESR (r = 0.29; P < 0.0001), total joint count (r = 0.2; P < 0.05) and swollen joint count (r = 0.2; P < 0.05). No association between anti- *Proteus* IgA, IgM or IgG levels and the presence of shared epitope alleles was noted (data not shown). Drug treatment with either DMARDs or prednisolone did not influence *Proteus* antibody titres (data not shown). There was no difference in antibiotic used in either group. Interestingly, there was a tendency for higher anti- *Proteus* IgA levels to be found in patients who had radiographic erosions at 1yr when compared with patients who had no radiographic erosions [mean 0.14 ± 0.05 and 0.12 ± 0.05; median 0.13 (range 0.2) and 0.12 (range 0.26); P = 0.065]. No differences in IgG and IgM anti- *Proteus* antibody levels were seen in patients with and without erosions.

Because of the correlation between IgM anti- *Proteus* antibody levels and RF, it was necessary to examine whether the IgM RF was causing a false-positive result in the *Proteus* assay. Therefore, sera from 12 patients with the highest levels of RF who also had high anti- *Proteus* IgM antibody titres were selected and RF was removed by repeated absorption with latex beads coated with human IgG. In 10 patients’ sera this resulted in no significant drop in the anti- *Proteus* IgM antibody level. In one patient’s serum (patient 8) there was a significant drop in anti- *Proteus* IgM titre after removal of RF (Table 3). To further exclude the possibility that the *Proteus* assay was measuring the RF, the mean OD for the anti- *Proteus* IgM antibody of the RF positive patients was used as an arbitrary cut-off level to define high-positive anti- *Proteus* IgM patients. This new category comprised 58 patients, of whom 34 (59%) were patients with RA and 24 (41%) were patients with other forms of arthritis. Of the patients with high anti- *Proteus* levels, 38 (66%) were RF-positive and 20 (34%) patients were RF-negative. Similar results were obtained when anti- *Proteus* positivity was defined on the basis of elevated IgA levels or a combination of either IgA or IgM levels (data not shown). If RF was causing false-positive measures of IgM anti- *Proteus*, we would anticipate that all RF-positive individuals would be IgM anti- *Proteus*-positive, and this was not the case. Of the 41 RA patients with the lowest levels of IgM anti- *Proteus* antibodies, 11 had markedly elevated RF, with levels of 80–598 IU/ml.

**Table 2.** Correlation coefficients (r) for markers of disease activity with *Proteus* antibody serum levels

<table>
<thead>
<tr>
<th></th>
<th>Anti-Proteus IgA</th>
<th>Anti-Proteus IgM</th>
<th>Anti-Proteus IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgA</td>
<td>0.49**</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total IgM</td>
<td>–</td>
<td>0.45**</td>
<td>0.28**</td>
</tr>
<tr>
<td>Total IgG</td>
<td>0.21*</td>
<td>0.46**</td>
<td>0.04</td>
</tr>
<tr>
<td>RF</td>
<td>0.02**</td>
<td>0.11</td>
<td>0.16*</td>
</tr>
<tr>
<td>ESR</td>
<td>0.13*</td>
<td>–0.08</td>
<td>0.13*</td>
</tr>
<tr>
<td>CRP</td>
<td>0.2*</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>Total joint count</td>
<td>0.2*</td>
<td>0.11</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Pearson correlations between the different parameters are shown. *P < 0.05; **P < 0.0001.
RF was removed from sera by repeated adsorption with latex beads coated in human IgG. After centrifugation to remove the latex beads, the supernatant was measured for IgM antibody to P. mirabilis by ELISA, and for RF level by reaction with latex beads coated with human IgG.

**Anti-Proteus IgM and IgA antibodies were highest in sera positive for RF and anti-IgG-AGE antibodies**

Because the immune response to IgG damaged by AGE has been shown to be linked to the RF response [23, 24] in other cohorts, it was of interest to divide the patients in the present cohort according to those that were positive for both RF and anti-IgG-AGE antibodies; those positive for the RF alone; those positive for anti-IgG-AGE antibodies alone; and those negative for both RF and anti-IgG-AGE antibodies. Table 4 summarizes the results obtained from this stratified analysis. Anti-Proteus IgM and IgA antibody levels were more significantly elevated in patients who were positive for both RF and anti-IgG-AGE antibodies than in any of the other patient groups (Fig. 2). In contrast, anti-Proteus IgG antibody levels did not differ significantly in the different groups.

**Anti-IgM E. coli-specific antibody titres are observed in patients with RF-positive RA**

We investigated the immune response to E. coli, the principal bacterium associated with bacteruria in the population at large. As can be seen from Table 5, there were no significant differences in the IgG, IgM antibody responses to E. coli for any patient group. The IgA response was slightly elevated in the RA group when compared with the UA group. Interestingly, however, the RF-positive RA subgroup had a significantly elevated reactivity for IgM anti-E. coli compared with the RF-negative RA group [median (95% CI): 0.488 (0.593–1.021) and 0.288 (0.337–0.614); P = 0.023]. Of the RF-positive RA patients, 26 of 57 (46%) were IgM anti-E. coli positive compared with 13 of 49 (27%) for the RF-negative group (P = 0.047; Fisher’s exact test). There was a weak correlation between the total IgM and the IgM anti-E. coli response (r = 0.336; P = 0.0018), whereas the correlations between total IgA or IgG and the respective anti-E. coli subclasses were negligible (r = 0.26; P = 0.016 for IgA, and r = 0.159 for IgG). There was a correlation between RF and the IgM anti-E. coli response (r = 0.373; P < 0.0001); however, by using F(αβ) fragments of the anti-IgM used in the ELISA assay we had lowered the possibility of false positives caused by the RFs. Indeed, several individuals with very high RF titres were negative for IgM anti-E. coli antibodies. There was a significant correlation between the IgM and IgA anti-E. coli responses, with r = 0.55; P < 0.0001, which is expected given the mucosal site of infection by these bacteria. There was no correlation observed between the IgA anti-E. coli response and the IgM RF.

Interestingly, there was a significant correlation between the IgM anti-P. mirabilis response and the IgM anti-E. coli response (r = 0.49; P = 0.0001), but only in the RF-positive RA patient subgroup, linking them as infectious organisms that colonize the mucosal surfaces. There was no significant correlation between the IgA anti-E. coli and IgA anti-P. mirabilis responses (r = 0.073). The elevations in the IgM response may suggest recent infections. As with the Proteus infections, there was a highly significant correlation between the IgM response to E. coli and the presence of anti-IgG-AGE antibodies [median (95% CI) for anti-E. coli response in those that had anti-IgG-AGE antibodies, 0.724 (0.662–1.133); in those that lacked anti-IgG-AGE antibodies, 0.288 (0.347–0.652); P = 0.0003]. There were no significant correlations between any disease activity scores, markers of inflammation or erosions or the presence of the shared epitope with the IgM immune response to E. coli. There was a weak association between the IgA anti-E. coli response and the use of prednisolone (r = 0.245; P = 0.0112). However, prednisolone use did not affect the IgM anti-E. coli response or the IgA or IgM anti-P. mirabilis response. We investigated the frequency of the antibody response to either or both of E. coli and P. mirabilis in the three patient groups, and found that 55% of RA patients had an IgM response to either or both bacteria, whereas the frequencies were lower in both the SpA (36%) and UA (34%) patients. Interestingly, in the RA patients there was a trend for elevated IgM responses being higher in females (39/70 or 56%) compared with males (18/37 or 49%). This is consistent with the fact that bacteruria and/or urinary tract infection is much more common in females than males.

**Differences in antibody responses to other potential arthritogenic pathogens were not seen in the different disease groups**

Analysis of the presence of antibodies to other potential arthritogenic pathogens, including C. trachomatis (IgG and IgM), C. jejuni (total Ig), S. typhi (IgG, IgM, IgA), S. flexneri (total Ig), Y. enterocolitica (IgG, IgM, IgA), and parvovirus B19 (IgG, IgM), was made to determine whether antibody titre differences could be detected in the different disease groups: RF-positive RA, RF-negative RA, SpA and UA. No significant differences in the frequency of antibody presence or levels (data not shown for antibody levels) were observed for any of the anti-pathogen antibodies listed (Table 6).
Discussion

Multiple cross-sectional studies in various ethnic populations have demonstrated that elevated antibody titres to \textit{P. mirabilis} are seen in patients with RA \cite{6-13}. However, the patients examined in these studies had longstanding disease, and control subjects were either healthy or comprised patients with reactive arthritis or osteoarthritis. Thus, the patient populations were not appropriate for the evaluation of the diagnostic and prognostic significance of raised antibody production to \textit{P. mirabilis}. We therefore examined the prevalence of anti-\textit{Proteus} antibodies and their prognostic and diagnostic value in a diverse cohort of patients with recent onset of inflammatory arthritis.

In our cohort of patients with recent onset of synovitis, we demonstrate an association between RF-positive RA and anti-\textit{Proteus} antibodies, specifically IgM and IgA but not IgG. We also examined the titres of the IgG anti-\textit{Proteus} antibodies (data not shown) and there was no significant association found in this early synovitis cohort for the different patient groups or subgroups, unlike what was reported previously \cite{6, 9}. Because IgG has a long half-life in serum (about a month) compared with IgM (about a week), it is not as reflective as the IgM levels of acute changes. Although the detection of IgM anti-\textit{Proteus} antibodies could indicate a recent infection, elevations of both IgM and IgA antibodies are associated with an immune response in the mucosa. Elevations of both IgA and IgM in our patient cohort may therefore reflect an infection at a mucosal surface. Since patients from all groups had similar levels of anti-\textit{P. mirabilis} IgG antibodies, it is evident that most of the individuals in the cohort had previously encountered this ubiquitous bacterium.

Since both \textit{P. mirabilis} and \textit{E. coli} are indigenous enterobacteria that inhabit our colons, a perturbation at that site could stimulate the antibody response. However, it also may be that the elevated serum levels of these antibodies in patients with RA may reflect an asymptomatic bacteruria. Indeed, it has been shown that \textit{P. mirabilis} can be isolated more frequently from the urinary tract of women and men with RA than from controls \cite{15, 17}. Furthermore, a significant positive correlation ($r$ = 0.714) between anti-\textit{Proteus} antibody levels in the serum and \textit{Proteus} colony-forming units in the urine of RA patients has been reported \cite{17}. Of RA patients with bacteria in the urine, \textit{P. mirabilis} was twice as frequently present as \textit{E. coli}, and represented 52\% of the infections \cite{15}. This is in contrast to urinary tract infections in the population at large, where \textit{E. coli} is the most frequent pathogen \cite{26}. These bacteriurial infections in the patients with RA were asymptomatic \cite{17}.

An examination of the \textit{E. coli} immune response in our cohort indicated that the levels of all three isotypes were very similar in the patient groups, consistent with previous reports \cite{11, 27-30}, only the IgA anti-\textit{E. coli} response being higher in the RA patients when compared with the undifferentiated arthritis group in the present study. We did find, however, a significant elevation in the IgM response to \textit{E. coli} in the subgroup of early RA patients that were RF-positive. Senior et al. \cite{7} previously reported a slight elevation of antibodies to \textit{E. coli}, \textit{Klebsiella} and \textit{Pseudomonas} in RF-positive RA patients over that found in RF-negative RA patients with longstanding disease, but the differences

![Figure 2](https://academic.oup.com/rheumatology/article-abstract/44/11/1433/1784683/fig2)

\textbf{FIG. 2.} Levels of anti-\textit{Proteus} IgG, IgM and IgA antibodies as determined by ELISA in patient groups subdivided according to positivity for RF and/or anti-IgG-AGE antibodies. Grey box represents range; + within box represents mean; horizontal bar represents median; solid black circles indicate outliers. 95\% confidence levels and outliers are indicated. *$P$ = 0.0002; **$P$ = 0.015 comparisons were made between all four groups.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Isotype & RA & SpA & UA & $P$ \\
\hline
IgG & 0.255 (0.216–0.349) & 0.181 (0.163–0.388) & 0.265 (0.200–0.394) & NS \\
IgA & 0.191* (0.264–0.474) & 0.155 (0.133–0.597) & 0.094* (0.120–0.262) & *$P$ = 0.011 \\
IgM & 0.389 (0.520–0.787) & 0.316 (0.308–0.611) & 0.261 (0.318–0.603) & NS \\
\hline
\end{tabular}
\caption{The median immune response to \textit{E. coli} (95\% CI) in the patient groups as measured by ELISA}
\end{table}

\textit{NS}, not significant.
C. trachomatis mice [38]. It is possible that IgG-coated different bacterial products, such as lipopolysaccharide, peptidoglycan and Cpg DNA. These receptors play an important role in the host’s innate immune response and can contribute to the inflammatory response. It is possible that IgG-coated P. mirabilis or IgG-coated E. coli containing a number of different pathogen-associated molecules could trigger one or more of the toll-like receptors expressed by B cells and, together with the cross-linking of the RF expressed by B cells, lead to the activation of RF-positive B cells. Since it appears that B-cell activation is an outcome of infection by one of these Gram-negative bacteria, particularly in those susceptible to RA, it is possible that the link is due to a bacterial product that directly stimulates B cells. This might be OmpA, a 39-kDa outer membrane protein of P. mirabilis, which is a murine B-cell mitogen [37]. Interestingly, a previous study has shown that lipopolysaccharide can stimulate RF production in mice [38].

The striking association of the anti-Proteus and the anti-E. coli response and IgM antibodies to IgG that were damaged by AGE, however, is novel. It is likely that these bacterial infections, even when subclinical, can stimulate a local inflammatory response, which could lead to IgG damage. We have shown previously that the anti-IgG-AGE response is linked to the RF response both in this cohort and in others [19, 23, 24]. It was previously proposed that molecular mimicry might explain the association between Proteus infection and RA. It had been suggested that an extra-articular infection might trigger arthritis through the mimicry of two different sets of bacterial proteins and host proteins [39]. The mimicked proteins postulated to play a role included the epitope motif EQRRRAA of HLA DRB1*0401, which is similar to the motif ESRRAL found in P. mirabilis haemolysin, and the epitope LRELI on type XI collagen shared with P. mirabilis urease [18, 35, 40, 41]. There is as yet neither conclusive epidemiological data nor direct proof that these bacteria or bacterial proteins induce arthritis. Moreover, in the present study no association was found between the shared HLA epitope linked to RA and the presence of elevated levels of P. mirabilis antibodies.

The possibility of other potentially arthritogenic bacteria playing a role in the development of early synovitis in the cohort was investigated, but there was no evidence that prior infection with any of these was more prevalent in the RA group with and without RF than in the other groups.

Our study indicates that, in patients with RA of less than 1 yr, elevated levels of anti-Proteus IgM and IgA antibodies are associated with RA. Since IgG antibodies specific for these bacteria are also present, we cannot conclude that it is a primary infection that is associated with the onset of RA. Rather, it may be that individuals who develop RA have a propensity for recurrent subclinical bacterial infections, but how this relates to RA pathogenesis is not known as yet. In the case of the E. coli infections in RA, prednisolone use may be a contributing factor that needs to be considered. It is of interest that the anti-Proteus IgA but not the anti-E. coli antibodies appeared to be of some clinical significance, because higher levels of such antibodies were detected in individuals who had erosions compared with those that did not. In addition, there was a weak association of the IgA but not the IgM anti-Proteus antibodies with ESR and both total joint and swollen joint counts. The active inflammation induced by the bacterial infection may be important in the light of the association with anti-IgG-AGE antibodies. How the P. mirabilis or even the E. coli infection or the antibodies, or both, contribute to the disease process is still unresolved, but such infections do appear to be an early event in the disease course of seropositive RA.

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>RF+ RA (n = 75)</th>
<th>RF+ RA (n = 38)</th>
<th>SpA</th>
<th>UA (n = 90)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis (IgM)</td>
<td>4 (5)</td>
<td>2 (5)</td>
<td>4 (9)</td>
<td>4 (4)</td>
<td>0.28</td>
</tr>
<tr>
<td>C. trachomatis (IgG)</td>
<td>20 (27)</td>
<td>8 (21)</td>
<td>13 (30)</td>
<td>26 (29)</td>
<td>0.8</td>
</tr>
<tr>
<td>C. jejuni (IgG)</td>
<td>4 (5)</td>
<td>7 (18)</td>
<td>7 (16)</td>
<td>7 (8)</td>
<td>0.5</td>
</tr>
<tr>
<td>S. typhi (IgG)</td>
<td>1 (1)</td>
<td>2 (5)</td>
<td>0 (0)</td>
<td>6 (7)</td>
<td>0.15</td>
</tr>
<tr>
<td>S. typhi (IgM)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>4 (4)</td>
<td>0.48</td>
</tr>
<tr>
<td>S. typhi (IgA)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>4 (4)</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>S. flexneri (IgG)</td>
<td>5 (7)</td>
<td>5 (13)</td>
<td>5 (12)</td>
<td>16 (18)</td>
<td>0.21</td>
</tr>
<tr>
<td>Y. enterocolitica (IgG)</td>
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<td>0</td>
<td>2 (5)</td>
<td>7 (8)</td>
<td>0.35</td>
</tr>
<tr>
<td>Y. enterocolitica (IgM)</td>
<td>3 (4)</td>
<td>1 (3)</td>
<td>5 (12)</td>
<td>3 (3)</td>
<td>0.16</td>
</tr>
<tr>
<td>Y. enterocolitica (IgA)</td>
<td>1 (1)</td>
<td>2 (5)</td>
<td>5 (12)</td>
<td>5 (6)</td>
<td>0.41</td>
</tr>
<tr>
<td>Parvovirus B19 (IgG)</td>
<td>39 (53)</td>
<td>15 (39)</td>
<td>29 (67)</td>
<td>48 (53)</td>
<td>0.09</td>
</tr>
<tr>
<td>Parvovirus B19 (IgM)</td>
<td>3 (4)</td>
<td>4 (11)</td>
<td>5 (12)</td>
<td>9 (10)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

P-values were not significant.

**Table 6.** Frequency of antibody responses to potentially arthritogenic pathogens in the different patient groups.

Acknowledgements

We acknowledge the invaluable contributions of Marianna Crane, Cheryl Yarboro and Drs Thurayya Arayssi, Jose Pando, Percio Gulko, Richard Siegel, Michael Froncek and Robert Ortmann in the clinical evaluations of the patients, Sheila Laku, Nicole Saba, Sharon Suson, C. Deville and Joseph Hoxworth for...
their excellent technical help, and Dr Robert Wesley for help with the statistical analyses. We thank Drs Michael Ward and Peter Lipsky for their insightful review of the manuscript.

The authors have declared no conflicts of interest.

References


