Chapter 5
Antibodies to *Proteus* in Rheumatoid Arthritis Patients from Bermuda and from Hertfordshire in England

Contents

A. Ebringer, *Rheumatoid Arthritis and Proteus*,
DOI 10.1007/978-0-85729-950-5_5,

Bermuda and Hertfordshire: Introduction

In 1992, during one of his visits back to Bermuda, Dr. Clyde Wilson met the physician Dr. Henry Subair who expressed an interest in the work of the 'Immunology Unit' at King's College in London. He mentioned that he had patients suffering from rheumatoid arthritis especially women and would be willing to participate in a study to investigate whether his Bermudian patients had similar antibodies to the ones described in English patients with rheumatoid arthritis from London.

It was decided to compare Bermudian rheumatoid arthritis patients with a group of English rheumatoid arthritis patients attending the Lister Hospital in Stevenage under the medical care of Dr. Allan Binder. The Lister Hospital has 600–700 beds and is located in Hertfordshire which is a county of south-east England, lying just north of Greater London. Most of the rheumatoid arthritis patients in the Lister hospital came from northern Hertfordshire, namely, Stevenage, Hitchin and Letchworth.

Both groups of rheumatoid arthritis patients were studied under blind conditions in that the person doing the antibody assays did not know which samples came from Bermuda or Hertfordshire and which came from patients or healthy donors.

The antibody studies were carried out by two different techniques ELISA and immunofluorescence.

Bermuda: Location and History

Bermuda is a small but prosperous island located some 1,000 miles north-east from Miami in the Atlantic. It was discovered in 1505 by the Spanish explorer Juan de Bermudez who gave the island its name. The island was settled by England in 1609, making it the oldest British overseas territory. It has a mild subtropical climate which accounts for its attraction to visitors. It is a centre of finance and tourism which provides

its multi-ethnic population of over 60,000 with a high standard of living.

It has one of the highest GDP (Gross Domestic Product) per capita in the world. Its old capital of St.George's was established in 1612 and is one of the oldest continuously inhabited English-speaking town in the Americas. The current capital is Hamilton where Dr. Henry Subair has his medical practice in the King Edward VII Memorial Hospital.

Bermuda has at its head a Governor appointed by the Crown, but since 1960, it is essentially a self-governing territory and forms part of the 'Caribbean Community'.

Patients and Controls

All the rheumatoid arthritis sera from both Bermuda and Hertfordshire were selected on the basis of clinical assessment of disease activity by the attending physicians and an elevated erythrocyte sedimentation rate (ESR) greater than 15 mm/h and a C-reactive protein level (CRP) greater than 10 mg/l.

It had previously been reported that patients with rheumatoid arthritis in the active phase of the disease, as measured by C-reactive protein levels, have higher levels of anti-*Proteus* antibodies compared to patients with inactive rheumatoid arthritis (Deighton et al. 1992).

Sera from Hamilton, Bermuda

Sera were obtained from 34 patients with active rheumatoid arthritis (5 male/29 female) attending the King Edward VII Memorial Hospital in Hamilton, Bermuda with a mean age of 56.9 years (range = 32–79 years) having a mean erythrocyte sedimentation rate (± standard error) of 40.5 ± 5.1 mm/h. The female/male ratio in the rheumatoid arthritis patients from Bermuda was 5.8. Samples were also obtained from 33 healthy controls (5 male/28 female) having a mean age of

57.1 years (range: 32–79 years). Erythrocyte sedimentation rates were not measured in the controls.

Sera from Stevenage, Hertfordshire

Sera were also collected from 34 patients with active rheumatoid arthritis (8 male/26 female) attending the Rheumatology Department of the Lister Hospital in Stevenage, Hertfordshire with a mean age of 59.0 years (range = 21–83 years) having a mean erythrocyte sedimentation rate (± standard error) of 49.6 ± 4.8 mm/h. The female/male ratio in the rheumatoid arthritis patients from Hertfordshire was 3.3. Erythrocyte sedimentation rates were not measured in the controls.

Preparation of the Bacteria for the ELISA Study

Bacterial isolates of *Proteus mirabilis* and *Escherichia coli* were obtained from the Department of Microbiology at King's College and prepared as previously published.

The cultures were grown aerobically in 250-ml conical flasks on an orbital shaker for 16–18 h in nutrient broth (Oxoid 13 g/l). Inoculation was performed by the addition of one loopful of bacteria from a nutrient agar plate. The cells were harvested by centrifugation at 4°C at 6,000 rpm for 20 min (MSE 18.6 × 250 ml rotor).

Bacterial isolates of three components of normal bowel flora were also obtained. A faecal sample was collected from a healthy adult and processed with careful attention to anaerobic technique. Isolates were identified by gram reaction, morphology and end product analysis by gas liquid chromatography (Borriello et al. 1978). Selected biochemical tests were also carried out using the rapid identification system (Rapid ID-32A, API Biomérieux).

The three normal bow el flora isolates were *Eubacterium* sp (Strain NF-iii), *Peptostreptococcus* sp (NF-ii-a) and *Bacteroides fragilis* (NF-vii).

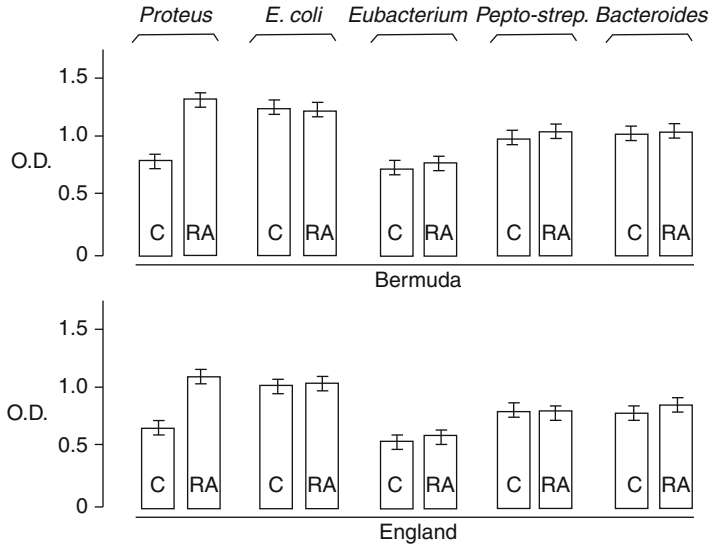

FIGURE 5.1 Antibody titres (mean ± standard error) measured by ELISA to the indicated organisms in 34 Bermudian and 34 English patients with rheumatoid arthritis and 33 Bermudian and 30 English controls (*OD* optical density) (Reprinted from Subair et al. (1995), with kind permission from The Journal of Rheumatology)

ELISA Studies with Five Bacteria: *Proteus mirabilis*, *Escherichia coli* and Three Normal Bowel Flora Isolates *Eubacterium*, *Peptostreptococcus* and *Bacteroides*

ELISA investigations were carried under code using the method of Khalafpour (Khalafpour et al. 1988).

The Bermudian rheumatoid arthritis patients with active disease had an elevated level mean (± standard error) of IgG *Proteus* antibodies of 1.31 ± 0.04 OD units when compared to the level in Bermudian control subjects of 0.98 ± 0.04 OD units and this difference was highly significant ($t = 6.07$, $p < 0.001$) (Fig. 5.1).

TABLE 5.1 Comparison of anti-*Proteus mirabilis* antibody titers (optical density or OD units) (Mean ± standard error) in male and female rheumatoid arthritis (RA) patients and controls from Bermuda

Sex	Number	Status	Antibody level	Statistical significance
Male	5	RA	1.14 ± 0.08	$P < 0.001$
	5	Controls	0.79 ± 0.09	
Female	29	RA	1.34 ± 0.04	$P < 0.001$
	28	Controls	1.01 ± 0.04	

The Hertfordshire rheumatoid arthritis patients with active disease also had an elevated level mean (± standard error) of IgG *Proteus* antibodies of 1.12 ± 0.03 OD units when compared to the level in English control subjects of 0.67 ± 0.03 OD units and this difference was again highly significant ($t = 9.77$, $p < 0.001$) (Fig. 5.1).

However, there was no significant elevation in IgG antibody titres in Bermudian or English patients with rheumatoid against *Escherichia coli* or total Ig against the three normal flora isolates, *Eubacterium* sp., *Peptostreptococcus* sp. and *Bacteroides fragilis*.

There was also a significant elevation in *Proteus* antibody titres in Bermudian men and women with rheumatoid arthritis when compared respectively to healthy Bermudian men and women (Table 5.1).

There was also a significant elevation of *Proteus* antibody titres in English men and women from Hertfordshire suffering from rheumatoid arthritis when compared to healthy English men and women respectively (Table 5.2).

There was no significant difference between men and women with rheumatoid arthritis either from Bermuda or Hertfordshire compared to their respective controls, when tested against the four other bacteria, namely *Escherichia coli* and the three normal bowel flora isolates *Eubacterium*, *Peptostreptococcus* and *Bacteroides fragilis*.

TABLE 5.2 Comparison of anti-*Proteus mirabilis* antibody titers (optical density or OD units) (Mean ± standard error) in male and female rheumatoid arthritis (RA) patients and controls from England

Sex	Number	Status	Antibody level	Statistical significance
Male	8	RA	1.09 ± 0.03	$P < 0.001$
	15	Controls	0.65 ± 0.06	
Female	29	RA	1.13 ± 0.04	$P < 0.001$
	28	Controls	0.71 ± 0.04	

Indirect Immunofluorescence Studies with *Proteus* in Both Bermudian and Hertfordshire Rheumatoid Arthritis Patients

Indirect immunofluorescence studies were carried out on the same 34 Bermudian patients with rheumatoid arthritis and controls but compared to a new batch of 30 rheumatoid arthritis from Hertfordshire.

The reason for using new sera in the English test and controls between ELISA and indirect immunofluorescence was due to inadequate volumes of sera available for both assays.

Indirect immunofluorescence was only used with *Proteus mirabilis* which was cultured as described previously. However, before dilution, a 1-ml aliquot of the stock bacterial solution was mixed with 1 ml of glutaraldehyde (0.25% v/v Sigma) in PBS to fix the bacteria.

The resulting suspension was then left for 10 min, and after this time, the dilution process was carried as before. Aliquots of 10 μl of the diluted bacterial suspension were added to each of the eight wells on the glass slide (ICN Flow Labs). The slides were then incubated at 30°C for 20 min to allow the bacteria to adhere to the glass slide. A 10-μl aliquot of test or control serum serially diluted from 1:10 through to 1:1,280 with *Proteus mirabilis* as the antigen in PBS (0.15 M, pH 7.4)

was added to each well and incubated in a damp box for 1 h. Each serum sample was tested in duplicate. The slides were then washed in a bath of PBS, continually stirred with a magnetic flea for 30 min at room temperature. After washing, the slides were carefully dried all around the wells using a cotton bud. Then 15 μl of rabbit anti-human IgG antibody conjugated to fluorescein isothiocyanate (FITC) (Dako Ltd), diluted 1:20 in PBS, was added to each test area. The slides were then incubated for a further 30 min at room temperature in a damp box followed by washing for 1 h, dried, 10 μl of mounting fluid (Sigma) was added to each test area, a cover slip applied and fixed using clear nail varnish around the edges.

Viewing of the test area was carried out using an immunofluorescence microscope (Leitz Dialux 20) with a 100× Leitz lens under water immersion. Each sample was scored between 0 and 8 $\log_2$ dilution units depending on the end point of fluorescence. No fluorescence at a dilution of 1: 10 scored a value of zero, fluorescence at 1: 10 scored 1, fluorescence at 1: 20 scored 2 and so on, up to a value up to a value of 1: 1,280 which scored 8. The results were expressed as anti-*Proteus* antibody titre ± standard error (IIFA units).

All runs were carried out under code in that the microscopist was not aware of the status of the sera under examination.

The indirect immunofluorescence studies confirmed the results obtained by the ELISA technique in that both the Bermudian and Herfordshire rheumatoid arthritis patients had elevated levels of antibodies to *Proteus mirabilis* when compared to their respective controls.

The 34 Bermudian rheumatoid arthritis patients with active disease had an elevated level mean (± standard error) of IgG antibodies to *Proteus mirabilis* of 6.06 ± 0.28 IIFA units when compared to the level in 33 in Bermudian control subjects of 2.86 ± 0.21 and this difference was highly significant ($t = 9.26, p < 0.001$) (Fig. 5.2).

The 31 rheumatoid arthritis patients with active disease from Hertfordshire had an elevated level mean (± standard

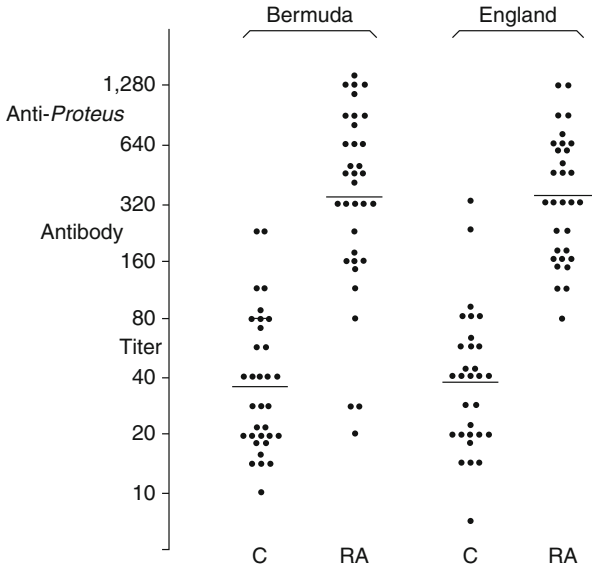

FIGURE 5.2 IgG antibody titres to *Proteus mirabilis* in 34 Bermudian and 31 English patients with rheumatoid arthritis and 33 Bermudian and 30 English controls measured by indirect immunofluorescence assay. (Bar = mean). Each *dot* represents the serum of either a rheumatoid arthritis patient or a blood donor subject (Reprinted from Subair et al. (1995), with kind permission from The Journal of Rheumatology)

error) of IgG antibodies to *Proteus mirabilis* of 6.06 ± 0.20 when compared to the level in 30 blood donor subjects of 2.88 ± 0.22 and again this difference was highly significant ($t = 10.94, p < 0.001$) (Fig. 5.2).

Clinical Implications

The results, by using two different techniques, show that both Bermudian and English rheumatoid arthritis patients from northern Hertfordshire have specific antibody elevations against *Proteus mirabilis*.

Furthermore, the ELISA studies show that this elevation is not present against *Escherichia coli* or three normal bowel bacteria. This confirms our previous report and the findings of the Newcastle group in England (Deighton et al. 1992) regarding the specificity of the anti-*Proteus* antibody elevation in rheumatoid arthritis patients.

The demonstration of a significant IgG antibody to *Proteus mirabilis* but not to the four other bacteria clearly eliminates the suggestion that rheumatoid arthritis patients have a 'leaky gut'. The 'leaky gut' hypothesis proposes that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDS) causes increased gut permeability to bowel bacteria or antigens and this might explain the elevation in anti-*Proteus* antibodies (Bjarnason et al. 1984).

If NSAID-induced gut ulceration was responsible for the increased level of anti-*Proteus* antibodies found in rheumatoid arthritis patients, then an antibody elevation to common bowel bacteria such as *Escherichia coli* or the three normal bowel isolates would also have been detected. The number of microorganisms per gram of faeces is of the order of 10^{12} for anaerobic bacteria, 10^{8} for *Escherichia coli* but only 10^{7} microbes per gram of faeces for *Proteus mirabilis* (Hentges 1983; Drasar and Barrow 1985).

Clearly, the lack of any immune response to *Escherichia coli* and the three normal bowel bacteria suggests that NSAID-induced increased gut permeability is not the explanation for the elevation of anti-*Proteus* antibodies found in rheumatoid arthritis patients.

Proteus mirabilis can be isolated from about 10% of patients with a urinary tract infection (Guenzel 1986). Since women suffer from 'urinary tract infections' more frequently than men, this could explain the higher prevalence of rheumatoid arthritis in the female population throughout the world. Indeed it has been reported that rheumatoid arthritis patients suffer from an increased incidence of 'urinary tract infections' (Tishler et al. 1992).

The results of the Bermudian and Hertfordshire study have been published, and a copy of the paper has been deposited in the Historical Archives of Bermuda (Subair et al. 1995).

The data presented here show that there are specific and significant antibody elevations against *Proteus mirabilis* in rheumatoid arthritis patients in both the Western (Bermuda) and Eastern Hemispheres (Northern Hertfordshire, England).

References

Bjarnason I, Williams P, So A, Zanelli GD, Levi AJ, Gumpel JM, et al. Intestinal permeability and inflammation in rheumatoid arthritis: effects of non-steroidal anti-inflammatory drugs. Lancet. 1984;2:1171–3.

Borriello SP, Hudson MJ, Hill MJ. Investigation of the gastrointestinal bacterial flora. Clin Gastroenterol. 1978;7:329–49.

Deighton CM, Gray J, Bint AJ, Walker DJ. Specificity of the *Proteus* antibody response in rheumatoid arthritis. Ann Rheum Dis. 1992;51:1206–7.

Drasar BS, Barrow PA. Intestinal microbiology. 3rd ed. Wokingham: Van Nostrand Reinhold; 1985.

Guenzel MN. *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* and *Proteus*. In: Baron S, editor. Medical microbiology. 2nd ed. Menlo Park: Addison Wesley; 1986. p. 495–506.

Hentges DJ. Human intestinal microflora in health and disease. Chapter 1. New York: Academic; 1983.

Khalafpour S, Ebringer A, Abuljadayel I, Corbett M. Antibodies to *Klebsiella* and *Proteus* microorganisms in ankylosing spondylitis and rheumatoid arthritis patients measured by ELISA. Br J Rheumatol. 1988;27 Suppl 2:86–9.

Subair H, Tiwana H, Fielder M, Binder A, Cunningham P, Ebringer A, et al. Elevation in anti-*Proteus* antibodies in patients with rheumatoid arthritis from Bermuda and England. J Rheumatol. 1995;22:1825–8.

Tishler M, Caspi D, Aimog Y, Segal R, Yaron M. Increased incidence of urinary tract infection in patients with rheumatoid arthritis and secondary Sjögren's syndrome. Ann Rheum Dis. 1992;51:604–6.