Chapter 4
Antibodies to *Proteus* in Irish Patients with Rheumatoid Arthritis

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Ireland: An Introduction

Early in 1985, Mr. Alex Whelan, the chief technician from St. Vincent's Hospital in Dublin, in Ireland, came to the Department of Immunology at the Middlesex Hospital to study under Professor Ivan Roitt and to extend his knowledge of immunological techniques. He became familiar with the work of the Immunology Unit at King's College, but the patients with ankylosing spondylitis and rheumatoid arthritis

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patients were treated in the Department of Rheumatology at the Middlesex Hospital which was located a floor below the Department of Immunology.

When told that elevated levels of antibodies to *Proteus mirabilis* had been found in rheumatoid arthritis patients, Mr. Alex Whelan expressed the intention that he would propose an investigation of rheumatoid arthritis patients, under the care of Dr. Barry Bresnihan attending his hospital in Dublin for the presence or absence of antibodies to *Proteus mirabilis*.

Dublin: Location and History

Dublin is the capital and the largest city of the Republic of Ireland. It has a population of over one million and is located on the eastern side of the island. The name is derived from the Irish name 'Dubh Linn' which means 'black pool'. The town was established by the Norse Vikings in 841 AD. It was sacked by the Celtic king Brian Boru who defeated the Norse at the Battle of Clontarf in 1014. The Norman invasion of Ireland in 1170 opened up a relentless struggle over the centuries with the Anglo-Norman nobility which ended with the partition of Ireland in 1922 and the setting up of the Republic.

Patients and Controls

Patients with rheumatoid arthritis attending the Rheumatology Clinic of St. Vincents Hospital in Dublin were studied.

Patients with active rheumatoid arthritis, that is, those who were deemed to require sodium aurothiomalate or lymphoid irradiation, were investigated for the presence of antibodies to *Proteus mirabilis* and compared to patients with systemic lupus erythematosus or sarcoidosis, as well as healthy controls.

All 29 patients had definite or classical rheumatoid arthritis as defined by the American Rheumatism Association (Ropes et al. 1958). Ten rheumatoid arthritis patients were

treated with gold sodium thiomalate, each patient receiving a cumulative dose of 1 g. Nineteen rheumatoid arthritis patients received lymphoid irradiation therapy (Hanly et al. 1986). These were randomly chosen to receive total doses of either 750 rad (9 patients) or 2,000 rad (10 patients) lymphoid irradiation. Radiotherapy was given on an outpatient basis four times weekly, using an 8 meV linear accelerator. Lymph nodes in the upper half of the body, which included the cervical, mediastinal and hilar lymph nodes, were encompassed in a mantle field and overall midline dose was given in 10 fractions. Without interruption, the lymph nodes in the lower half of the body, including the para-aortic, iliac and inguinal lymph nodes (inverted Y-field), received a similar midline dose in 10 fractions. The overall treatment was usually completed in 5–6 weeks. The spleen was not included in the radiation portal. Haemoglobin levels, white cell counts and platelet counts were monitored regularly during treatment.

In one premenopausal female patient, the pelvic lymph nodes were shielded to protect the ovaries.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assays were performed either by the Biodot Microfiltration apparatus (Biorad) or the conventional 96 well microtitre plates (Nunc). Bacterial isolates of *Proteus mirabilis* were obtained from the Department of Microbiology, St. James Hospital in Dublin and grown on nutrient agar plates (Oxoid). The organisms were harvested and washed in 0.2 molar phosphate-buffered saline (PBS) pH 7.4 by centrifugation at 2,500 g for 20 min at 4°C.

Proteus sonicate was prepared by fixing the bacteria in 1% formaldehyde in PBS for 20 min. After washing twice in PBS, the organisms were sonicated and bacterial debris pelleted. The supernatant was removed and stored at 4°C. The *Proteus* sonicate was adjusted to give an optical density (OD) reading

of 0.66 at 280 nm in 0.02 tris-buffered saline (BS) at pH 7.5 and 100 μl of this was incubated on nitrocellulose membrane for 10 min using the Biodot Microfiltration apparatus. After each incubation, the membrane was washed in 0.05% Tween-TBS (TTBS). The remaining binding sites were blocked by applying 100 μl of 3% bovine serum albumin (BSA) in TTBS to each well. The wells were washed a further five times and peroxidase-conjugated affinity purified goat anti-human IgG (Biorad) diluted 1/1,000 was then applied in 100 μl volumes for 30 min at room temperature. The nitrocellulose sheet was then removed from the apparatus, washed and incubated for 3 min in an acetate buffer 0.02 M, pH 5.1 containing 3-amino-9-ethylcarbazole (EAC) in 0.02% H_2O_2. The reaction was stopped by washing with distilled water, and the sheet was then allowed to dry overnight in the dark. Each lane was scanned using a transmittance/reflectometer scanning densitometer in the reflectance mode.

When microtitre plates were used, *Proteus* sonicate was adjusted to give an optical density (OD) of 1.6 at 280 nm while whole organisms were adjusted to a concentration of 6.4×10^6 organisms/ml in carbonate/bicarbonate buffer pH 9.6. Hundred microliter aliquots of either sonicate or whole *Proteus* was then added to the microtitre wells and incubated overnight at 4°C. Wells coated with sonicate were washed twice with PBS/Tween and blocked with 3% BSA in PBS for 1 h at room temperature, whereas the wells coated with whole *Proteus* were blocked with 1% gelatin for the same period after the initial two washes.

Serum samples were diluted 1/50 in PBS/Tween for the sonicate assay and 1/25 when using whole *Proteus* bacteria. Hundred microliter volumes of each dilution were added to each well for 30 min and incubated for 37°C. Following three washes, commercially available peroxidase-conjugated rabbit anti-human IgG (Dako) was diluted 1/500 in PBS/Tween and 100 μl volumes added. After 30 min incubation at 37°C, the plates were washed again in PBS/Tween. Hundred microliter of substrate *o*-phenylenediamine (OPD) was then added to

each well and the reaction stopped after 15 min with the addition of 2.5 M H_2SO_4. The microtitre plates were read on a Dynatech spectrophotometer at wavelength 490 nm.

Results of the Assays

Results with Sonicated Proteus Antigen

When sonicated *Proteus* organisms were used as the antigen to detect antibodies in rheumatoid arthritis patients or control subjects, prior to treatment using the dot or microtitre ELISA assays, no significant difference was found between the two groups.

However, in rheumatoid arthritis patients after gold treatment, a fall in anti-*Proteus* antibodies was observed using both of these assay systems. This reduction reached significance with the microtitre ELISA technique ($p < 0.02$). No fall in antibody levels was seen in patients after the lymphoid irradiation.

Results with Whole Proteus Bacteria

However, when whole *Proteus* bacteria was used as a target, there was a significant difference in antibody levels ($p < 0.01$) between controls and rheumatoid arthritis patients before treatment.

Again, there was a significant fall in these antibody levels after gold therapy ($p < 0.01$), but there was no fall observed after lymphoid irradiation.

The levels of antibodies to *Proteus* in rheumatoid arthritis patients were significantly elevated ($p < 0.001$) when compared to healthy blood donors as well as when compared to the three disease groups: coeliac disease, sarcoidosis, systemic lupus erythematosus patients (Fig. 4.1).

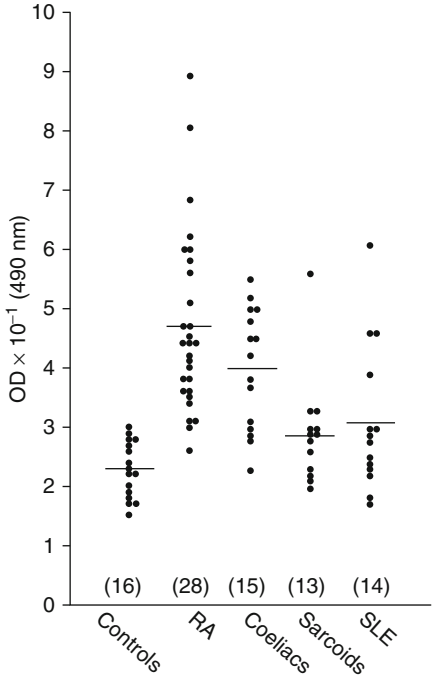

FIGURE 4.1 IgG anti-*Proteus mirabilis* antibody levels to whole *Proteus* bacteria as antigen using microtitre ELISA plates. The groups studied include normal controls ($n=16$); active rheumatoid arthritis (28); coeliac disease ($n=15$); sarcoidosis ($n=13$) and systemic lupus erythematosus ($n=14$) patients. Each *dot* represents either a control subject or a patient (Reprinted from Rogers et al. 1988, with permission from Oxford University Press)

Of the three disease control groups, only patients with coeliac disease had significantly elevated levels of antibodies against *Proteus* when compared to healthy blood donors ($p<0.01$) but the level observed was nevertheless lower than that seen in patients with rheumatoid arthritis.

Clinical Implications and Discussion

Patients with active rheumatoid arthritis patients showed significant elevations in IgG antibody against whole *Proteus* bacteria when compared to patients with coeliac disease, sarcoidosis and systemic lupus as well as to healthy blood donors.

There was also a rise in the anti-*Proteus* antibody titre in coeliac disease when compared to healthy controls and this may reflect some gut mucosal damage. The presence of increased α-gliadin antibodies in rheumatoid arthritis patients has been reported (O'Farrell et al. 1986).

The results with sonicated *Proteus* antigen were equivocal which suggests that the assay conditions may have not been optimized for this study.

The results in the rheumatoid arthritis pre- and post-irradiation lymphoid irradiation showed no correlation between clinical improvement and *Proteus* antibody levels.

However, in the gold treated group, there was a reduction in *Proteus* antibody levels following drug therapy. The Dublin group had previously reported that IgG, IgM and IgM RF levels fall following gold therapy (Hassan et al. 1984; Hanly et al. 1985). Thus, the reduced anti-*Proteus* antibody level may be a result of the general reduction in immunoglobulins.

The possibility of 'rheumatoid factor' amplifying the results by binding to specific *Proteus* antibodies in the assay systems was investigated.Rheumatoid arthritis sera were absorbed to remove 'rheumatoid factor' using latex particles coated with human IgG. The results of these studies did not reveal any interference in the assay systems.

In conclusion, increased anti-*Proteus* antibody levels were found in Irish patients with rheumatoid arthritis, to a lesser extent in patients with coeliac disease when compared to patients with sarcoidosis or systemic lupus erythematosus or healthy blood donors. These results confirm the results obtained by the London group.

These results were presented by Mr. Alex Whelan at the 'Second International Symposium' held at the Middlesex Hospital on 14–15 April 1987 (Ebringer and Shipley 1988).

During the discussion at this meeting, Mr. Alex Whelan expressed the following comment:

> The whole story about *Proteus* has potential interest only if you find crossreactivity to other agents. There appears to be an indication for a possible aetiological agent coming from the gut. If you then ask the question, which has probably been asked many times over the last two centuries, if there is an agent in rheumatoid arthritis, is it very rare or very common?
>
> If it is very rare, we would possibly have found it. If it is very common, it is because we all have it. Then if you ask the question what does genetic susceptibility mean, unless someone gives me a better answer, I think "molecular mimicry" stands out OK (Rogers et al. 1988).

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