Chapter 3
HLA-DR1/4 and Antibodies to *Proteus* in London

Contents

The Middlesex Hospital in London

The first Middlesex Hospital opened in 1745 as the Middlesex Infirmary in Windmill street London W1. The second Middlesex Hospital opened in 1757 in Mortimer street and

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was incorporated by an Act of Parliament in 1836. The new building was opened in 1935, by the Duke of York, later to become King George VI.

The hospital lobby contained four large paintings, entitled 'Acts of Mercy' by Frederick Cayley Robinson, completed in 1920. After the merger of the Middlesex Hospital with University College Hospital in 1994, the paintings were acquired by the Wellcome Collection.

The Middlesex Hospital was where Dr. WSC 'Will' Copeman had his early appointment as Senior Consultant in Rheumatology in the Arthur Stanley Institute before he wrote his 'Textbook of Rheumatic Diseases'.

Professor Eric Bywaters, the doyen of academic rheumatologists, graduated from the Middlesex Hospital.

The post-war services in rheumatology were provided by Dr. Oswald Savage. The Department of Rheumatology at the Middlesex Hospital was founded by Dr. Archibald Cabbourn Boyle, known as 'Bill' and was located in Arthur Stanley House, after it merged with the Arthur Stanley Institute, in Tottenham street, next to the main hospital.

Arthur Stanley House was opened by Her Majesty the Queen in 1965 and became a centre dedicated to the care of rheumatic patients.

The building also contained a physiotherapy and rehabilitation section as well as a Department of Immunology, under Professor Ivan Roitt.

Bill Boyle was a gifted teacher, and by the time of his retirement he had had a hand in training one third of the rheumatologists in the UK and many from overseas, especially Australasia.

Dr. Mary Corbett was appointed as a second Consultant in Rheumatology and started longitudinal studies on rheumatoid arthritis, known as 'Rheumatoid Arthritis Prospective Studies' or RAPS for short.

She emphasised that early treatment was paramount to prevent the development of irreversible bony changes and deformities. She approved the studies on antibodies to *Proteus* which were carried out on her rheumatoid arthritis patients attending the weekly 'Gold Clinic'.

Professor David Isenberg was appointed Professor of Rheumatology for the merged Middlesex University College Hospitals and collaborated in some studies in both ankylosing spondylitis and rheumatoid arthritis patients carried out by the King's College group. Dr. Michael Shipley was appointed Consultant in Rheumatology and provided clinical advice for the 'Ankylosing Spondylitis Research Clinic' and support in running two International Symposia held at the Middlesex Hospital in 1983 and 1987. Professor Jonathan Edwards contributed to the international symposia and showed that chemical ablation of B cells led to improvement in patients with rheumatoid arthritis.

The 'Ankylosing Spondylitis Research Clinic of the Middlesex Hospital'

In 1973, two seminal observations were published one from the Westminster Hospital in London and the other one from Terasaki's group in Los Angeles, that over 95% of patients with ankylosing spondylitis carried the major histocompatibility marker, HLA-B27, whilst it was present in only 8% of the general population. Clearly, here was a puzzle that required some form of explanation.

Dr. D.C.O. James, the immunologist involved in the discovery of HLA-B27 at the Westminster Hospital, came to the immunology lectures at Queen Elizabeth College, now part of King's College, and discussed this problem.

The suggestion from our group was that there was probably some form of 'molecular mimicry' between an unknown microbe and HLA-B27.

This was based on the precedent of rheumatic fever and Sydenham's chorea being caused by anti-*streptococcal* antibodies following an upper respiratory tract infection such as tonsillitis.

It was decided to study this question and Dr. Bill Boyle was approached with the suggestion that this could be

investigated in an 'Ankylosing Spondylitis Research Clinic'. He gave his approval and supported the clinic with financial resources. The clinic was started by my brother, Dr. Roland Ebringer who was in charge of the clinical studies till 1980 when he returned to Australia.

The immunological studies were carried out at Queen Elizabeth College and the clinical studies at the Middlesex Hospital. Some 900 ankylosing spondylitis patients were seen at the clinic between 1975 and 2002 when the clinic was closed.

In 1975, molecular mimicry was demonstrated between the bowel microbe *Klebsiella* and HLA-B27. Faecal studies showed that *Klebsiella* microbes could be isolated from active ankylosing spondylitis patients and later elevated levels of antibodies to *Klebsiella* could be demonstrated by several techniques.

When 2 years later, Professor Stastny from Dallas showed that rheumatoid arthritis patients were found more frequently in carriers of HLA-DR4; it was considered that the same explanation might apply to this observation as it did for *Streptococcus* and rheumatic fever or *Klebsiella* and ankylosing spondylitis.

Dr. Mary Corbett gave her approval that her rheumatoid arthritis patients could be investigated by the same methods as the patients in the 'Ankylosing Spondylitis Research Clinic'.

Investigation into a Possible Link Between HLA-DR4 and Bacteria

Serum was obtained from a rabbit prior to immunisation to be used as a control sample. Then the rabbit was immunised with 'Ficoll'-separated lymphocytes from two 40-ml samples of venous blood obtained from a patient with severe rheumatoid arthritis, whose HLA-DR tissue type was DR4,X and who was rheumatoid factor–positive. Rabbit serum taken before and after four immunisations was tested by immunodiffusion against soluble extracts from 18 different bacteria.

Table 3.1 Microorganisms used in immunodiffusion studies

Microorganism	Culture reference number
Gram-negative bacteria	
Enterobacter cloacae	QEC B27
Shigella sonnei	QEC B21
Salmonella typhimurium	QEC B22
Escherichia coli	QEC B35
Pseudomonas aeruginosa	QEC B15
Proteus vulgaris	QEC B11
Proteus mirabilis	QEC B17
Alcaligenes faecalis	QEC B18
Gram-positive bacteria	
Bacillus subtilis	QEC S6
Streptococcus faecalis	QEC D2
Streptococcus pyogenes	QEC D10
Streptococcus viridans	QEC D15
Streptococcus lactis	QEC D8
Staphylococcus albus	QEC C1
Staphylococcus aureus	QEC C22
Staphylococcus aureus	St. Stephen's.
Streptococcus pyogenes	St. Stephen's
Streptococcus pneumoniae	St. Stephen's
Yeast	
Candida albicans	St. Stephen's

QEC Queen Elizabeth College collection, St. Stephen's Hospital

Microorganisms were obtained from the Microbiology Department at Queen Elizabeth College and from the Department of Microbiology at St. Stephen's Hospital (Table 3.1).

Gram-positive and gram-negative bacteria were plated on blood and MacConkey agar, incubated at 37°C for 18 h. Individual colonies were subcultured and incubated at 37°C for a further 48 h.

Pure bacterial broth cultures were centrifuged, washed three times with phosphate-buffered saline (pH 7.4) containing 0.08% sodium azide, resuspended, and ultrasonicated. The supernatants were used as antigen solutions for immunodiffusion studies.

Immunodiffusion plates containing 1% agar (Oxoid) and 0.1% sodium azide were prepared to a depth of 1 cm, a centre well and four peripheral wells, each 5 mm from the centre well were cut. Rabbit serum was placed in the centre well and bacterial or yeast sonicates in the peripheral wells.

The rabbit antiserum gave five precipitin lines against *Enterobacter cloacae*, *Salmonella typhimurium*, *Alcaligenes faecalis*, as well as against *Proteus mirabilis* and *Proteus vulgaris*. However, the pre-immunisation serum also produced precipitin lines against the first three microbial sonicates, namely *Enterobacter cloacae*, *Salmonella typhimurium* and *Alcaligenes faecalis*.

However, the interesting observation was that the HLA-DR4-immunised rabbit serum produced precipitin lines against *Proteus* bacteria, microorganisms which are the second commonest cause of urinary tract infections, especially in the upper urinary tract. It became apparent that a urinary tract infection could readily explain the preponderance of rheumatoid arthritis in women.

Proteus bacteria are ubiquitous in nature: They are found in soil, on vegetables, in water, sewage, mammalian gut and vagina.

These bacteria have also been incriminated in opportunistic infections especially in wounds, burns, throat and ear infections as well as in bronchitis and chest infections.

The suggestion arises that *Proteus* bacteria may be involved in this disease, and if this hypothesis is correct, then antibodies to this microbe should be demonstrable in rheumatoid arthritis patients.

Patients and Controls

Sera from Rheumatoid Arthritis Patients

Serum samples were obtained from 30 rheumatoid arthritis patients attending the weekly 'Gold Clinic' at the Middlesex Hospital.

It was considered that rheumatoid arthritis requiring second-line therapy represented a group with more active disease than rheumatoid arthritis patients being treated with non-steroidal anti-inflammatory drugs.

There were 14 male and 16 women. Their mean age was 59 years (Range: 40–78 years) and the diagnosis was made according to the American Rheumatism Association criteria (Ropes et al. 1958).

All serum samples were investigated for C-reactive protein, IgG and IgA by the single radial immunodiffusion method of Mancini.

The hospital service measured the erythrocyte sedimentation rate (ESR) of all patients by the method of Westergren.

The mean (± standard error) erythrocyte sedimentation rate (ESR) in the rheumatoid arthritis was 28.2 ± 5.2 mm/h and the mean (± standard error) C-reactive protein level was 22.7 ± 5.6 μg/ml (Table 3.2).

Sera from Ankylosing Spondylitis Patients

Serum samples were obtained from 52 patients with ankylosing spondylitis, satisfying the New York criteria (Bennet and Wood 1968) and attending the 'Ankylosing Spondylitis Research Clinic' of the Middlesex Hospital.

Ankylosing spondylitis patients with serum IgA greater or equal to 3 g/l were allocated to the active ankylosing spondylitis group and those with serum IgA less than 3 g/l and erythrocyte sedimentation rate less than 15 mm/h were placed in the inactive group. Ankylosing spondylitis patients with a serum IgA less than 3 g/l but with an erythrocyte sedimentation rate

Table 3.2 Characteristics of the study groups

	Controls	Active AS	Inactive AS	RA
Number	41	24	28	30
M/F	23/18	20/4	21/7	14/16
Age range	20–50	28–62	24–69	40–78
Mean age	ND	41	44	59
ESR mm/h	ND	41.6 ± 5.0	5.7 ± 1.0	28.2 ± 5.2
CRP μg/ml	1.6 ± 0.6	35.9 ± 5.8	3.8 ± 0.8	22.7 ± 5.6
IgG g/l	12.4 ± 0.4	20.6 ± 0.1	14.0 ± 0.8	15.2 ± 0.9
IgA g/l	1.96 ± 0.1	4.74 ± 0.2	1.74 ± 0.1	2.55 ± 0.2

AS ankylosing spondylitis, *RA* rheumatoid arthritis, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *ND* Not done, Mean ± standard error, *M/F* Male/Female, Mean age: years

above 15 mm/h were excluded from the study. Previous studies had shown that active ankylosing spondylitis patients had elevated levels of serum IgA (Cowling et al. 1980).

Control sera were samples obtained from 41 healthy blood donors.

Statistical Analysis of Patient Groups and Controls

The mean (± standard error) erythrocyte sedimentation rate in the rheumatoid arthritis group was 28.2 ± 5.2 mm/h and this was significantly higher ($p < 0.001$, $t = 4.17$) than the level of 5.7 ± 1.0 mm/h found in the inactive ankylosing spondylitis group.

The mean (± standard error) erythrocyte sedimentation rate in the active ankylosing spondylitis was 41.6 ± 5.0 mm/h and this was significantly higher ($p < 0.001$, $t = 7.67$) than the level of 5.7 ± 1.0 mm/h found in the inactive ankylosing spondylitis group.

For C-reactive protein levels, all three patient groups, active ankylosing spondylitis ($p < 0.001$, $t = 7.60$), inactive ankylosing spondylitis ($p < 0.05$, $t = 2.23$) and rheumatoid arthritis ($p < 0.001$, $t = 5.85$), had significantly higher levels than the one of 1.6 ± 0.6 μg/ml measured in the controls.

Furthermore, the inactive ankylosing spondylitis group had significantly lower C-reactive protein level compared to the active ankylosing spondylitis group ($p < 0.001$, $t = 5.87$) and the rheumatoid arthritis group ($p < 0.001$, $t = 3.23$).

The mean (± standard error) serum IgG in active ankylosing spondylitis was 20.60 ± 0.15 g/l and this was significantly higher than the level of 14.04 ± 0.82 g/l found in inactive ankylosing spondylitis ($p < 0.001$, $t = 3.93$), the level of 15.20 ± 0.94 g/l found in the rheumatoid arthritis group ($p < 0.005$, $t = 3.15$) and the level of 12.44 ± 0.44 g/l found in the controls ($p < 0.001$, $t = 6.28$).

The mean (± standard error) serum IgA of the active ankylosing spondylitis was 4.74 ± 0.24 g/l and this was significantly higher than the level of 2.55 ± 0.21 g/l found in the rheumatoid arthritis group ($p < 0.001$, $t = 6.82$) or the level of 1.96 ± 0.15 g/l found in the control group ($p < 0.001$, $t = 10.35$).

The mean (± standard error) serum IgA of the rheumatoid arthritis group was 2.55 ± 0.21 g/l and this was significantly higher than the level of 1.74 ± 0.08 g/l found in the inactive ankylosing spondylitis group ($p < 0.001$, $t = 3.53$) or the level of 1.96 ± 0.15 g/l found in the control group ($p < 0.025$, $t = 2.34$).

Coombs Agglutination Assay

Proteus mirabilis (B17) and *Klebsiella pneumoniae var oxytoca* (MX100) were obtained from the Department of Microbiology at Queen Elizabeth College. The cultures were maintained on nutrient agar slopes at room temperature.

Nutrient broth (Oxoid) 13 g/l of water was autoclaved at 121°C, 1.05 kg/cm^2 for 15 min. A starter culture was prepared in 25 ml of autoclaved nutrient broth in a 250-ml conical flask and inoculated from the agar slope, incubated at 37°C for 6 h and agitated at 250 rpm on an orbital shaker.

Shake-flask cultures were prepared by inoculating 200 ml of nutrient broth, in a 2-l conical flask with 10-ml culture from the starter broth, incubated at 37°C and shaken for 16 h at 250 rpm on an orbital shaker.

Overnight cultures were centrifuged at 2,500 × g for 15 min, washed three times with phosphate-buffered saline and the pellet resuspended in 25-ml phosphate-buffered saline.

The concentration of the bacteria was adjusted to give an optical density of 1.64 in a Spectrophotometer 1800 at 540 nm, since this had been found to give optimum agglutination. It was equivalent to 1.57×10^{12} cells/ml for *Klebsiella* and 2.05×10^{11} cells/ml for *Proteus* microorganisms, when measured in a Coulter counter (Model 2B1).

The agglutination assay was carried out as follows: 100 µl volumes of bacterial suspension were added to 100 µl doubling dilutions of test serum in Dreyer's tubes. The mixtures were incubated at 37°C for 2 h, then 350-µl phosphate-buffered saline was added and the tubes were centrifuged for 20 min (1,500 × g, Mistral 6 l) at 4°C.

The supernatant was decanted, the pellet resuspended in 350-µl fresh phosphate-buffered saline, and the washing repeated twice. After the final wash, the pellet was resuspended in 100 µl one-tenth dilution in phosphate-buffered saline of Coombs rabbit anti-human immunoglobulin reagent. The tubes were incubated at 37°C for 2 h, then at 4°C overnight and the agglutination end-point determined.

All test serum samples were coded and end-points read blind, in that the tester was unaware whether patient or control sera were being investigated.

Results of Coombs agglutination Assay

Three separate agglutination studies were carried out against whole *Proteus mirabilis* and *Klebsiella pneumoniae var oxytoca* microorganisms. There were approximately 40 serum samples in each run, all tests were carried out blind and all samples came from different individuals. Therefore, each

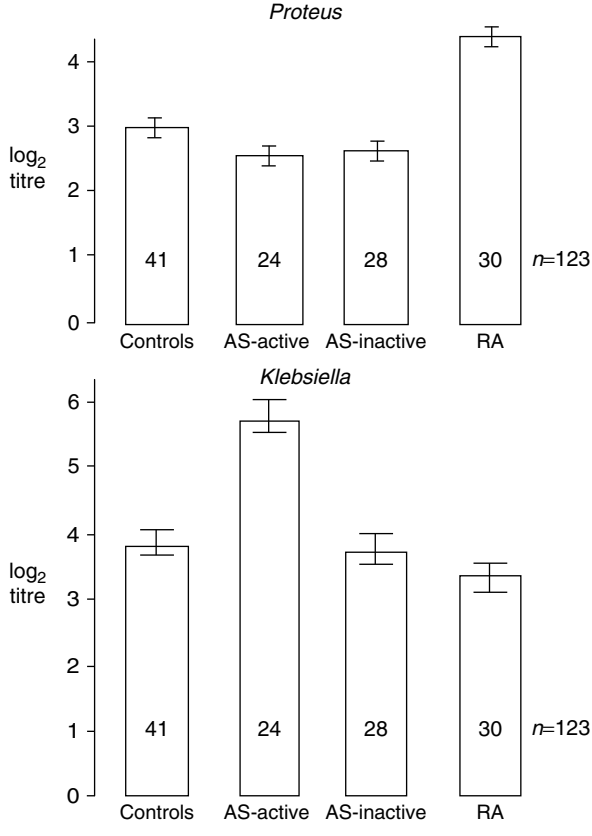

FIGURE 3.1 Antibody agglutination titres (mean ± standard error) from three pooled runs. Number of subjects in each group is indicated (Reprinted from Ebringer et al. (1985), with permission from Elsevier)

coded run was an independent investigation and the pooled results are shown (Fig. 3.1).

The mean (± standard error) *Proteus mirabilis* agglutination titre in the rheumatoid arthritis patients was 4.50 ± 0.18 $\log_2$ dilution units and this was significantly higher than the level of 3.08 ± 0.17 $\log_2$ dilution units found in the active ankylosing spondylitis group ($p < 0.001, t = 5.69$), the titre of 2.68 ± 0.16 $\log_2$

dilution units found in the inactive ankylosing spondylitis patients ($p < 0.001$, $t = 7.60$) or the level of 2.63 ± 0.16 $\log_2$ dilution units found in the healthy controls ($p < 0.001$, $t = 9.27$).

The mean (± standard error) *Klebsiella pneumoniae* agglutination titre in the active ankylosing spondylitis patients was 5.80 ± 0.20 $\log_2$ dilution units and this was significantly higher than the level of 3.79 ± 0.21 $\log_2$ dilution units found in the inactive ankylosing spondylitis group ($p < 0.001$, $t = 6.26$), the titre of 3.67 ± 0.24 $\log_2$ dilution units in the rheumatoid arthritis patients ($p < 0.001$, $t = 7.22$) or the level of 3.85 ± 0.15 $\log_2$ dilution units in the healthy controls ($p < 0.001$, $t = 7.61$).

Clinical Implications and Discussion

The data presented here show that there are specific and significant antibody elevations against the urinary microbe *Proteus mirabilis* in rheumatoid arthritis patients attending the 'Gold Clinic' of the Middlesex Hospital in London when compared to active and inactive ankylosing spondylitis or healthy blood donors.

There are also specific and significant antibody elevations against the commensal bowel microbe *Klebsiella pneumoniae* in active ankylosing spondylitis patients from London when compared to rheumatoid arthritis patients or healthy blood donors.

The high level of *Proteus* antibodies in rheumatoid arthritis was not due to nonspecific effects of high immunoglobulin levels, since the patients with active ankylosing spondylitis had higher serum levels of IgG and IgA than the rheumatoid arthritis patients, yet their levels of *Proteus* antibodies were similar to those of inactive ankylosing spondylitis patients and healthy controls. To some extent, the two groups of patients with inflammatory arthritis acted as reciprocal controls to one another, in that rheumatoid arthritis patients had high levels of antibodies to *Proteus* but not against *Klebsiella* and active ankylosing spondylitis patients had high titres against *Klebsiella* but not against *Proteus*.

Inactive ankylosing spondylitis patients and healthy controls did not have high antibody titres against either microorganism.

It is relevant to note that inactive ankylosing spondylitis did not have elevated levels of anti-*Klebsiella* antibodies despite having bony changes and sacro-iliitis, features characteristic of the disease. The elevated antibody level to *Klebsiella* microbes is not associated with the phenotype of the disease but with the presence of inflammation.

These observations were published in 1985 and are the first report that active rheumatoid arthritis patients have elevated levels of antibodies to the microbe *Proteus mirabilis* which is known to cause upper urinary tract infections (Ebringer et al. 1985). Further studies are required to determine whether similar observations can be made in rheumatoid arthritis patients from other centres and different countries.

The rabbit antiserum produced against human HLA-DR4 lymphocytes indicated that some bacterial antigens especially from *Proteus* bacteria may carry epitopes resembling human HLA sequences. Clearly such a hypothesis requires also further investigations.

Proteus bacteria are widely distributed in the environment and can be isolated from soil, sputum and urinary cultures. Many different microbiological agents have been suggested in the past as possible exogenous factors in rheumatoid arthritis, such as diphtheroids, L-phase variants, mycoplasmas and a variety of viruses, but further examination failed to confirm the original observations (Marmion 1976). In a survey using direct agglutination of 30 microbiological agents in 22 newly diagnosed rheumatoid arthritis patients, some patients had responses against herpes virus hominis and *Proteus OXK* (Chandler et al. 1971).

One serious problem with these previous investigations is that an assumption is being made, that the external microbiological agent would target the joint and could be isolated from the synovial cavity. However, the possibility that an autologous agent, such as an antibody made by the

rheumatoid arthritis patient being responsible for the joint inflammation, was not considered as a possibility. Yet this is exactly what happens in rheumatic fever: An infection by *Streptococcus pyogenes* located in the tonsils and upper respiratory tract evokes anti-bacterial antibodies which not only bind to the microbe wherever it is located but also to cross-reacting self-antigens found in the heart causing rheumatic fever and to the basal ganglia of the brain and giving rise to Sydenham's chorea.

During his first year residency, the author of this book witnessed a spectacular case of a 16-year-old female rheumatic fever patient with a pronounced heart murmur and severe chorea which prevented her from walking or going to the toilet and the attending senior physician made the confident claim that he would cure her in 1 week with the use of penicillin antibiotics. On the subsequent ward-round, 1 week later, the patient was running around filling the water bottles of the other elderly stroke female patients in the ward whose clinical status had not changed. If rheumatic fever could be caused by cross-reactive autologous antibodies evoked by an infection, why not other diseases?

Conclusions

Longitudinal studies are required in rheumatoid arthritis patients to assess whether antibiotic intervention will reduce the anti-*Proteus* antibody titre and modify the clinical outcome of the arthritic disease. The role of *Proteus* in rheumatoid arthritis patients merits further study.

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