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The detection of raised levels of IgM to *Proteus mirabilis* in sera from patients with rheumatoid arthritis

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Summary. An analysis by ELISA of 100 rheumatoid factor (RF)-positive sera selected at random from a collection of sera from patients with various auto-immune diseases and joint pains, and 100 RF-negative sera from the same collection matched by patient age and gender, showed that the RF-positive sera had highly significantly ($p < 0.0001$) raised levels of IgM antibody, but not IgG antibody, to *Proteus mirabilis* over those of the RF-negative sera. This response was subsequently found to be associated with sera from patients who clinically had rheumatoid arthritis (RA). Sera from the RA patients had significantly greater amounts ($p = 0.026$) of IgM antibody to *P. mirabilis* than to the other organisms tested and these values were also highly significantly different ($p < 0.0001$) from *P. mirabilis* IgM antibody levels in matched RF-negative sera. Sera from RA patients also had significantly greater amounts of IgA to *P. mirabilis* ($p < 0.0001$) and greater amounts of IgM to *Escherichia coli* ($p < 0.0001$) and *Klebsiella pneumoniae* ($p < 0.0001$) than those in matched RF-negative sera. Other classes of antibody to these organisms and all classes of antibody to *Pseudomonas aeruginosa* were not raised in the sera of RA patients over those of RF-negative controls. The IgM response in RA patients was not specific for only one O serotype of *P. mirabilis* but was associated with all 11 different O serotypes of *P. mirabilis* tested and those of other *Proteus* spp. Moreover, the IgM antibodies to *Proteus* spp. appeared to be independent from C-reactive protein and RF. Fourteen of 27 sera from RA patients had an IgM antibody that reacted with an internal 90-kDa protein of *P. mirabilis*. This antibody was not found in RF-positive sera of patients with Sjögren's disease or systemic lupus erythematosus, nor in other RF-negative sera, nor in those of healthy people, nor in those with osteoarthritis or ankylosing spondylitis. IgM antibodies capable of reacting with the haemolysin protein Hpm B of *P. mirabilis* were not found in sera of RA patients of unknown HLA status.

Introduction

Rheumatoid arthritis (RA) is an auto-immune disease that affects 1–3% of the population and is commoner in women (particularly middle-aged and elderly women) than men. It presents as an inflammatory polyarthritis, initially usually of small peripheral joints but later of other joints and organs. In most cases, rheumatoid factors (RFs)—antibodies to the Fc region of IgG—can be found in the serum and synovial fluid of patients. However, RF formation is not restricted to RA but is found also in a number of diverse chronic infectious diseases and some other auto-immune diseases such as systemic lupus erythematosus (SLE) and Sjogren's disease. RF reacts with IgG to form immune complexes which activate complement and trigger inflammatory processes.

Although there is a strong genetic predisposition to RA associated with HLA-DR4,[1,2] it is believed that there are other factors involved in triggering the disease. One of these may be microbial. Although a number of micro-organisms have been implicated,[3–6] one which many investigators have linked with RA is *Proteus mirabilis*, the organism which, after *Escherichia coli*, is most frequently associated with urinary tract infections, particularly of the upper tract and in middle aged and elderly women.[7]

Ebringer and colleagues first showed that RA patients had raised levels of antibody specific for *P. mirabilis* and that antisera to HLA-DR4 lymphocytes reacted only with *Proteus* spp. and not with 16 other bacteria of various gram-positive and gram-negative genera.[8] Employing a variety of techniques, they,[9,10]

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and others[11,12] working independently, subsequently confirmed these findings. It was also shown that raised antibody levels to *Proteus* spp. appeared to be specific for RA for they were not found in other auto-immune conditions such as systemic lupus erythematosus, sarcoidosis[11] or ankylosing spondylitis,[8,9] nor was the response the outcome of increased permeability of the gut wall to intestinal bacteria through medication.[13] It was suggested that RA is a proteus-reactive arthritis triggered by recurrent or asymptomatic urinary tract infections through antibodies to *Proteus* spp. cross-reacting with cells expressing HLA-DR4.[10] Supporting this hypothesis was the more frequent isolation of *Proteus* spp. from the urine of RA patients than healthy controls.[14] However, others[15] have not been able to confirm this nor have proteus antibodies been found in urine from RA patients more frequently than in that from healthy controls.[16]

The aims of this project were to verify a raised antibody response specifically to *Proteus* spp. and specifically for RA patients, to determine if the response was specific for a particular serotype of a *Proteus* sp. and to investigate the nature of the antibody response and the antigen(s) to which it was reacting.

Materials and methods

Bacteria

Eleven strains of *P. mirabilis* representing the 11 commonest O serotypes of strains associated with proteus urinary tract infections (UTI)[17] and 11 strains of *E. coli* (kindly donated by Dr P. Gomes, Department of Medical Microbiology, University of Dundee) representing the 11 commonest O serotypes associated with *E. coli* UTI[18] were studied. These are listed in table I. Other bacteria used included seven clinical isolates of *P. vulgaris* of serotypes O1 (two strains), O3, O8, O19, O21 and O28, seven clinical isolates of *P. penneri* of unknown O serotype and, from laboratory stock cultures, one clinical isolate of *Pseudomonas aeruginosa* and one of *Klebsiella pneumoniae*. The strains were identified by standard biochemical tests.

Table I. *The P. mirabilis* and *E. coli* strains used and their O serotypes

P. mirabilis strain no.	O serotype	*E. coli* strain no.	O serotype
PB6	O3	74744/77	O1
PB4	O6	7540/78	O2
PB 15	O10	24607/77	O4
F 67	O11	15964/77	O6
PB 48	O13	NCTC 9007	O7
SW 39	O23	NCTC 9008	O8
SW 49	O24	NCTC 9009	O9
PB 7	O27	NCTC 9011	O11
PB 13	O28	NCTC 9022	O22
SW 74	O29	NCTC 10430	O25
SW 79	O30	35000/78	O75

E. coli strains bearing either the plasmid pWPM 100 carrying the *P. mirabilis* haemolysin genes *hpm*A and *hpm*B or the plasmid pWPM 109 carrying *hpm*B[19,20] were donated by Dr R. A. Welch, Department of Medical Microbiology, University of Wisconsin, Madison, WI 53706, USA.

Culture of strains

The strains were grown overnight at 37°C on Cystine Lactose Electrolyte Deficient (CLED) medium (Oxoid CM 301). The cells were harvested and suspended in phosphate-buffered saline, pH 7·2, containing sodium azide 0·04% (PBS-Az) and washed in the same solution. The washed cells were resuspended in PBS-Az to an OD_{550} of 1·0 and stored as standard suspensions at 4°C.

Measurement of rheumatoid factor (RF)

Serum (50 μl) diluted 20-fold in glycine-saline buffer, pH 8·2, was mixed with 50 μl of neat RF latex reagent (Biostat Diagnostics, Stockport) (latex particles coated with human IgG) on a dark tile. The tile was rocked for 2 min and examined for agglutination of latex particles. Positive and negative control sera were included in each batch of tests. Agglutination indicated that the serum had an RF content of $\geqslant$ 20 IU/ml. Doubling dilutions of such positive sera in buffer from 1 in 20 were re-tested. The RF concentration (IU/ml) was equal to the highest dilution that gave visible agglutination.

Sera

From a large collection of sera from patients with various auto-immune diseases and joint pains, 100 test sera (referred to as RF-positive) that had RF levels of $\geqslant$ 475 IU/ml were selected at random and blindly regarding the donors' clinical symptoms. The control sera (referred to as RF-negative) comprised 100 sera from the collection selected randomly with respect to patient symptoms from those without RF (RF level 0 IU/ml) but which were matched as closely as possible to those of the test sera with respect to patient age and gender. A number of sera which were RF-negative were also obtained from healthy volunteers with no auto-immune disease or joint pains.

ELISA protocol

One hundred-μl volumes of either a 1 in 10 dilution of the standard suspensions of bacteria in PBS-Az, or PBS-Az alone (control), were added to flat-bottomed wells of microtitration plates (Nunc, Maxisorp F8) which were covered and left overnight at room temperature. The contents of the wells were then removed and discarded and the wells were washed

three times in PBS containing Tween-20 0·05% (PBST). PBST (200 μl) containing bovine serum albumin 0·05% was added to each well and incubated for 1 h at room temperature. The contents of the wells were then removed and discarded and the wells were washed three times in PBST. Serum (100 μl) diluted 1 in 100 in PBST was added to each well. The plates were incubated at room temperature for 2 h and the well contents were removed and discarded. The wells were washed three times in PBST and then 200 μl of goat anti-human alkaline phosphatase-conjugated IgM (μ chain specific) or IgA (α chain specific) or IgG (γ chain specific) (Sigma reagent numbers A-3275, A-3400 and A-3150 respectively) diluted 1 in 1000 in PBST were added to each well. After incubation for 1·5 h at room temperature, the well contents were removed and discarded and the wells were washed three times in PBST. Two hundred μl of *p*-nitrophenyl phosphate 2 mg/ml in substrate buffer (100 mM sodium bicarbonate, 1 mM magnesium chloride, pH 10·2) were added to each well and the plates incubated at 37°C for 30 min. The OD_{405} of each well of the plate was then measured immediately by a Dynatech MR 5000 plate reader.

C-reactive protein (CRP) measurements

These were made on 20 μl of serum by a fluorescent polarisation immunoassay based on the TDX system (Abbott Laboratories).

Haemolysin assay

E. coli strains bearing plasmids pWPM 109 and 100 which had been cultured overnight at 37°C in Luria nutrient broth (LB) containing, respectively, either chloramphenicol 30 μg/ml or carbenicillin 50 μg/ml were diluted 100 times into fresh antibiotic-supplemented LB and incubated with shaking at 37°C. At intervals, 50 μl of the culture or of the supernate from a portion of the culture centrifuged at 11600 *g* for 2 min, or of sterile LB (control), were added to a microcentrifuge tube holding 1 ml of washed horse erythrocytes 2% in saline. The tubes were incubated in a water bath at 37°C for 15 min and then centrifuged at 11600 *g* for 30 s. The haemolytic activity of the sample was assessed by measuring the amount of free haemoglobin in the supernate by determining its absorbance at 540 nm against that of the control.

SDS-PAGE and immunoblotting

Suspensions of washed bacterial cells were mixed with equal volumes of sample buffer (0·125 M Tris-HCl, pH 6·8, SDS 4%, glycerol 20%, mercaptoethanol 10% and a trace of bromophenol blue dye), boiled for 5 min and applied to a stacking gel of acrylamide 4% in 0·125 M Tris-HCl, pH 6·8, SDS 0·1% over a resolving gradient gel of acrylamide 5–15% in 0·375 M Tris-HCl, pH 8·8, SDS 0·2%. The upper tank buffer was 0·053 M Tris, 0·052 M glycine, SDS 0·1%, pH 8·9, and the lower tank buffer was 0·1 M Tris-HCl, SDS 0·1%, pH 8·1. Electrophoresis was performed at 25 mA in the cold until the dye front reached the bottom of the gel. The separated proteins were transferred from the gel to nitrocellulose by electrophoresis in methanol 10% containing 25 mM Tris and 190 mM glycine at 4°C for 16 h at 10 V. The membrane was then stained either with Gold Stain (Protogold, British Biocell International, Cardiff) or with Ponceau S dye 0·2% in trichloroacetic acid 3% and the positions of reference protein standards were marked. After washing the membrane in PBS, it was agitated for 2 h in PBS containing dried milk powder (Marvel) 5%. Strips of the membrane were then cut and each was agitated in PBS-Marvel containing a 1 in 100 dilution of the patient's serum for 2 h at room temperature. The strips were washed thoroughly in several changes of PBS and then agitated in PBS-Marvel containing a 1 in 1000 dilution of goat anti-human IgM (μ chain specific) (or other appropriate anti-human antibody class) labelled with alkaline phosphatase (Sigma) for 2 h at room temperature. The immunoblots were subsequently developed in 30 ml of buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9·5) containing 100 μl bromochloro-indolyl phosphate (50 mg/ml in dimethyl formamide) and 200 μl nitroblue tetrazolium (50 mg/ml in dimethyl formamide 70% in water).

Statistics

The significance of differences between ELISA results was examined by determination of two-tailed p values by the Mann-Whitney test with the aid of Graphpad Instat software (San Diego, CA, USA). The correlation of antibody, CRP and RF levels was examined by calculation of correlation coefficients (r) by an Inplot program.

Results

In the development of the ELISA protocol, each of the 11 O serotypes of *P. mirabilis* was tested against one strongly RF-positive and one RF-negative serum selected from each group of 100 sera. Because *P. mirabilis* SW 74 (O29) (subsequently referred to as "*Proteus*"), gave the highest OD value with the selected RF-positive serum and the lowest OD value with the selected RF-negative serum, it was selected as the standard *Proteus* strain for ELISA tests.

The ELISA results of IgM, IgA and IgG antibody levels to *Proteus* in 100 RF-positive and 100 RF-negative sera are presented in fig. 1 and table II. They showed that RF-positive sera could be distinguished from RF-negative sera; the former had highly significantly ($p < 0·0001$) raised IgM levels, and also, but to a lesser extent ($p = 0·034$), significantly raised IgA levels to *Proteus*. However the levels of IgG to *Proteus* in the RF-positive sera were not significantly different ($p = 0·12$) from those in the RF-negative sera.

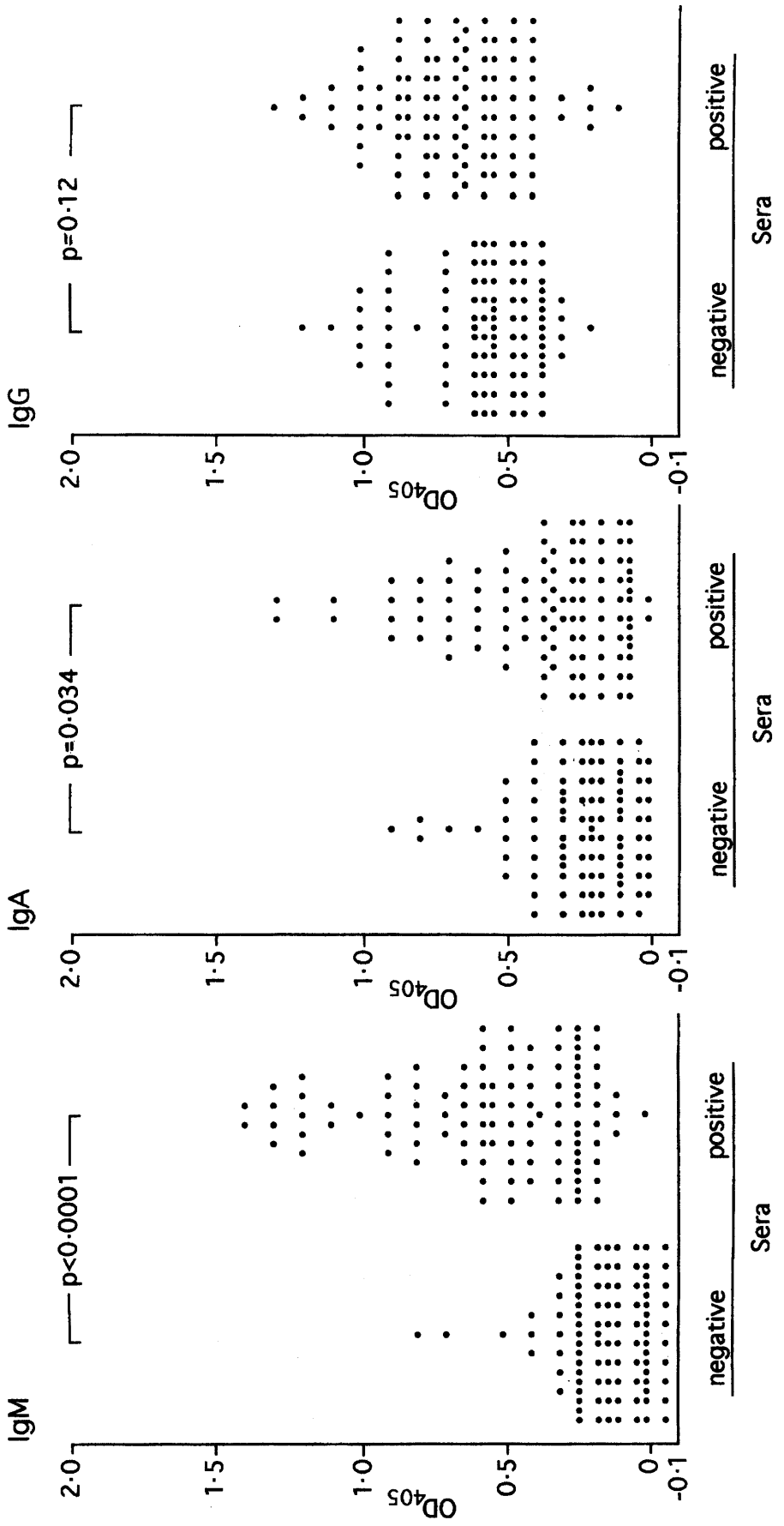

Fig. 1. ELISA measurements of IgM, IgA and IgG antibody levels to *P. mirabilis* in 100 RF-positive and 100 matched RF-negative sera.

Table II. Mean ELISA values (OD_{405}) of different classes of antibodies to different organisms found in RF-positive and -negative sera from the mixed diagnosis pool and from 27 RF-positive RA patients and 27 matched RA-negative patients

Organism	RF positive sera			RF negative sera		
	Number of sera	Antibody class	Mean ELISA OD(SD)	Number of sera	Mean ELISA OD(SD)	p value
P. mirabilis O29 SW74	100	IgM	0·49 (0·35)	100	0·13 (0·13)	< 0·0001
	100	IgA	0·33 (0·28)	100	0·24 (0·17)	0·034
	100	IgG	0·60 (0·31)	100	0·59 (0·19)	0·12
	50	IgM	0·55 (0·31)	50	0·11 (0·12)	< 0·0001
	50	IgA	0·27 (0·21)	50	0·13 (0·097)	< 0·0001
	50	IgG	0·33 (0·20)	50	0·35 (0·19)	0·48
E. coli O22 NCTC 9022	50	IgM	0·42 (0·21)	50	0·10 (0·11)	< 0·0001
	50	IgA	0·039 (0·030)	50	0·025 (0·026)	0·0073
	50	IgG	0·32 (0·38)	50	0·31 (0·26)	0·91
K. pneumoniae	50	IgM	0·31 (0·15)	50	0·12 (0·12)	< 0·0001
	50	IgA	0·18 (0·13)	50	0·13 (0·095)	0·0395
	50	IgG	0·28 (0·14)	50	0·25 (0·11)	0·502
Ps. aeruginosa	50	IgM	0·15 (0·095)	50	0·11 (0·091)	0·080
	50	IgA	0·08 (0·017)	50	0·07 (0·016)	0·104
	50	IgG	0·15 (0·12)	50	0·11 (0·089)	0·0745
	From RA patients			**From RA-negative patients**		
P. mirabilis O29 SW 74	27	IgM	0·67 (0·33)	27	0·07 (0·05)	< 0·0001
	27	IgA	0·22 (0·15)	27	0·09 (0·061)	< 0·0001
	27	IgG	0·27 (0·12)	27	0·24 (0·098)	0·308
E. coli O22 NCTC 9022	27	IgM	0·36 (0·20)	27	0·076 (0·059)	< 0·0001
	27	IgA	0·030 (0·016)	27	0·024 (0·020)	0·137
	27	IgG	0·27 (0·13)	27	0·33 (0·17)	0·139
K. pneumoniae	27	IgM	0·29 (0·14)	27	0·095 (0·073)	< 0·0001
	27	IgA	0·16 (0·086)	27	0·12 (0·079)	0·061
	27	IgG	0·27 (0·023)	27	0·24 (0·019)	0·308
Ps. aeruginosa	27	IgM	0·14 (0·086)	27	0·10 (0·091)	0·177
	27	IgA	0·085 (0·018)	27	0·074 (0·021)	0·263
	27	IgG	0·14 (0·12)	27	0·096 (0·089)	0·0657

To determine whether raised IgM and IgA levels to *Proteus* in RF-positive sera were specific for this organism, 50 sera chosen at random from both the RF-positive and RF-negative pools of sera were tested by ELISA for IgM, IgA and IgG antibodies to *Proteus*, *E. coli* NCTC 9022 (serotype O22) (this strain consistently gave the highest OD value of the *E. coli* strains with RF-positive sera and the lowest OD value with RF-negative sera), *K. pneumoniae* and *Ps. aeruginosa*. It was found (table II) that for all these organisms, the highest levels of IgM and IgA antibodies found in RF-positive sera were those to *Proteus* and that for this organism, the levels of both these antibody classes in RF-positive sera were significantly different ($p < 0·0001$) from those in RF-negative sera. RF-positive sera also had highly significantly ($p < 0·0001$) different levels of IgM antibodies to *E. coli* and *K. pneumoniae* and significantly different levels of IgA antibodies to *E. coli* ($p = 0·0073$) and to *K. pneumoniae* ($p = 0·0395$) from those of RF-negative sera. However, the antibody levels in the RF-positive sera to these organisms and to *Ps. aeruginosa* were much lower than those to *Proteus*. IgG antibody levels to *Proteus* and the other organisms were not significantly higher in RF-positive sera than in RF-negative sera.

When an assessment was made of the clinical diagnosis for as many as possible of the patients from whom the 50 RF-positive sera with high IgM levels to *Proteus* had been obtained, 27 patients with rheumatoid arthritis were identified. When the sera from these 27 RA patients were compared by ELISA with 27 sera matched for age and gender from the RF-negative pool, the above findings were confirmed. Moreover, the differences between the levels of IgM antibodies to *Proteus* in the RF-positive sera of the 27 RA patients and those to the other organisms were now even greater than those which had been found among the 50 RF-positive sera of all types of patient. The results are presented in table II. The difference in the IgM levels in sera from the 27 RA patients between *Proteus* and *E. coli* was significant ($p = 0·026$), between *Proteus* and *K. pneumoniae* was very significant ($p = 0·0012$) and between *Proteus* and *Ps. aeruginosa* was highly significant ($p < 0·0001$).

It was noted that many RA patients who had high IgM levels to *Proteus* did not have correspondingly high levels of IgA to *Proteus* and *vice versa*. However, there was a significant correlation ($r = 0·34$, $p = 0·0128$) between these classes of antibody when their levels were compared for the RA group as a whole. Furthermore, although many RA patients had high IgM levels to *Proteus* and low IgM levels to *E. coli* and *vice versa*, there was also a very significant correlation ($r = 0·59$, $p < 0·0001$) between IgM levels to these organisms among the RA patients.

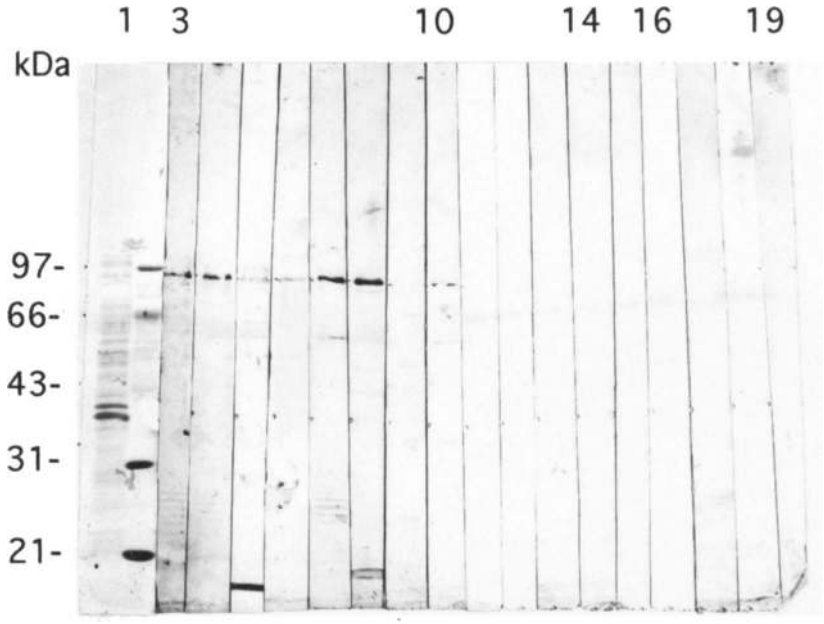

Fig. 2. Immunoblot of lysates of *P. mirabilis* SW 74 serotype O29 stained with gold (lane **1**) or blotted with sera from different patients and then with goat anti-human μ chain-specific immunoglobulin labelled with alkaline phosphatase and developed. A 90-kDa protein was detected in most of the sera from only RA patients (**3–10**) and not in RF-negative patients' sera (**11–14**) nor in those with osteoarthritis (**15, 16**) nor in those with ankylosing spondylitis (**17–19**); mol. wt (kDa) protein markers (**2**).

Further analysis of the sera from the 27 RA patients with raised *Proteus* IgM levels showed that although all the patients had CRP levels > 10 mg/L, there was no correlation between IgM levels to *Proteus* and CRP levels ($r = 0{\cdot}148$, $p = 0{\cdot}463$), no correlation between CRP and RF levels ($r = -0{\cdot}158$, $p = 0{\cdot}433$) and no correlation between IgM levels to *Proteus* and RF levels ($r = -0{\cdot}279$, $p = 0{\cdot}158$).

When the 27 RF-positive sera from the RA patients and the 27 matched RF-negative control sera were examined by ELISA against the 10 other O serotypes of *P. mirabilis* strains and the 10 other O serotypes of *E. coli* strains (see table I), it was found that the raised IgM antibody levels found in RF-positive sera but not in the RF-negative controls were specific not only for *P. mirabilis* O29 and *E. coli* O22, but that the IgM levels were also raised to the other O serotypes of *P. mirabilis* and *E. coli* tested. Further tests showed that the levels of IgM antibodies in the RF-positive sera, but not in the RF-negative control sera, were also raised to all of the other strains of *Proteus* spp. tested, which included four strains of different O serotypes of *P. vulgaris* and four strains of *P. penneri* of unknown serotype.

To determine the antigen(s) inducing the raised antibody response to *Proteus* in RA patients, cell lysates were prepared from all the strains used. In addition, cell lysates were prepared from cultures of *E. coli* bearing pWPM 100 when they were found to be actively haemolytic (mid-late log phase of growth) and secreting haemolysin into the supernate. This indicated that at the time of sampling both HpmA and HpmB were being produced.[19] Although similar cultures of *E. coli* bearing pWPM 109 and the *E. coli* parent strain lacking both plasmids were not haemolytic, cell lysates of these strains were also prepared. After the lysates had been electrophoresed, they were treated with: the RF-positive sera from RA patients; the matched RF-negative sera; other control sera from healthy individuals; and sera from patients with osteoarthritis and ankylosing spondylitis and other auto-immune diseases. Subsequent immunoblots were developed in reactions with goat anti-human alkaline phosphatase-conjugated IgM, IgA and IgG.

There appeared to be no correlation between the IgM ELISA response and the amount or number of antigens detected by immunoblotting. Although among the different bacteria tested a number of different antigens were found that reacted with different classes of antibody in the different sera, there were few which were restricted to *Proteus* or *Proteus* and *E. coli* that reacted only with most of the RA patients' sera and not the other sera. However, a 90-kDa protein was found in *P. mirabilis* which reacted with an IgM antibody in sera from 14 of the 27 RA patients. This IgM antibody was not present in any of the RF-negative sera, nor in those of healthy controls, nor in those from patients with osteoarthritis or ankylosing spondylitis, nor in sera from nine RF-positive patients with Sjögren's disease or systemic lupus erythematosus (fig. 2). Subsequent analysis showed the same antigen to be present in all of the 11 different O serotypes of *P. mirabilis* studied. Sera from RA patients which had this IgM antibody reacted not only with the 90-kDa *P. mirabilis* protein but also with an 80-kDa protein present in all of the 11 different O serotypes of *E. coli* (fig. 3). IgM antibodies to both the 90-kDa *P. mirabilis* protein and the 80-kDa *E. coli* protein were removed by absorption of sera with the supernate of sonicated *P. mirabilis* cells but not by

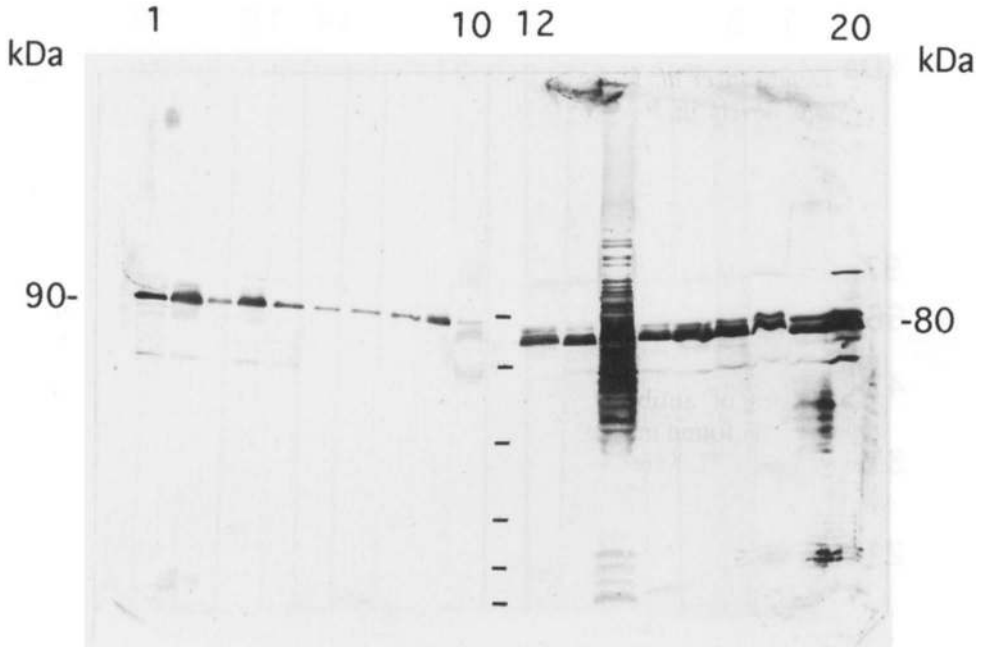

Fig. 3. Immunoblot of lysates of different O serotypes of *P. mirabilis* (lanes **1–10** serotypes O3–O30; O29 not shown) and *E. coli* (**12–20**, serotypes O1–O25; O9 and O75 not shown) blotted with serum 1703 from a patient with RA and then with goat anti-human μ chain-specific immunoglobulin labelled with alkaline phosphatase and developed; mol. wt protein markers (**11**). The RA serum detected the 90-kDa protein in all the different O serotypes of *P. mirabilis* and an 80-kDa protein in all the different O serotypes of *E. coli*.

absorption with suspensions of intact *P. mirabilis* cells. This indicated that the IgM antibody detected exclusively in sera from RA patients reacted to an internal protein antigen common to both *P. mirabilis* and *E. coli* strains of many different O serotypes.

An IgM antibody capable of reacting with HpmB (64 kDa) in lysates of *E. coli* bearing pWPM 100 or pWPM 109 was not found in any of five sera examined from RA patients with high RF titre and high IgM levels to *Proteus*.

Discussion

The first evidence of a possible link between RA and *Proteus* spp. was the finding of Ebringer *et al.*[8] that RA patients had raised levels of antibody specifically to *Proteus* spp. Others independently have confirmed this.[11,12] However, some have found it to be particularly true only for those RA patients, often referred to as those with "active" RA, who have raised (> 10 mg/L) CRP levels.[12,15] This may be misleading, for raised proteus antibody and CRP levels may simply reflect a recent proteus urinary tract infection rather than RA activity.[21]

The class of antibody raised to proteus in RA patients was reported by some[9,11] to be IgG and by others that it was not IgA in the 10 sera they examined.[21] However, these reports provide no evidence of class specificity for the antibodies detected. The results of the present study, which are based on the use of conjugated affinity purified class-specific anti-immunoglobulin reagents, are different from these. It was not confirmed that RA patients have higher IgG levels to *Proteus* than those of controls even though the RA patients studied had CRP levels > 10 mg/L. However, both IgM, and to a lesser extent IgA, levels to *Proteus* were found to be highly significantly raised in RA patients when compared with those of controls and the levels of these antibodies to *Proteus* were much higher than those to the other organisms tested. These results have also been confirmed with conjugated class-specific monoclonal antibodies (results not shown). The use of non-class specific antibodies in ELISA tests is more likely to detect IgM, because of its polymeric structure, than IgA or IgG. This interpretation of the findings of others[9,11,21] would make their results consistent with ours. Furthermore, we found that the response in RA was not specific for a particular serotype of *P. mirabilis* but was directed against many different O serotypes of this and other *Proteus* spp. The antibody response appeared to be distinct from RF, for no correlation was found between the *Proteus* IgM level and the RF titre. In this matter, the results were in agreement with those of others.[9,11,12]

The reason for the presence of raised IgM levels to *Proteus* spp. in RA patients is not understood. It is unlikely that all had either current or recurrent or asymptomatic proteus urinary tract infections, but this was not investigated because of the retrospective nature of the study. Although it is theoretically possible that these results could arise by IgM RF binding to IgG antibodies which are specific for proteus antigens, it is thought that this is a most unlikely explanation. Most sera from RA patients had IgG antibodies to *Proteus*, yet there was no correlation between RF titre and IgM levels to *Proteus*. Moreover, several sera from patients with primary Sjögren's disease or SLE which had high RF titres also had high

levels of IgG antibodies to *Proteus* but low levels of IgM antibodies to *Proteus*.

Although at one time Deighton *et al.* found that changes in proteus antibody levels in RA patients correlated strongly with corresponding changes in CRP,[12] the same group later were unable to find any correlation between activity of clinical RA disease (including CRP levels) and proteus antibody levels.[15] Our results, which showed no correlation between either CRP level or RF titre and level of *Proteus* IgM, support their latter observation.

The levels of all classes of antibodies to *Ps. aeruginosa* and of most classes of antibody to *K. pneumoniae*, organisms frequently found in faeces and also occasionally associated with UTI, were not raised in RA patients over those of controls. Although others have found the raised antibody levels in RA patients to be specific for *Proteus* spp., we found that RA patients had also, though to a lesser extent, raised IgM levels to *E. coli* and *K. pneumoniae*. It is unlikely that this is the outcome of increased gut permeability to these enteric bacteria through medication for RA[13] because there was no correlation in many RA patients between their IgM response to *Proteus* and that to *E. coli*.

In attempting to identify the antigen(s) common to many *Proteus* strains that might provoke the above immune response in RA patients, attention has been given to proteus haemolysin. It is known that most strains of *P. mirabilis* and many of other *Proteus* spp. produce a calcium-independent haemolysin[22] determined by *hpm*A, the gene for a 166-kDa haemolysin protein and *hpm*B, the gene for a 63-kDa protein, probably located in the outer membrane, that is necessary for the secretion and activation of HpmA.[19] The predicted amino acid sequences of HpmA and HpmB are similar to those of their corresponding proteins ShlA and ShlB of the haemolysin of *Serratia marcescens*.[19] It has been reported[23] that the amino acid sequence ESRRAL situated close to the N terminal of HpmB (and also found in ShlB), resembles structurally the amino acid sequence EQRRAA found in the β chain of HLA types associated with RA but not in those not linked with the disease. IgG (but not IgA or IgM) antibodies to a synthetic peptide containing the ESRRAL sequence were found to be raised in patients with active RA over those of healthy controls and those with ankylosing spondylitis.[24]

However, the results of this study have not been able, at this stage, to support this link between HpmB and RA. None of several sera from RA patients with high RF titres and raised *Proteus* IgM levels reacted with HmpB in immunoblots of lysates of an *E. coli* strain actively haemolytic through carriage of *hpm*A and *hpm*B on plasmid pWPM 100, or of an *E. coli* strain carrying *hpm*B only on plasmid pWPM 109. However, the HLA status of the RA patients whose sera were tested was not known. In conclusion, we have identified a 90-kDa protein present in all O serotypes of *P. mirabilis* examined that reacted with an IgM antibody found to be present in many sera from RA patients but not in RF-positive sera from patients with other auto-immune diseases and in RF-negative controls.

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References

1. Stastny P. Mixed lymphocyte culture typing cells from patients with rheumatoid arthritis. *Tissue Antigens* 1974; **4**: 571–579.
2. Lanchbury JSS. Molecular genetics of the HLA-D region component of inherited susceptibility to rheumatoid arthritis. *Br J Rheumatol* 1988; **27**: 171–175.
3. Dearlove SM, Neumann V, Barr K *et al.* Antibody levels to *Cl. perfringens* and response to sulphasalazine in patients with rheumatoid arthritis (RA). *Br J Rheumatol* 1990; **29** (Abstract, Suppl II): 3.
4. Zborovsky A, Lempert B, Baranovskaya N, Babayeva A. The clinical significance of immune reactions with some streptococcal antigens in rheumatoid arthritis. *J Rheumatol* 1990; **17**: 874–879.
5. Tsoulfa G, Rook GA, Van-Embden JD *et al.* Raised serum IgG and IgA antibodies to mycobacterial antigens in rheumatoid arthritis. *Ann Rheum Dis* 1989; **48**: 118–123.
6. Harris ED. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* 1990; **322**: 1277–1289.
7. Senior BW. The special affinity of particular types of *Proteus mirabilis* for the urinary tract. *J Med Microbiol* 1979; **12**: 1–8.
8. Ebringer A, Ptaszynska T, Corbett M *et al.* Antibodies to Proteus in rheumatoid arthritis. *Lancet* 1985; **2**: 305–307.
9. Ebringer A, Cox NL, Abuljadayel I *et al. Klebsiella* antibodies in ankylosing spondylitis and *Proteus* antibodies in rheumatoid arthritis. *Br J Rheumatol* 1988; **27** Suppl II: 72–85.
10. Ebringer A, Khalafpour S, Wilson C. Rheumatoid arthritis and Proteus: a possible aetiological association. *Rheumatol Int* 1989; **9**: 223–228.
11. Rogers P, Hassan J, Bresnihan B, Feighery C, Whelan A. Antibodies to *Proteus* in rheumatoid arthritis. *Br J Rheumatol* 1988; **27** Suppl II: 90–94.
12. Deighton CM, Gray JW, Bint AJ, Walker DJ. Anti-*Proteus* antibodies in rheumatoid arthritis same-sexed sibships. *Br J Rheumatol* 1992; **31**: 241–245.
13. McDonagh J, Gray J, Deighton CM, Sykes H, Walker DJ. Patient medication does not explain elevated anti-*Proteus* antibodies (Abs) in rheumatoid arthritis (RA). *Br J Rheumatol* 1992; **31** (Abstracts Suppl): 3.
14. Wilson C, Corbett M, Ebringer A. Increased isolation of *Proteus mirabilis* species from rheumatoid arthritis patients compared to osteoarthritis patients and healthy controls. *Br J Rheumatol* 1990; **29** (Abstracts Suppl II): 99.
15. McDonagh J, Gray J, Sykes H, Walker DJ, Bint AJ, Deighton CM. Anti-*Proteus* antibodies and *Proteus* organisms in rheumatoid arthritis: a clinical study. *Br J Rheumatol* 1994; **33**: 32–35.
16. Deighton CM, Gray J, McDonagh J, Walker DJ. Failure to detect urinary anti-*Proteus* antibodies (Abs) more frequently in rheumatoid arthritis (RA) patients compared with controls. *Br J Rheumatol* 1992; **31** (Abstracts Suppl I): 4.
17. Larsson P, Olling S. O antigen distribution and sensitivity to the bactericidal effect of normal human serum of *Proteus* strains from clinical specimens. *Med Microbiol Immunol* 1977; **163**: 77–82.
18. Sussman M. *Escherichia coli* in human and animal disease. In: Sussman M (ed) The virulence of *Escherichia coli*. Review and methods. Special publications of the Society for General Microbiology. No. 13. London, Academic Press. 1985: 7–45.
19. Uphoff TS, Welch RA. Nucleotide sequencing of the *Proteus mirabilis* calcium-independent hemolysin genes (*hpm*A and

*hpm*B) reveals sequence similarity with the *Serratia marcescens* hemolysin genes (*shl*A and *shl*B). *J Bacteriol* 1990; **172**: 1206–1216.

20. Welch RA. Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect Immun* 1987; **55**: 2183–2190.
21. Murphy EA, Mowat L, Sturrock RD. Antibodies to *Proteus* in rheumatoid arthritis. *Br J Rheumatol* 1991; **30**: 390.
22. Senior BW, Hughes C. Production and properties of haemolysins from clinical isolates of the *Proteeae*. *J Med Microbiol* 1988; **25**: 17–25.
23. Ebringer A, Cunningham P, Ahmadi K, Wrigglesworth J, Hosseini R, Wilson C. Sequence similarity between HLA-DR1 and DR4 subtypes associated with rheumatoid arthritis and proteus/serratia membrane haemolysin. *Ann Rheum Dis* 1992; **51**: 1245–1246.
24. Ebringer A, Wilson C, Ahmadi K *et al.* Molecular similarity and immune response to a *Proteus mirabilis* membrane haemolysin antigenic determinant in rheumatoid arthritis which is homologous with the HLA-DR1/DR4 susceptibility sequence. *Br J Rheumatol* 1993; **32** (Abstract Suppl 2): 10.