

# Rheumatoid arthritis patients have elevated antibodies to cross-reactive and non cross-reactive antigens from *Proteus* microbes

T. Rashid<sup>1</sup>, K.S. Jayakumar<sup>2</sup>, A. Binder<sup>2</sup>, S. Ellis<sup>2</sup>, P. Cunningham<sup>1</sup>, A. Ebringer<sup>1,3</sup>

<sup>1</sup>School of Biomedical and Health Sciences, Kings College London, London; <sup>2</sup>Department of Rheumatology, Lister Hospital, Stevenage; <sup>3</sup>Department of Rheumatology, Middlesex Hospital, UCL School of Medicine, London, U.K.

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## Abstract

### Background and objective

Although a large number of independent studies have shown a paramount role for *Proteus mirabilis* in the aetio-pathogenesis of rheumatoid arthritis (RA), this hypothesis is still controversial among rheumatologists. The main obstacle to its acceptance is the impression that increased *Proteus* antibodies in RA patients is a secondary phenomenon, occurring as the result of cross-reactivity between bacterial and self-antigens. To shed light on this problem, we examined the link between antibodies to various cross-reactive and non cross-reactive antigenic peptides from *P. mirabilis* and analysed the relationship between these antibodies and disease severity in patients with RA.

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### Methods

Using the ELISA method, serum samples from 70 RA patients and 20 healthy controls were screened for total and class-specific antibodies against three human cross-reactive and non-crossreactive synthetic peptides from *P. mirabilis* haemolysin, urease C and urease F enzymes. An antibody index, which comprised the total concentration of antibodies against these peptides in each sample, was correlated with the biochemical parameters of disease activity and/or severity, such as the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and rheumatoid factors (RF). Furthermore, anti-peptide antibody indices were evaluated among RA patients with different levels of disease activity as defined by ESR and CRP.

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### Results

Significantly elevated levels of total and class-specific IgG antibodies against the 3 *Proteus* peptides were observed among RA patients compared to healthy controls ( $p < 0.001$ ). Active RA patients had elevated IgM antibodies against all peptides compared to healthy subjects ( $p < 0.001$ ). However, no such elevation was observed in IgA anti-peptide antibodies in RA patients. A positive correlation was observed between the antibody indices and ESR ( $p < 0.001$ ) and CRP ( $p < 0.01$ ) concentrations, but not the RF status or disease duration. Furthermore, more than 90% of active RA patients showed positive values for the *Proteus* anti-peptide indices.

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### Conclusion

The elevated levels of antibodies against *Proteus* antigenic epitopes (which are cross-reactive or non cross-reactive with human tissue antigens) observed indicates that this enhanced bacterial immune response in RA patients is specifically triggered by *Proteus* microbes. Furthermore, the correlation of anti-peptide antibody indices with the biochemical markers of disease activity indicates that these antibodies exert damaging cytotoxic effects on joint tissues during the course of the disease.

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### Key words

Rheumatoid arthritis, *Proteus mirabilis*, anti-peptide antibodies.

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Please address correspondence to:  
Professor Alan Ebringer, Division of  
Health and Life Sciences, Infection and  
Immunity Group, King's College London,  
150 Stamford Street, London SE1 9NN,  
UK.

E-mail: alan.ebringer@kcl.ac.uk

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## Introduction

Rheumatoid arthritis (RA) is a chronic and disabling joint disease that affects millions of people throughout the world. The disease shows a prevalence in middle-aged females and presents as a progressive inflammatory symmetrical polyarthritis of the small joints (1). RA has a major impact on the physical and mental health status of patients (2), with associated long-term morbidity, mortality and health care costs (3).

RA has a strong genetic component, which has been demonstrated by familial and twin studies (4), especially in its association with HLA-DRB1 haplotypes (5). The association of RA with HLA-Dw4 was first reported in the mid-1970s (6). It was shown later that Caucasian RA patients exhibit certain HLA-DR4 subtypes such as HLA-DRB1\*0401, and -DRB1\*0404, but not -DRB1\*0402 or DRB1\*0403, while other racial and ethnic groups exhibit non-DR4 HLA genes, such as HLA-DR1 (DRB1\*0401) in Jewish, HLA-DR6 (DRB1\*0901) in Chilean, and HLA-DR10 (DRB1\*1001) in Spanish populations (7). Overall, more than 90% of patients with RA possess one of these alleles (8). This linkage has been attributed to the presence of a group of conserved amino acid sequences, EQK/RRAA, comprising residues 69-74 in the third hypervariable region (HVR3) or DRB1 chain, which are shared between different types and subtypes of the HLA-DRB1 haplotypes and which has been given the name of "shared epitope" (SE) (9).

The role of genes alone is not sufficient to fully explain the aetiopathogenesis of RA, however. In a study of monozygotic twins with RA, less than 20% were found to be susceptible to development of the disease (10), and it appears that other non-genetic, environmental – mainly microbial – factors are likely to be involved in the development of this condition.

Among the various bacterial and viral pathogens, *Proteus mirabilis* could contribute to the aetiopathogenesis of RA. The evidence for this can be summarized as follows.

1. *Proteus* microbes were isolated more frequently among patients with RA

than healthy controls (11, 12).

2. A significant positive correlation was observed between increased urinary *Proteus* isolation and the levels of antibodies against these microbes (13).
3. Increased humoral immune responses against *Proteus* antigens have been observed consistently in patients with RA between the years 1985 (14) and 2005 (15). During this period more than 1,300 patients with RA from 15 different countries have been investigated by different independent groups. Nearly all of these studies have shown elevated antibodies to *Proteus*, but not to other microbes in the sera of patients with RA compared to those with other diseases or corresponding healthy subjects (16). Specifically, elevated levels of anti-*Proteus* antibodies were found in RA patients, but not in those with AS (14, 17-20), sarcoidosis (21), Crohn's disease and ulcerative colitis (22), osteoarthritis (23), systemic lupus erythematosus (24), or in patients with spondyloarthropathies and undifferentiated arthropathies (15).
4. Various molecular homologies and cross-reactivities have been found between different proteins from *Proteus* and self-antigens. For example, *Proteus* haemolysins possess "ESR-RAL" amino acid sequences which share biochemical similarities with the "EQRRRAA" sequence present in the "SE" moiety (25). The *Proteus* urease enzyme shares "IRRET" amino acid sequence homology with the "LRREI" motif which is present in type XI collagens (26). Furthermore, features of immunological cross-reactivity have been shown to occur between *Proteus* and self antigenic peptides (27).
5. Antibodies in the sera of patients with active RA were found to have significantly increased cytotoxicity effects against red blood cells from sheep coated with the synthetic peptides from *Proteus* or cross-reactive self-antigens, but not against those coated with control peptides (28).

Our study was carried out to investigate whether RA patients possess antibodies against a new antigenic motif

of the *Proteus* microbe and to determine whether *Proteus* anti-peptide antibodies correlate significantly with biochemical disease activity and/or the severity markers of RA.

## Material and methods

### Patients and controls

Serum samples were taken from a total of 70 patients with RA who were attending the Rheumatology Clinic at Lister Hospital in Stevenage, UK. All RA patients fulfilled the American College of Rheumatology (ACR) diagnostic criteria (29). The female to male ratio was 3.6:1, whilst the mean (range) age at baseline was 64 (33-81) years. Rheumatoid factors were positive in 46 patients and the mean (range) disease duration was 12.9 (0.5-41) years.

Sera from 20 age- and sex-matched control subjects, who were healthy donors from "London Blood Bank" were included in this study. The mean (range) age of these individuals was 62 (29-75) years, and the female to male sex ratio was 4:1. The general characteristics of the study groups are shown in Table I.

### Defining disease activity in RA patients

RA patients were arbitrarily divided into active, probably active, and inactive groups as defined by the levels of C-reactive protein (CRP) and/or the erythrocyte sedimentation rate (ESR).

Active RA patients

(CRP  $\geq$  15 mg/l and ESR  $\geq$  30 mm/h).

Probably active RA patients

(CRP  $\geq$  15 mg/l or ESR  $\geq$  30 mm/h).

Inactive RA patients

(CRP < 15 mg/l and ESR < 30 mm/h).

### Peptide sequence database analysis

In order to find a novel amino acid motif that was specific to the *Proteus* microbe and not present within human body tissues, a computer search was carried out using the GenBank and SwissProt databases. The urease enzyme molecule was selected for this study because it is not present in *Escherichia coli*, a microorganism responsible for a significant proportion of cystitis and urinary tract infections (UTI). A sequence of six charged amino acid, the "KELR-

QEER" motif, with a high hydrophilicity index was identified at positions "90-97" from the urease F enzyme of *Proteus mirabilis* bacteria. This sequence was confirmed to be absent in the synovial and other tissues targeted by RA in the human body.

### Peptide synthesis

Three synthesized amino acid peptides included in this study were provided by New England Peptide, Inc. (Gardner, USA). The peptides were analysed by high performance liquid chromatography, and all had a purity of at least 90%. Each of these peptides was 15 mer in length and all were synthesized from amino acid sequences present in *Proteus mirabilis* enzymes (Table II).

1. *Proteus* haemolysin (HpmB):

H2N-NGSSESRRALQDSQR-OH

2. *Proteus* urease C (UreC):

H2N-FAESRIRRETIAAED-OH

3. *Proteus* urease F (UreF):

H2N-ASRETKELRQEERQP-amide

The proline amino acid at the end of the urease F peptide sequences was difficult to link to the oxide group, and therefore an amide motif was used instead.

### Enzyme immunosorbent assay (ELISA)

Anti-peptide antibodies were measured using the ELISA method. Briefly, each well of a flat-bottom polystyrene mi-

crotitre plate (Nunc, Maxisorp), apart from duplicate wells which received buffer only, were coated with 200  $\mu$ l of the synthetic peptide (25  $\mu$ g/ml) prepared in carbonate buffer (0.05 M Na<sub>2</sub>CO<sub>3</sub>, 0.05 M NaHCO<sub>3</sub>, pH 9.6), and incubated overnight at 4°C. The unbound peptides were discarded from the plates, which were flickered gently on a soft tissue, and then each well was overlaid with 200  $\mu$ l of 1% bovine serum albumin (Sigma) diluted in phosphate buffered saline containing 0.05% Tween-20 (PBS-T). The plates were then incubated for 1 hour at 37°C. After washing the plates in PBS-T three times for a period of 3 minutes each, each well received 200  $\mu$ l of coded serum samples diluted to 1/200 in PBS-T and the plates were incubated for 2 hours at 37°C. The washing procedure was repeated 3 times, and then to each well was added either 200  $\mu$ l of rabbit anti-human peroxidase-conjugated IgM ( $\mu$ -chain specific) or IgG ( $\gamma$ -chain specific) or IgA ( $\alpha$ -chain specific) or anti-total ( $\mu$ -,  $\gamma$ -,  $\alpha$ - and light-chain specific) immunoglobulins (Dakopatt, Ltd), diluted 1/500 in PBS-T. The plates were then incubated at 37°C for 90 minutes. After washing the plates 3 times as before, 200  $\mu$ l of the enzyme substrate solution, 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid) (Sigma), 0.5 mg/ml in citrate phosphate buffer, pH

**Table I.** The general characteristics of the RA patients studied. They have been subdivided into groups based on their disease activity.

|                                         | Rheumatoid arthritis patients |               |                       |                |            | Healthy controls<br>20 |
|-----------------------------------------|-------------------------------|---------------|-----------------------|----------------|------------|------------------------|
|                                         | All<br>70                     | Active<br>21  | Probably active<br>24 | Inactive<br>25 |            |                        |
| Number                                  | 70                            | 21            | 24                    | 25             |            |                        |
| Age (yrs.) at diagnosis<br>mean (range) | 64 (38-81)                    | 64 (33-80)    | 65 (39-81)            | 63 (40-79)     | 62 (29-75) |                        |
| Female: Male                            | 55:15                         | 16:5          | 21:3                  | 18:7           | 16:4       |                        |
| Disease duration (yrs.)<br>mean (range) | 12.90 (0.5-41)                | 13.46 (1-34)  | 13.63 (0.6-41)        | 11.64 (0.5-35) | N/A        |                        |
| ESR (mm/hr) mean (SD)                   | 38.01 (24.36)                 | 63.14 (18.46) | 36.32 (12.81)         | 14.38 (6.30)   | ND         |                        |
| CRP (mg/dl) mean (SD)                   | 26.42 (40.78)                 | 61.81 (58.23) | 16.92 (15.68)         | 4.96 (2.24)    | ND         |                        |
| RF +ve (%)                              | 46 (66)                       | 11 (52)       | 17 (71)               | 18 (72)        | ND         |                        |
| Immunosuppressors (%)                   | 33 (47)                       | 11 (52)       | 11 (46)               | 11 (44)        | N/A        |                        |
| DMARDs (%)                              | 24 (34)                       | 5 (24)        | 10 (42)               | 9 (36)         |            |                        |
| Combined (%)                            | 13 (19)                       | 5 (24)        | 3 (12)                | 5 (20)         |            |                        |

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; DMARD: disease modifying anti-rheumatic drugs; ND: not done; N/A: not applicable.

**Table II.** Characteristics of the *Proteus mirabilis* amino acid peptides and their cross-reactivities with human tissue antigens.

| Peptide | Bacterial source | Amino acid sequences (position)                          | Cross-reactivity with human tissue | Human source              | Amino acid sequences (position) |
|---------|------------------|----------------------------------------------------------|------------------------------------|---------------------------|---------------------------------|
| 1       | Haemolysins HpmB | H2N-NGSSES <b>RR</b> ALQDSQR-OH <sup>a</sup> (28-42)     | Yes                                | HLA-DRB1 molecules        | EQK/RRAA (69-74)                |
| 2       | Urease C         | H2N-FAESR <b>IR</b> RETIAED-OH (332-346)                 | Yes                                | $\alpha$ 2 (XI) collagens | LRREI (421-425)                 |
| 3       | Urease F         | H2N-ASRET <b>KELRQ</b> EEERQP-amide <sup>b</sup> (85-99) | No                                 | N/A                       | N/A                             |

<sup>a</sup> Identical amino acids (in bold); <sup>b</sup> non-identical, charged amino acids (in bold and underlined).

A: alanine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; I: isoleucine; K: lysine; L: leucine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine. N/A: not available.

4.1, containing 0.98 mM H<sub>2</sub>O<sub>2</sub> (Sigma) was added to each well, and the plates were kept at room temperature in a dark place for 20 minutes, after which the reaction was stopped with 100  $\mu$ l of 2 mg/ml sodium fluoride diluted in distilled water. The absorbance or optical density (OD) values were then measured at a wavelength of 630 nm using a microtitre plate reader (Dynatech MR 600).

Each serum sample was tested in duplicate and the plates were coded so that the examiner did not know whether the tested sera had been taken from diseased or control individuals.

#### Determinations of ESR, CRP and RF

The erythrocyte sedimentation rate (ESR) (mm/hr) and C-reactive protein (CRP) (mg/dl) for each patient was determined by using the Westergren and the immuno-turbidimetric methods, respectively. The status and titre of each patient's rheumatoid factor (RF) was established at the time of serum collection using laser nephelometry.

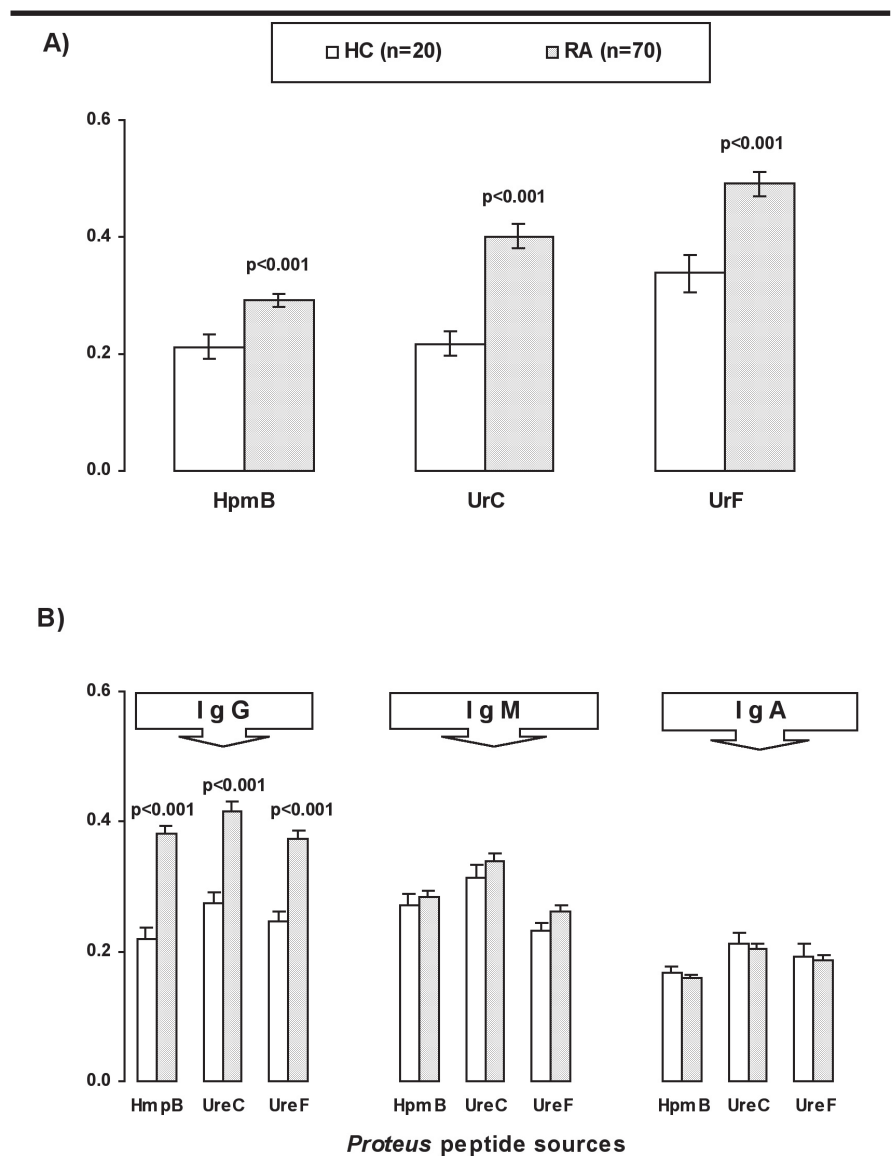
#### Statistical analysis

A comparative analysis of the mean OD units for each class-specific antibody titre was carried out between RA patients as a whole and those with active, probably active or inactive disease status and healthy control subjects using a two-tail Student's t-test. The relationship between peptide antibody levels and each of the biochemical markers of disease activity or severity, such as CRP, ESR, RF, as well as the disease duration, were estimated in the RA patients using Pearson's correlation coefficient

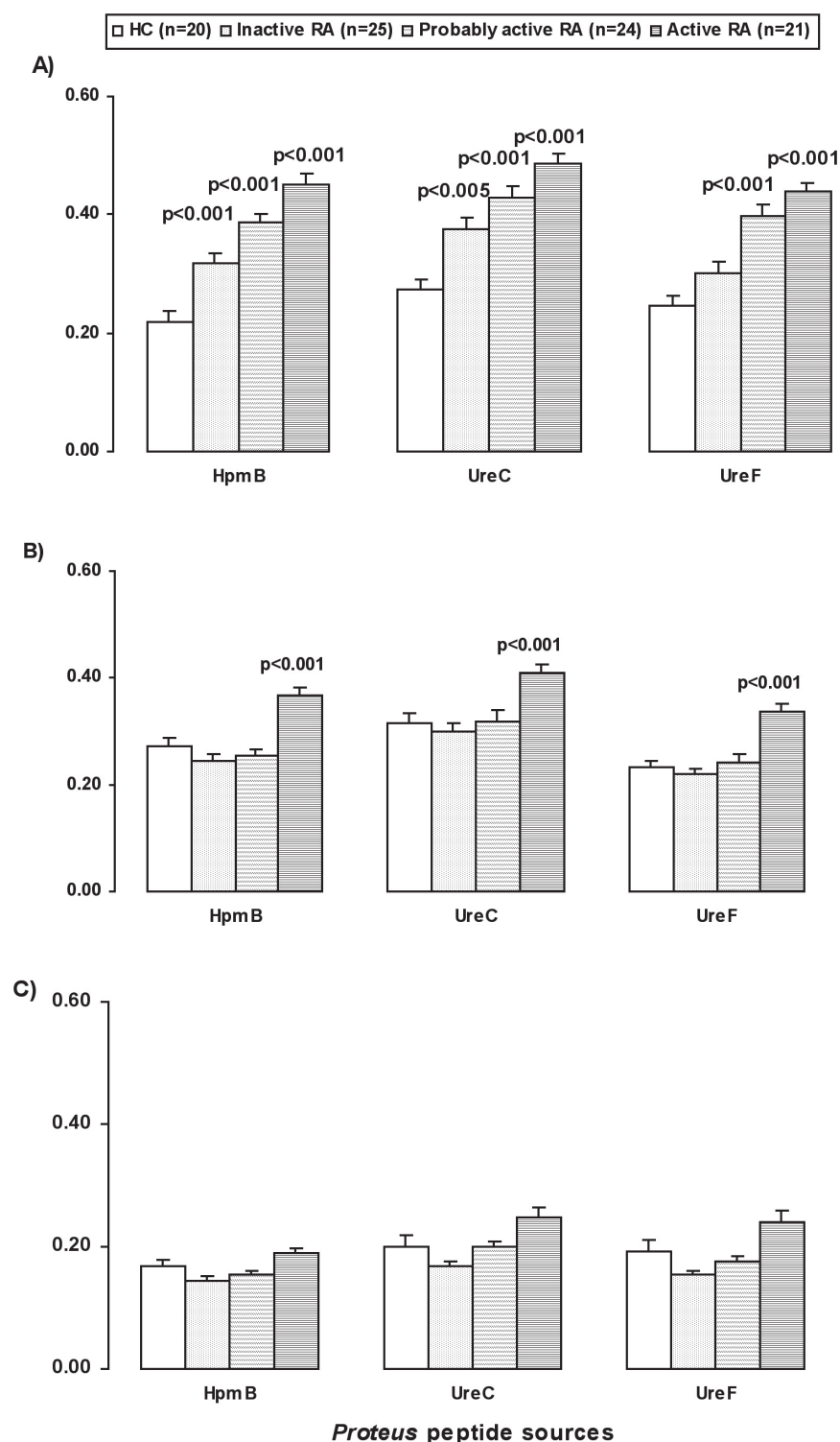
[r]. Furthermore, the relationship between the disease status and positive or negative RF in these patients was es-

tablished using the chi square test with or without Yates correction.

To study the relationship between RA



**Fig. 1.** Total (A) and IgG (B) *Proteus* anti-peptide antibodies in patients with rheumatoid arthritis (RA) and healthy controls (HC).



**Fig. 2.** IgG (A), IgM (B) and IgA (C) *Proteus* anti-peptide antibodies in rheumatoid arthritis (RA) patients with different disease activity status compared to healthy controls (HC). For the definition of patient's disease activity classification, see the text.

disease activity and the OD levels of antibodies to all three peptides, an antibody index was determined for each serum sample using the following formula, as described previously (30) but with the addition of data results from

antibodies against the new Urease F peptide:

Anti-peptide antibody index = (Antibodies against HpmB x 10) (Antibodies against UreC x 10) (Antibodies against UreF x 10).

## Results

### Total *Proteus* anti-peptide antibodies

When total immunoglobulins against the synthetic peptides from haemolysin (HpmB), urease C (UreC) and urease F (UreF) *P. mirabilis* enzymes were investigated, significantly elevated levels of antibodies against HpmB ( $t = 3.65$ ;  $p < 0.001$ ), UreC ( $t = 5.54$ ;  $p < 0.001$ ) and UreF ( $t = 4.02$ ;  $p < 0.001$ ) peptides were observed in patients with RA compared to healthy control subjects (Fig. 1A).

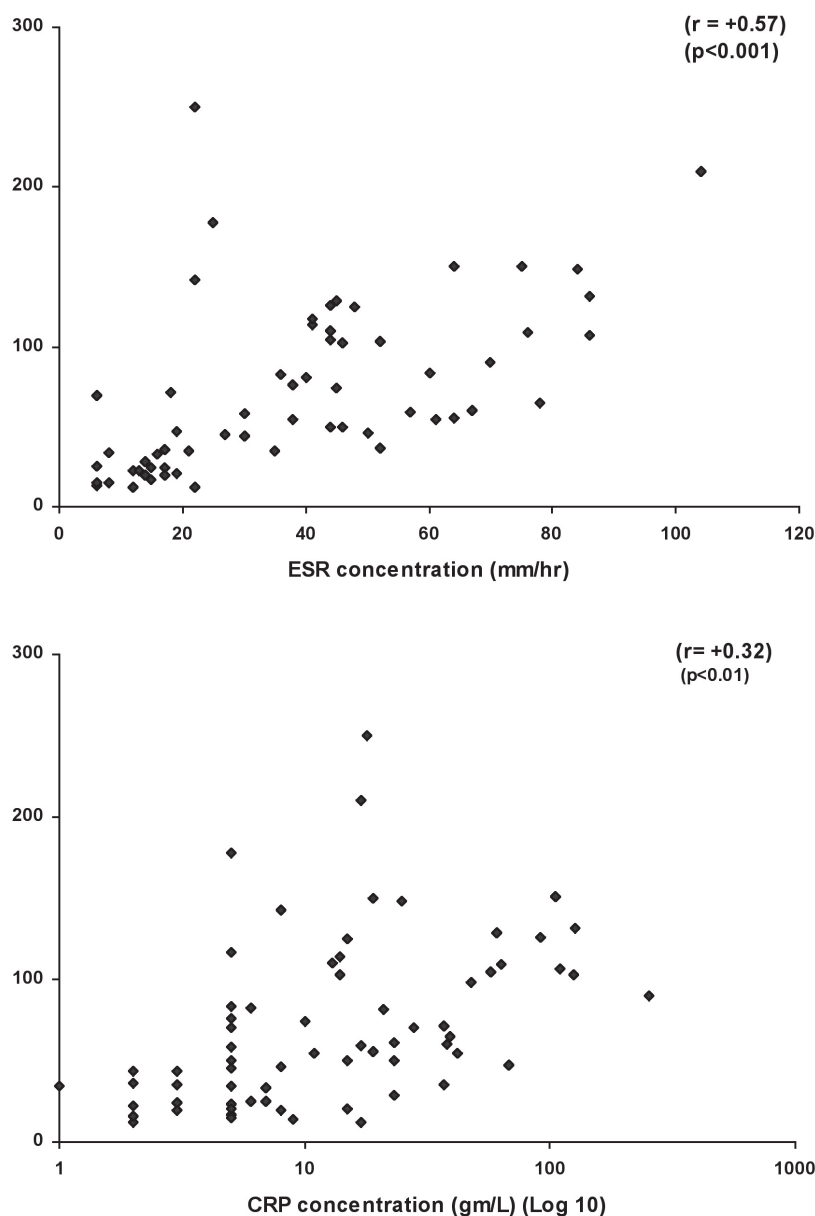
### Isotypic *Proteus* anti-peptides antibodies

When class-specific immunoglobulins were screened against the synthetic peptides from *P. mirabilis* enzymes, significantly elevated levels of IgG antibodies to HpmB ( $t = 6.80$ ;  $p < 0.001$ ), UreC ( $t = 5.72$ ;  $p < 0.001$ ) and UreF ( $t = 5.12$ ;  $p < 0.001$ ) were observed in patients with RA when compared to healthy subjects. No such differences were detected between patient and healthy individuals for IgM and IgA isotypic antibodies against any of the tested peptides (Fig. 1B).

### Disease activity and *Proteus* isotypic anti-peptide antibody levels

When RA patients were grouped by disease activity (active, probably active, and inactive disease), patients with active disease had significantly elevated levels of IgG antibodies against HpmB ( $t = 9.59$ ;  $p < 0.001$ ), UreC ( $t = 8.69$ ;  $p < 0.001$ ) and UreF ( $t = 9.37$ ;  $p < 0.001$ ) *Proteus* peptides compared to healthy controls. When probably active RA patients were compared to healthy subjects, significant differences were detected among IgG antibodies to HpmB ( $t = 7.65$ ;  $p < 0.001$ ), UreC ( $t = 5.49$ ;  $p < 0.001$ ) and UreF ( $t = 6.23$ ;  $p < 0.001$ ) peptides. When the inactive RA patient group was compared to healthy subjects, significantly elevated IgG antibodies were detected against HpmB ( $t = 3.82$ ;  $p < 0.001$ ) and UreC ( $t = 3.09$ ;  $p < 0.005$ ), but not UreF ( $t = 1.82$ ) *Proteus* peptides (Fig. 2A).

When IgM antibodies against these *Proteus* peptides were screened, active RA patients had significantly el-



**Fig. 3.** Correlation between IgG *Proteus* anti-peptide antibody indices and erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) concentrations in patients with rheumatoid arthritis (n = 70). For the definition of the *Proteus* anti-peptide indices, see the text.

elevated titres of IgM antibodies against HpmB ( $t = 4.39$ ;  $p < 0.001$ ), UreC ( $t = 3.88$ ;  $p < 0.001$ ) and UreF ( $t = 4.84$ ;  $p < 0.001$ ) *Proteus* peptides compared to healthy control subjects. No significant differences were observed in the IgM antibodies against any of the *Proteus* peptides in the probably active and the inactive RA patient groups compared to healthy controls (Fig. 2B). No significant differences in the IgA antibodies against synthetic *Proteus* peptides were observed between any of the three groups of RA patients and healthy subjects (Fig. 2C).

#### *Correlation between the IgG Proteus anti-peptide antibody index and disease activity parameters, RF status and disease duration*

The production of IgG antibodies against all three *Proteus* peptides (anti-peptide antibody index) was calculated for each serum sample, and their correlations with various disease activity and severity parameters were examined. Significant correlations were observed between the anti-peptide *Proteus* antibody indices and both ESR ( $r = 0.57$ ,  $p < 0.001$ ) and CRP ( $r = 0.32$ ,  $p < 0.01$ ) concentrations (Fig. 3). However, no correlations

were observed between these antibody indices and the RF status or the disease duration in RA patients. In addition, no significant correlation between RF and the disease activity status was found.

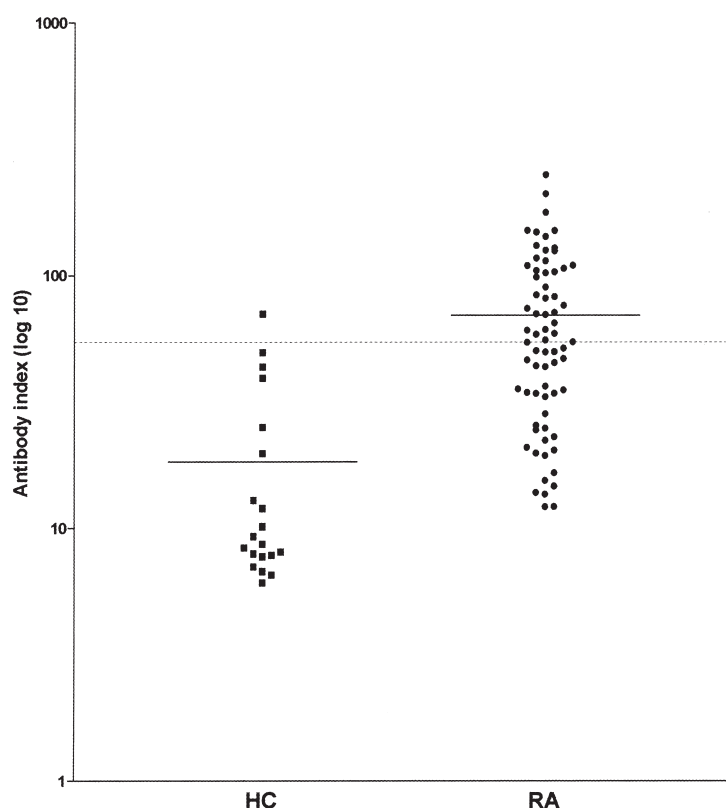
#### *Determination of RA patients with positive cut-off values for IgG anti-Proteus antibody index levels*

The IgG *Proteus* anti-peptide antibody index for RA patients was designated as 'positive' if it exceeded the 95% confidence limit (mean + 2 standard deviations) of the healthy controls. Overall, 50% of the RA patients were found to have a positive *Proteus* anti-peptide antibody index (Fig. 4). However, when subdivided by disease activity status, it was observed that 19 (91%) of 21 active, 15 (63%) of 24 probably active and 3 (12%) of 25 inactive patients with RA had positive anti-*Proteus* antibody index values (Fig. 5).

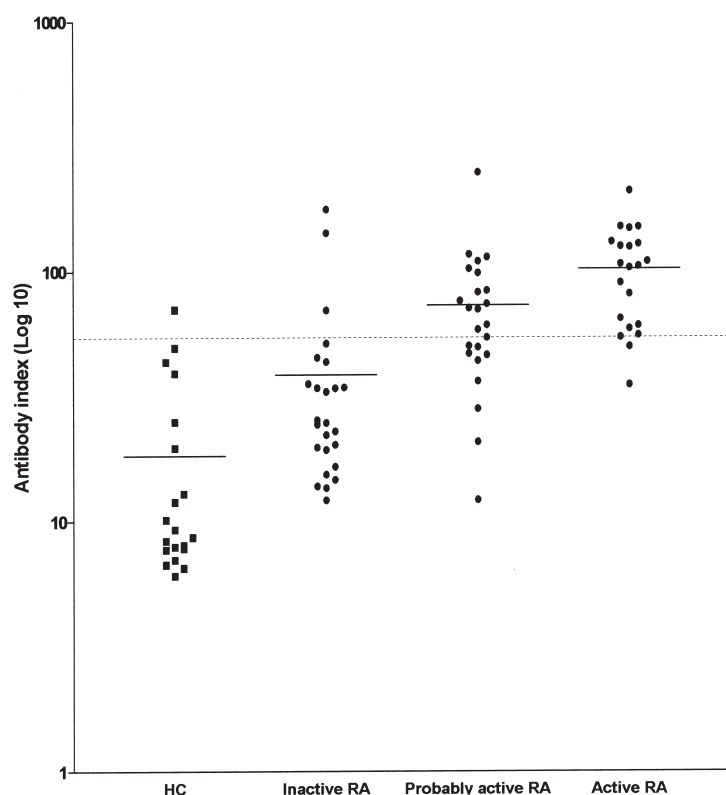
#### **Discussion**

Our findings of significantly elevated antibodies against *Proteus mirabilis* antigenic peptides in patients with RA add support to the results of studies by our and other independent groups from many countries (16, 24, 31). In this study we found that active, probably active and inactive RA patients all had significantly elevated levels of IgG antibodies against haemolysins, as well as two other urease synthetic peptides (one human cross-reactive and another non cross-reactive) from *Proteus mirabilis* when compared to healthy controls. Furthermore, active RA patients showed significantly increased titres of IgM antibodies to the three *Proteus* peptides compared to healthy control subjects. However, analogous differences in the levels of IgA antibodies to these peptides were not detected.

Our study shows that elevated *Proteus* anti-peptide antibody indices are significantly correlated with increased levels of CRP and ESR, which could indicate that these biological markers of disease activity arise from the effect of pathological damage to the synovial tissues via antibody-mediated cytotoxicity reactions (28). However, no significant correlations were observed between elevated antibody indices and



**Fig. 4.** Positive cut-off values of *Proteus* IgG anti-peptide antibody indices in patients with rheumatoid arthritis (RA) and in healthy controls (HC) (solid line = means; dotted line = 95% CL of HCs). For the definition of cut-off values, see the text.



**Fig. 5.** Positive cut-off values of *Proteus* IgG anti-peptide antibody indices in rheumatoid arthritis (RA) patients with different disease activity status and in healthy controls (HC) (solid line = means; dotted line = 95% CL of HCs)

RF levels or the disease duration.

Most recently, a multi-centre prospective study involving five centres from the USA, Canada and the UK was carried out on 246 patients with inflammatory arthritis of less than one year's duration. Newkirk *et al.* found that IgM and IgA anti-*Proteus* and IgA anti-*Escherichia coli* antibodies were significantly higher in patients with RF-positive RA compared to those with RF-negative RA, spondyloarthropathies or undifferentiated arthritis (15). However, there were no significant differences in the isotypic antibodies to *Chlamydia*, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* and potentially arthritogenic parvovirus B19 microbes. The possibility that the increased levels of anti-*Proteus* antibodies could reflect high RF titres among RF-positive patients with RA was excluded in the study when Newkirk *et al.* failed to detect a reduction in the levels of antibodies to *Proteus* after the absorption of RF. The raised levels of IgM antibodies to *E. coli* reported by Newkirk *et al.* and by Aoki (32) are not, however, in agreement with the results of other studies (19, 20, 24, 33-35). The reason for the elevated levels of anti-*Proteus* IgA and IgM but not IgG in cases of early RA is that these patients are more likely to be exposed to *Proteus* infections for the first time, but repeated bouts of infections – whether apparent or subclinical – will eventually lead to a shift from excess IgA and IgM to IgG anti-*Proteus* antibody production, as shown in our study.

The detection of relatively low OD values in the class-specific antibodies in our study could be due to the fact that more than 65% of the RA patients examined were receiving one or two of the most widely used immunosuppressive agents, which could have lowered the overall levels of immunoglobulins in their sera. This finding could also explain why Newkirk *et al.* (15) reported considerably higher OD values for IgM anti-*Proteus* antibodies in RA, as these patients were studied during early stages of the disease and were more likely to have received anti-inflammatory rather than immunosuppressive drugs. In another recent study, a group from

Los Angeles reported a higher incidence of IgA anti-*Proteus* antibodies in RA patients than in healthy controls. Several molecules, however, were identified in the fumarate reductase A-chain of *P. mirabilis*, in particular one with a molecular weight of 66 kDa to which the RA patients exhibited a reduced IgA immune response compared to controls (36). It has been suggested that the occurrence of selective holes in the IgA immune repertoire for these antigens in *Proteus* bacteria could explain the increased likelihood that RA patients will harbour this microbe and hence the greater chance of infection and the consequent enhancement of anti-*Proteus* antibody responses.

*P. mirabilis* has a propensity to settle in the upper part of the urinary tract, particularly in the kidneys (37). This particular characteristic, together with the tendency to form struvite urinary stones as a consequence of urease-mediated urea hydrolysis (38), make it difficult to detect these microbes by ordinary microbiological methods. It also explains why they are not easily eradicated by host immune cells or anti-microbial measures.

The *Proteus* microbe is a urinary pathogen and commonly second to *E. coli* in causing UTI (39), especially among women. RA patients were found to have UTI (40) or *Proteus* bacteriuria (12) more frequently than controls. These observations could explain the higher prevalence of RA among women.

The new *Proteus* bacterial antigen used in our study is formed of charged amino acids, which are more likely to be expressed on the surface of urease enzyme molecules and which could also be immunogenic, as shown by the elevated levels of IgG and IgM antibodies to this particular epitope, particularly in patients with active RA. Enhanced humoral immune responses to the novel antigenic profile of the *Proteus* urease enzyme, which does not share similarities with any other self antigenic synovial molecule, challenges the notion that raised anti-*Proteus* antibodies in RA patients is a secondary phenomenon resulting from damage to the synovial tissues by autoantibodies generated via mechanisms other than bacterial triggers.

Based on the cumulative evidence for the role of *Proteus* microbes in the development of RA, it is logical to suggest that patients with RA, especially during the very early stages of the disease before irreversible pathological lesions have developed, can be treated with anti-*Proteus* measures including a vegetarian diet (41), antibiotics, and a high daily intake of cranberry juice (42). These anti-microbial treatments could be instituted in conjunction with currently recommended anti-rheumatic drugs. In order to study the possible benefits of anti-*Proteus* treatment in the management of RA, prospective longitudinal controlled studies need to be carried out in patients with early RA.

In conclusion, this study shows that patients with RA have elevated levels of antibodies to various antigens from *Proteus* microbes. To investigate this immunological link, further studies should be carried out using western blot techniques against these, as well as other microbial antigens, in patients with RA compared to other disease and healthy controls.

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