STUDIES ON SECRETOR STATUS, 
FAECAL FLORA AND THE HUMORAL 
IMMUNE RESPONSE 
IN 
ANKYLOSING SPONDYLITIS 

Gillian W. Smith 

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DECLARATION

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself or under my direct supervision.

Edinburgh, February, 1993

Gillian W. Smith
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First and foremost, I would like to express my appreciation to all the patients who attended my research clinic, for without them this work would have been impossible. Many thanks also to Prof George Nuki, Dr TM Chalmers and Dr NP Hurst of the Rheumatic Diseases Unit, Western General Hospital, Edinburgh for permission to include their patients in this study, and to Prof Nuki for helpful discussions and critical comments.

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I am indebted to all my friends and colleagues who cooperated with my request for (at times unsavory) samples to form my control population – and who remain friends and colleagues!

I acknowledge with thanks Lederle Laboratories, Gosport, Hampshire who funded me for most of my research time.
I dedicate this work to my mother and the memory of my father.
ABSTRACT

STUDIES ON SECRETOR STATUS, FAECAL FLORA AND THE HUMORAL IMMUNE RESPONSE IN ANKYLOSING SPONDYLITIS.

It has been suggested that ankylosing spondylitis (AS) is a form of reactive arthritis similar to that observed among patients with genitourinary or intestinal infections. This hypothesis was supported by several studies in which some, but not all, found an increased isolation rate of *Klebsiella* species from the stools of AS patients. The strongest association known of a human leucocyte antigen (HLA) with disease is observed with HLA-B27 and AS. One hypothesis proposed to explain this association is molecular mimicry, in which cross-reactivity between HLA-B27 and arthritogenic bacteria leads to a cross-reacting immune response and inflammation. Non-secretion of ABO blood group substances, an autosomal recessive characteristic, is another factor associated with susceptibility to disease, particularly to infection of mucosal surfaces. In a study in 1987, non-secretors were found to be over-represented in patients with AS, evidence which supported the hypothesis of an infectious aetiology in AS.

The aims of this work were to reassess the association of non-secretion with AS; to study the faecal flora in a cohort of patients with spondyloarthropathy; to determine the prevalence of bacteria expressing antigens cross-reactive with the HLA-B27 antigen; to examine the humoral immune responses of patients and controls to their own faecal flora; and to correlate these findings with the disease activity of the patients as assessed clinically and by the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) as laboratory parameters of inflammation.

The proportion of non-secretors in this AS population was identical to that in the control population when determined by haemagglutination inhibition assay (HAI) and confirmed by enzyme-linked immunosorbent assay (ELISA) for Lewis antigens. Careful scrutiny of the secretor status in a group of patients examined in both present and initial studies revealed that 27% of non-secretors in the previous survey had been wrongly typed and were indeed secretors. Enzymatic degradation of blood group antigens in some saliva specimens could explain this discrepancy and might have resulted from inadequate cooling prior to
processing and storage in the previous study. Although there was a direct correlation between clinical disease activity and ESR and CRP for all patients with spondyloarthropathy and for AS patients alone, there was no evidence that non-secretors experienced more severe disease than secretors by either clinical or laboratory indices.

Analysis of stool samples from AS patients and controls showed no significant differences in types of organisms isolated. *Klebsiella* species were isolated in 8.5% of patients' and 19.4% of controls' stools. The presence of a HLA-B27 cross-reactive epitope on enteric bacteria from patients' and controls' stools was sought by immunoassay and flow cytometry; however, none of the bacteria examined expressed epitopes cross-reactive with that to which the monoclonal was produced.

Previous work examining the humoral response to enteric bacteria has largely concentrated on measuring serum antibody levels to laboratory strains of bacteria which might not be part of the faecal flora of any of the patients under investigation. In this study an ELISA was developed to measure specific antibody levels to autologous enteric bacteria harvested from faeces. The concentration of serum IgM reactive with self gut bacteria was significantly lower in patients compared with controls.
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ABBREVIATIONS

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAU</td>
<td>Acute anterior uveitis</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AS</td>
<td>Ankylosing spondylitis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumen</td>
</tr>
<tr>
<td>CMB</td>
<td>Cooked meat broth</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>Enthesis index</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMS</td>
<td>Early morning stiffness</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HAI</td>
<td>Haemagglutination inhibition assay</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSB</td>
<td>High salt buffer</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphocyte transformation</td>
</tr>
<tr>
<td>MC27</td>
<td>Mouse monoclonal anti-HLA-B27 antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NSU</td>
<td>Non-specific urethritis</td>
</tr>
<tr>
<td>OMPG</td>
<td>Outer membrane peptidoglycan</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFD</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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Chapter One
General Introduction

Ankylosing spondylitis (AS) is a chronic rheumatic disease primarily affecting the spine and sacroiliac joints in which early inflammatory changes can be followed by progressive restriction of spinal movement, loss of normal posture and the development of a rigid, stooped spine. The name derives from the Greek words ankylos (bent or crooked) and spondylos (vertebra). The disease has a preponderance of males and, typically, has an insidious onset in the third decade presenting with low back pain and morning stiffness. Peripheral joint involvement affects up to 35% of patients during the course of the disease, is often asymmetrical and frequently affects the lower limb joints. Inflammation can also occur at extra-articular sites such as the eye, heart and lungs (Calin, 1989). In most patients the disease is characterised by exacerbations and remissions, tending towards fewer episodes of acute exacerbations after the age of 40 years.

1.1 Historical Review

A comprehensive examination of Egyptian mummified remains from the Roman period (200 AD) to the IIIrd Dynasty (2980-2900 BC) provides evidence of AS in the skeletons at that time (Ruffer and Rietti, 1912). The first description of AS was in 1693 by Bernard Connor who studied the fusion of the ilium, sacrum, vertebrae and ribs in a skeleton (cited by Bywaters, 1980). Arguably, the best early clinical description of AS was by Sir Benjamin Brodie in 1850 who described a 31 year old man with a 3 year history of 'pain referred to the spine from the neck downwards, but especially to the middle dorsal vertebrae' (Brodie, 1850). This pain was aggravated by sneezing and the patient had occasional pains in the pelvis and lower extremities. On bending 'he experienced a sense of stiffness of the spine, and there was scarcely any perceptible flexure of it, the stooping being apparently confined to the motion of the pelvis on the thigh'. He had lost weight and had intermittent fevers. He had an effusion of one knee and the spine was 'rigid and inflexible'. On subsequent review of this man over a 3 year period, Brodie noted that although there was no improvement in his spinal
mobility, he was generally much less symptomatic apart from occasional 'inflammation of the eye'.

In the 1890's, Strumpell in Germany, von Bechterew in Russia and Marie in France all published papers on the disease. Marie, with his pupil Leri, gave the characteristic clinical and pathological description as it is known today.

In the first and second world wars, X-ray screening highlighted the presence of this disease which had often been unrecognised by the individual.

### 1.2 Epidemiology of Ankylosing Spondylitis

#### 1.2.1 Ethnic and geographical distribution

Direct comparison of different studies on the prevalence of AS is difficult because of differences in survey methods, populations studied and diagnostic criteria employed. The prevalence of AS in the UK is about 1 per 2000 population (0.05%) (West, 1949; Wright and Moll, 1973) with a similar order of frequency (0.013%) determined from hospital records in the USA (Carter et al., 1979). Population surveys have given higher prevalence estimates of 0.2% in north-west England (Lawrence, 1977) and 0.23% in Hungary (Gomor et al., 1977).

Marked differences in the prevalence of AS are recognised in different racial and ethnic groups. The prevalence in the UK is similar to that in the Japanese (0.02-0.04%) (Sonozaki et al., 1975), is low in New Zealand Maoris (Rose and Isdale, 1963) and is rare in African blacks. No cases of AS were found in over 600 West African blacks (Muller et al., 1972) and only 1 case was identified in 1300 South African blacks (Solomon et al., 1975). Gofton et al. (1966) found no instance of sacroiliitis in 255 normal Jamaican males. Studies of Caucasians and Afro-American blacks showed that racial intermixture produced a prevalence of AS in Afro-Americans to be about one quarter that seen in whites (Baum and Ziff, 1971). In contrast, there is a high prevalence among several North American Indian populations in which AS affects 6.7% of males of the Haida tribe, 7.6% of male Bella Bella Indians, 5.9% of male Pima Indians, 3.1% of males of the Bella Coola tribe (Gofton et al., 1972) and 9% of the male Chippewa Indians (Arnett et al., 1982).
The racial differences in AS prevalence clearly relate to the frequency of HLA-B27 in these populations. The HLA-B27 gene is found mostly in Caucasoid and Mongoloid racial groups and is particularly common among the North American Indian tribes mentioned above. The gene is virtually absent in African negroids of unmixed ancestry and rare in Orientals.

1.2.2 Age and sex distribution

Probably the only true incidence survey of AS has come from Rochester, Minnesota, USA, in which the overall age-adjusted incidence rate for both sexes combined was 6.6 per 100,000 population per year (Carter et al., 1979). The age-specific incidence rates peaked in the 25-34 year old age group, while the maximum prevalence was in the 55-64 year old age group. This agrees with the observation several years earlier that the disease usually presents between the ages of 20-40 years with less than 5% beginning after the age of 50 years (Wilkinson and Bywaters, 1958).

The male to female ratio is approximately 4:1 in patient series (Wilkinson and Bywaters, 1958; Wright and Moll, 1973; van der Linden et al., 1984b) and in population studies (Carter et al., 1979). The higher ratio of 10:1 reported in other studies (West, 1949) indicates that the disease was previously underdiagnosed in females. One explanation for this is that chronic low back pain in young women was often attributed to pelvic pathology and not investigated radiologically to avoid possible irradiation damage to germ cells (Calin and Fries, 1975). Young men, however, might have sought medical advice earlier if back pain was interfering with work.

1.2.3 Morbidity and mortality

In terms of morbidity, AS patients are hospitalised infrequently and retain good functional capabilities. A 25-year follow-up study of 76 patients showed that 65% were still able to work full-time; approximately half of these had retained their original jobs, while the remainder had to change to a less physically demanding job. 5% of the patients had retired because of age and only 30% were unfit to work (Lehtinen, 1981).

Most, but not all, surveys have noted an increased mortality in AS patients. A study of over 14000 AS patients who had received radiotherapy reported a 1.8-
fold greater excess mortality than expected compared with national death rates. The excess deaths were attributed to the spondylitis itself, amyloidosis, leukaemia, aplastic anaemia, cancers originating in heavily-irradiated tissues and ulcerative colitis (Cour Brown and Doll, 1965). Radford and colleagues (1977) studied over 800 patients diagnosed during the same period but who did not receive radiotherapy and found a 1.6-fold increase in mortality compared with the general population. This was significant for males but not for females. The excess mortality was attributed to the disease itself and to ulcerative colitis, peptic ulcer disease, tuberculosis or other respiratory disease and nephritis. A survey of 151 Canadian World War II veterans with AS showed that there was an increased mortality only in those who had received radiotherapy (Kaprove et al., 1980). The causes of this excess mortality were due to lymphatic and haematological malignancies and an increase in cardiovascular, cerebrovascular, pulmonary and renal disease. In a study in Minnesota, the mortality in AS patients was not significantly different from that in the general population (Carter et al., 1979).

The causes of death in 79 Finnish patients with AS were examined by Lehtinen (1980). No comparisons were made with the national death rates in age-matched controls, therefore no conclusions can be drawn regarding mortality rates of AS patients compared with the general population. Almost every patient received radiotherapy for pain (albeit a small dose), but only 1 patient had a lymphoma and another had chronic lymphatic leukaemia. Renal failure due to amyloidosis was reported considerably more frequently (21.5%) than in other surveys.

A more recent retrospective appraisal of the effect of single treatment radiotherapy on survival of AS patients, showed that compared with the general population of England and Wales, there was a threefold increase in mortality due to leukaemia, a 30% increase in mortality for cancer of the colon and a 28% increase in mortality for cancers others than leukaemia or colon (Darby et al., 1987). In addition, there was a 51% increase in mortality for non-neoplastic conditions in AS patients.
1.3 Diagnosis

Criteria for diagnosing AS were first formulated in Rome in 1961 and were based on 5 clinical features and the presence of radiological sacroiliitis (Table 1.1). AS could be diagnosed if either 4 clinical findings, or a combination of 1 clinical feature and radiographic sacroiliitis existed. Criticisms of the Rome criteria are that limitation of movement is not defined, and reduced chest expansion is a late event in the course of the disease by which time the diagnosis should be obvious.

Table 1.1
The Rome criteria (1961)

1. Clinical criteria:
   a. Low back pain and stiffness for more than 3 months that is not relieved by rest;
   b. Pain and stiffness in the thoracic region;
   c. Limited motion in the lumbar spine;
   d. Limited chest expansion;
   e. History or evidence of iritis or its sequelae.

2. Radiologic criterion:
   a. X-ray showing bilateral sacroiliac changes characteristic of ankylosing spondylitis. (This would exclude bilateral osteoarthrosis of the sacroiliac joints).

A revised set of criteria was introduced in New York in 1966 (Table 1.2). Limitation of spinal mobility in three directions is a requirement as limitation of forward flexion alone can be seen in several low back conditions. Discomfort rather than pain might better describe what is experienced. Absent in the New York criteria is the relationship of pain to rest. Analysis of the New York criterion of pain in the dorso-lumbar spine was considered to lack specificity and the chest expansion criterion to lack sensitivity (van der Linden et al., 1984a). A modification of the New York criteria was proposed by these authors in an attempt to identify early cases of AS (Table 1.3). Interpretation of
radiological sacroiliac changes, however, is open to procedural and observer variability. An anteroposterior view is usually sufficient but special views of the sacroiliac joints are sometimes required.

Table 1.2
The New York criteria (1966)

A. Diagnosis

1. Limitation of motion of the lumbar spine in all three planes—anterior flexion, lateral flexion, and extension;
2. History or the presence of pain at the dorsolumbar junction or in the lumbar spine;
3. Limitation of chest expansion to 2.5cm or less, measured at the level of the fourth intercostal space.

B. Grading

Definite as:

Grade 3-4 bilateral sacroiliitis with at least one clinical criterion;

or

Grade 3-4 unilateral or Grade 2 bilateral sacroiliitis with clinical criterion 1 (limitation of back movement in all three planes) or with both clinical criteria 2 and 3 (back pain and limitation of chest expansion).

Probable as:

Grade 3-4 bilateral sacroiliitis with no clinical criteria.
Table 1.3
The modified New York criteria

Diagnosis

Clinical criteria:

1. Low back pain and stiffness for more than 3 months which improves with exercise, but not relieved by rest;
2. Limitation of the lumbar spine in both the sagittal and frontal planes;
3. Limitation of the chest expansion relative to normal values when corrected for age and sex.

Radiological criterion:

Sacroiliitis grade ≥ 2 bilaterally or sacroiliitis grade 3-4 unilaterally.

Grading

Definite ankylosing spondylitis:

If the radiological criterion is associated with at least one clinical criterion.

Probable ankylosing spondylitis:

Three clinical criteria are present;
or

The radiological criterion is present without any signs or symptoms satisfying the clinical criteria (other causes of sacroiliitis should be considered).

It is now recognised that AS can occur in the absence of sacroiliitis (Calin, 1979) and spondyloarthritis without sacroiliitis may be associated with the HLA-B27 antigen in relatives of those with AS (Khan et al., 1985). Lone sacroiliitis is uncommon and progression to ascending spinal disease is primarily a function of disease duration. Sacroiliitis and ascending spinal disease are indeed part of the same spectrum (Calin and Elswood, 1988).
HLA-B27 typing is not necessary for the diagnosis of AS which should be made on the existing clinical and radiological criteria (Calin, 1980).

1.4 **The Spondyloarthropathies**

Until 30 years ago, AS was known throughout different parts of the world as rheumatoid spondylitis; psoriatic arthropathy and arthritis associated with inflammatory bowel disease were thought of as variants of rheumatoid arthritis. In 1963, the American Rheumatism Association produced a classification in which rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, ulcerative colitis and Crohn's disease appeared as separate entities. This classification was based not only on clinical features, but also on the finding that rheumatoid factor, while present in the serum of over 70% of patients with rheumatoid arthritis, was absent in patients with these 'variant' diseases. The term 'seronegative' was coined to describe the phenomenon. The other finding common to these diseases is spondylitic involvement and 'spondylarthritis' was the term applied.

AS is the prototype of this group of inter-related disorders known collectively as the seronegative spondyloarthropathies. This concept was originally proposed by Moll and colleagues (Moll et al., 1974) and includes uncomplicated AS, Reiter's disease, psoriatic arthropathy, ulcerative colitis, Crohn's disease, juvenile chronic arthritis, Behcet's syndrome, Whipple's disease, reactive arthritis and acute anterior uveitis. All these conditions are characterised by the following: radiographic sacroiliitis (with or without ankylosing spondylitis); peripheral inflammatory arthritis; muco-cutaneous features such as oro-genital ulceration; inflammatory eye disease; and a tendency to familial clustering. Other common features are the absence of rheumatoid factor and the absence of subcutaneous nodules.

AS is considered to be primary if no other rheumatic disease is present, and secondary if the spondylitis is associated with inflammatory bowel disease, Reiter's syndrome or psoriasis.
1.5 PATHOLOGY

The essential pathological feature of the spondarthritides is an enthesopathy-inflammation occurring at the sites of attachment of tendons, ligaments and joint capsules to bone. Enthesopathy is not peculiar to AS. The most common cause for an isolated lesion is trauma and enthesitis has been recorded in schistosomiasis (Atkin et al., 1986) and leprosy (Atkin et al., 1990).

Inflammatory lesions of particular bone surfaces in AS eg., the tip of vertebral spinous processes, were first described nearly 40 years ago (Engfeldt et al., 1954). Further work by Ball (1971) showed that destruction of the chondrified and calcified parts of the ligament occurs leaving a small erosion or defect in the cortical bone. This becomes infiltrated by lymphocytes, plasma cells and a few polymorphonuclear cells which are arranged in a perivascular distribution. Around this area of erosion an osteitis develops. During the repair process, these areas of focal osteitis become filled by reactive or cancellous bone which is laid down in a finely fibrous connective tissue without preceding cartilage formation. This new bone joins the deeper bone to the eroded end of the ligament and so lies above the original bone surface forming a new enthesis. Re-modelling takes place with mature lamellar bone replacing the reactive bone forming a prominence or spur.

Erosive changes, when they occur at the sites of attachment of the outer fibres of the annulus fibrosus of the intervertebral disc to the vertebral body, result in syndesmophyte formation and eventually ankylosis of the spine (Bywaters, 1987). Similar lesions occur in the capsular attachments of the apophyseal and sacroiliac joints leading to ossification and ankylosis.

Destructive lesions of the vertebral bodies sometimes occur which often present with a sudden onset of localised back pain and in some there is a history of trauma (Cawley et al., 1972). These lesions may be localised defects in the cartilaginous end-plate and are due to discal herniae, or be present in the vertebral rim and due to focal osteoporotic defects of the subchondral plate.

Enthesopathy of more peripheral ligamentous insertions are common. Plantar fasciitis occurs particularly in Reiter's syndrome, is less frequent in AS and psoriatic arthritis and is due to inflammation at the insertion of the plantar
aponeurosis and long plantar ligaments into the calcaneum (Niepal and Sit'aj, 1979). Achilles tendon enthesitis and enthesopathies around the pelvis and upper femora are especially common in AS and are also seen in psoriatic arthritis and Reiter's syndrome.

The predilection of the disease process for the enthesis remains a mystery. One hypothesis proposed to explain this site as the disease 'target' is that as the enthesis is a site of transmission of stress/load forces, it is possible that trauma initiates the lesions in a genetically predisposed host (Wisnieski, 1984; Moll, 1987). In support of this is the observation that enthesitis is more common in the pelvis and lower limb than in the upper limb. Patients often associate injury with the development or flare-up in such lesions, but this has not been validated statistically. Trauma, by a shearing effect, could expose molecules on the surface of cells that form part of the enthesis and which were hidden prior to trauma. Antigens could also appear in the lesion from bleeding due to trauma. These previously-hidden molecules are now exposed and might associate with other cell surface antigens, or might have a structure that is similar to antigenic determinants present on enteric bacteria. In this way these host molecules could act as a target for an autoimmune response.

The synovial pathology of AS is very similar to that of rheumatoid arthritis (Cruikshank, 1951; Julkunen, 1966; Revell and Mayston, 1982). Proliferation of synovium occurs over the surface of the articular cartilage, granulation tissue destroys the cartilage and penetrates the underlying bone. The histological similarities originally led to the interpretation that rheumatoid arthritis and AS were variants of the same disease. It has subsequently been recognised that the pathological lesions represent a common reaction to different aetiological factors.

Immunohistological techniques have identified the presence of CD4-positive cells in the synovium of patients with AS. Monoclonal antibodies, including one directed to RFT2 antigen, have been used to detect T cell 'activation markers'. RFT2 is a 40kD molecule preferentially expressed by T cell blasts or activated T cells. 8% of the synovial membrane T cells from AS patients were RFT2-positive compared with 25% of those from patients with rheumatoid arthritis and less than 1% of synovial membrane T cells from normal individuals or patients with osteoarthritis or Reiter's syndrome (Poulter et al., 1985).
The influence of heredity was suggested from von Bechterew's original report in 1893 of first and second generation relatives of probands of AS patients also being affected (cited by Riecker et al., 1950). There were similar reports in the early part of the century of a familial occurrence of 'spondylitis rhizomelique'. The validity of these early reports, however, must be accepted with the reservations that the history was not typical in some cases, X rays were not available and the diagnosis of AS in relatives (with a few exceptions) relied on hearsay evidence. Several reports in the 1930s and 1940s noted a familial occurrence of the disease but these cases were relatively isolated and could hardly constitute significant evidence for the operation of genetic factors.

One pedigree described 5 affected individuals in 3 generations (Riecker et al., 1950) and another family study identified 8 affected individuals in 2 generations (Graham and Uchida, 1957). These observations supported an autosomal dominant gene transmission. In the study of Stecher et al. (1957) and that of de Blécourt et al. (1961) AS was found to be 30 times (Stecher et al., 1957) and 23 times (de Blécourt et al., 1961) more common among relatives of spondylitics than among relatives of healthy controls with no history of the disease. In a review of 10 series in which 960 AS patients were examined, 14% had 1 or more first-degree relatives with AS (Hochberg, 1984). This data was compatible with an autosomal dominant pattern of inheritance with incomplete penetrance.

The landmark discovery in 1973 of the strong association of the human leucocyte group A (HLA) antigen B27 with AS was the major development that allowed progress to be made in understanding the genetics of this disease (Brewerton et al., 1973b; Schlosstein et al., 1973). HLA-B27 is found in up to 95% of Caucasians with AS compared with 8% of the general population.

A number of studies compared the prevalence of AS in HLA-B27-positive relatives of probands with AS with the prevalence of disease in relatives of healthy HLA-B27-positive individuals. The entry criteria for disease phenotype varied for each study. This explains the considerable variation in frequencies of disease between studies; but, in each case, there was a greater frequency of disease in HLA-B27-positive relatives of AS patients than in HLA-B27-positive relatives of healthy controls (Lochead et al., 1983; Woodrow et al., 1983; Calin et al., 1983;
van der Linden et al., 1984b). Earlier studies had suggested that the risk of disease in relatives was greater for female probands than for males (Stecher et al., 1957). Calin and colleagues (1983), however, found that the risk was not significantly different for a female than it was for a male proband.

Data from twin studies suggest that genetic factors alone are not sufficient to explain the disease. In a review, Moesmann (1960) noted 9 monozygotic twins of which 3 were discordant for AS. In a later study of 3 monozygotic twins over 45 years of age, 2 were discordant for AS and in the third there was a marked difference in the severity of the disease (Eastmond and Woodrow, 1977). As the two discordant pairs were aged 48 and 52 years respectively, it is unlikely that the unaffected twin would subsequently develop the disease. This leaves room for the participation of other influences such as environmental factors.

There is no good evidence for an association of non-HLA-B27 B locus antigens conferring susceptibility to AS. A group of HLA-B locus antigens (B7, Bw22, B40) which form a cross-reactive group (B7-CREG) was found to be associated with HLA-B27-negative patients with spondyloarthropathy (Arnett et al., 1977). In contrast, Khan and colleagues (1983) did not find a significant association with the B27-CREG antigens in their series of HLA-B27-negative white patients with AS, Reiter's or in those with secondary AS.

Because of the existence of HLA-B27-negative AS patients and healthy HLA-B27-positive individuals, studies were done to determine other genetic loci contributing to susceptibility to AS. A study of haplotypes in HLA-B27 individuals with and without AS did not reveal any significant differences between the two groups (Lochead et al., 1983). This is evidence in favour of the HLA-B27 gene being the major HLA-linked susceptibility gene for AS.

HLA-A2 is increased in frequency in AS due to linkage disequilibrium with HLA-B27. HLA-Bw60 is thought to act in conjunction with HLA-B27 to increase susceptibility to AS by a factor of approximately 3 (Robinson et al., 1989).

A study to assess the relative importance of genetic and environmental factors in disease expression concluded that environmental factors play a greater role in determining the age of onset of AS, whereas genetic factors have more influence on progression of radiological signs and physical disability (Calin and Elswood, 1989).
1.7  ENVIRONMENTAL AND OTHER FACTORS PROPOSED TO BE INVOLVED IN THE AETIOLOGY OF ANKYLOSING SPONDYLITIS

1.7.1  Trauma

It was proposed by Wehrsig in 1910 that AS was the result of an abnormal reaction to trauma (cited by Moll, 1980). In recent years, there have been anecdotal reports of trauma being implicated in the initiation of both AS (Oliveri et al., 1988; Jacoby et al., 1985) and Reiter's syndrome (Masson et al., 1985; Wisnieski, 1984). In the case of spinal AS, physical injury tends to bring the condition to the patients' attention, probably through enforced immobilisation rather than provoking the disease (Jacoby, 1985). Oliveri et al. (1988) reported the development of a destructive peripheral arthropathy immediately after trauma in 2 HLA-B27-positive patients; 1 of these patients had a 4 year history of sacroiliitis but no peripheral joint symptoms until her injury. From his observations, Wisnieski (1984) suggested that new antigens might be exposed through trauma and lead to an abnormal autoimmune reaction in genetically predisposed individuals.

1.7.2  Metabolic abnormality

An association between hyperparathyroidism (diagnosed on somewhat loose grounds) and AS was noted (Funsten, 1933); 14/26 AS patients in this series were treated by parathyroidectomy and all but 1 improved. This vogue for parathyroidectomy, however, did not last long. Buckley (1945) postulated a defect in the metabolism of bone alkaline phosphatase causing a mobilisation of calcium from the bone and its deposition in the adjacent ligaments.

1.7.3  Viruses

In view of the association of AS with a HLA class I molecule (see below), a viral aetiology has been suggested. High levels of an adenylic acid polymer considered to be a marker for viral replication, have been found among patients with AS and psoriatic arthropathy but not in seropositive rheumatoid arthritis, acute chondrocalcinosis or healthy controls (Luxembourg et al., 1987). Raised IgG antibodies to measles virus have been detected in the sera of AS patients without iritis compared with hospitalised controls, but the significance of this is
not clear (Kalliomaki et al., 1983). So far, no virus has been conclusively shown to be the trigger for the development of AS.

1.7.4 Bacteria

Following the observation that acute rheumatic complaints and endocarditis can be preceded by a tonsillar infection (rheumatic fever), focal sepsis became a common explanation for several illnesses. In the investigation of chronic arthritis and nephritis it was recommended that a diligent search was made for septic foci in tonsils, gingiva, sinuses, pulmonary cavities, gall bladder, bowels, urinary and genital tract and that these foci be removed (Billings, 1912). It was suggested that as sacroiliitis was the likely result of some chronic infection, then eradication of this infection should reduce the possibility of ankylosis and spondylitis (Scott, 1936). Indeed, the sacroiliac joints of 3 patients were curetted; soft bone and granulation tissue but no pus was found. Attempts to grow cultures from the debris were abortive due to contamination (Scott, 1936).

In 1926 Marie suggested that AS was the result of gonococcal infection (cited by Romanus, 1953). In two studies chronic prostatovesiculitis was associated with AS: 89% of 114 AS patients in one study (Romanus, 1953); 83% of 54 spondylitics in another (Mason et al., 1958). A later study also supported urogenital infection as a cause of AS (Julkunen et al., 1966). It has been suggested (Olhagen, 1983) that the high proportion of prostatic infection in two of these studies (Romanus, 1953; Mason et al., 1958) was probably due to the fact that most of the patients considered had the onset of their disease before the advent of antibiotics.

Romanus (1953) suggested that the erosive and ankylosing changes of the sacroiliac joints previously demonstrated in paraplegics (Abramson and Kamberg, 1949) were due to chronic urinary tract infection (UTI). Subsequent studies have revealed that sacroiliitis is not more common in paraplegics with significant UTI, as manifest by persistent hydronephrosis, than in those without (Liberson et al., 1966).

Several bacteria have been well-documented to be associated with reactive arthritis. This term, coined by Ahvonen et al. (1969), describes the nonpurulent inflammatory arthritis which occurs in association with a definite or highly-suggestive precipitating genito-urinary or gastrointestinal infection. Reiter’s syndrome is the term originally applied to the triad of features of reactive
arthritis, urethritis and conjunctivitis. This classical definition of Reiter's syndrome has been replaced by more broadly-based criteria of an episode of arthritis lasting more than one month in association with urethritis or cervicitis (Willkens et al., 1981), or as defined by Fox et al. (1979) in Table 1.4. It must be borne in mind that while many Reiter's syndrome patients do fall into either the epidemic (or enteric) form or the endemic (or post-veneral) form, some are not so easily classified. Post-venereal Reiter's syndrome has been considered the more common form of Reiter's syndrome in Britain and the United States, whereas in Europe the post-dysenteric form is more common.

Table 1.4
Definition of Reiter's syndrome

Seronegative asymmetric arthropathy (predominantly lower limb)

Plus one or more of the following:

Urethritis/cervicitis
Inflammatory eye disease
Mucocutaneous disease: balanitis, oral ulceration, or keratoderma blenorraghia.

Exclusions:
Primary ankylosing spondylitis
Psoriatic arthropathy
Other rheumatic disease.

Bacteria implicated in the enteric form of reactive arthritis and Reiter's syndrome include Shigella flexneri (Paronen, 1948; Simon et al., 1981), Salmonella typhimurium (Vartiainen and Hurri, 1964), Salmonella enteritidis (Iveson et al., 1975), Yersinia spp (Ahvonen et al, 1969) and Campylobacter jejuni (Berden et al., 1979; Gumpel et al., 1981). Neither Shigella sonnei (which causes dysentery) nor enteropathic Escherichia coli have been associated with a reactive arthropathy or Reiter's syndrome. Noer et al. (1966) found an incidence of Reiter's syndrome of 1.5% in an epidemic of Shigella dysentery. It was later calculated that the risk of a HLA-B27-positive individual with shigellosis developing Reiter's was between 16% and 37% (Calin and Fries, 1976).
Reiter's syndrome develops in a small proportion of subjects with nonspecific urethritis (NSU), from 1% (Csonka, 1958) to 3% (Keat et al., 1978). Some of these cases have been associated with Chlamydia trachomatis. In a follow-up study of 531 men with NSU, arthritis developed in 16 and 5 of these were positive for chlamydia (Keat et al., 1978). 40% of the patients with arthritis were HLA-B27-positive compared with 6% in the total series and 20% of the HLA-B27-positive patients developed reactive arthritis. It was calculated that a HLA-B27-positive individual with NSU was ten times more likely to develop Reiter's syndrome than an HLA-B27-negative individual (Keat et al., 1978).

Reiter's syndrome, in common with AS, shares a strong association with HLA-B27; about 70-80% of Caucasians with Reiter's are HLA-B27-positive (Brewerton et al., 1973a; Fox et al., 1979). In blacks the link is less strong (Good et al., 1976). It is possible that HLA-B27-positive individuals might react to different organisms (or react in a different way to certain organisms) and develop either a reactive arthritis or Reiter's syndrome depending on other unknown genetic or environmental factors.

In contrast with reactive arthritis and Reiter's syndrome, there are no reports of a microorganism being directly implicated in the onset of AS. A case of prostatitis due to Trichomonas vaginalis (a protozoan) causing AS has been reported, but it likely that this patient had incomplete Reiter's syndrome (Kuberski, 1981).

1.7.5 HLA-B27 association

Human leucocyte antigens (HLA), originally defined as the major transplantation antigens, are the products of genes in the major histocompatibility complex (MHC) on chromosome 6. Over 60 diseases are associated with particular HLA alleles (Engelman et al., 1984). These include: putatively autoimmune diseases such as rheumatoid arthritis, Graves disease and Goodpasture's syndrome which have a primarily MHC class II association; diseases associated primarily with a HLA-B locus antigen such as AS, Reiter's syndrome, acute anterior uveitis and subacute thyroiditis; and diseases for which the actual susceptibility gene might lie outside the MHC but is in linkage disequilibrium with a particular HLA allele e.g., haemochromatosis
susceptibility gene is linked to HLA-A3. In 1973 the strong association between AS and HLA-B27 antigen was made.

1.7.5.1 The structure and function of HLA antigens

The HLA class I molecules consist of a polymorphic 45kD glycosylated heavy chain that spans the cell membrane, noncovalently linked to an invariant 11kD light chain, β2-microglobulin. The gene for β2-microglobulin is found outwith the MHC. The HLA class I genes are present at three loci and code for the heavy chain of the HLA-A, -B and -C molecules. The extracellular part of the heavy chain consists of 3 domains each of approximately 90 amino acids, α1, α2 and α3. The α3 domain is closest to the membrane and, with β2-microglobulin, shows structural homology to the constant region domains of immunoglobulins. The 3-dimensional structure of a class I molecule (HLA-A2), as visualised by X-ray crystallography, has revealed that the α1 and α2 domains are spatially related to form a platform of an 8-stranded beta-pleated sheet supporting 2 alpha helices (Bjorkman et al., 1987). In the centre of this platform lies a groove in which electron dense material lies. It is this groove which is thought to be the site where binding of antigen fragments for presentation to CD8-positive T cells occurs. Virtually all positions of high variability in HLA-A, -B and -C antigens are located in and around the groove. These amino acid substitutions are thought to influence the peptides that can interact with the HLA molecules and which might play a role in determining the T cell receptor repertoire.

The only known function of MHC class I antigens is to present peptides derived from endogenous antigens to specific receptors on CD8-positive (cytotoxic) T cells. Endogenous proteins are degraded into peptides in the cytoplasm of the antigen presenting cell (APC). These peptides enter the endoplasmic reticulum (ER) where they associate with class I molecules and β2-microglobulin and are transported through the Golgi apparatus to the cell membrane (Ploegh, 1990). Drugs that inhibit protein synthesis or block the transport of freshly synthesised proteins out of the ER inhibit presentation of antigen by class I molecules.

HLA class II molecules (HLA-DP, -DQ or -DR) are composed of two polypeptide chains of molecular weight 28kD and 32kD. Class II molecules present degraded forms of exogenous proteins to CD4-positive (helper) T cells. It is thought that exogenous antigen is internalised by endocytosis into the APC and degraded in lysosomes. The resultant peptides are then bound by class II molecules in an as
yet undefined compartment before being transported to the cell membrane. Class II-mediated antigen presentation, in contrast to that of class I, is unaffected by inhibitors of protein synthesis, but is sensitive to lysosomotropic agents, while class I-mediated presentation is not.

As has been stated, class I antigens present endogenous peptides. The question arises as to why there is an association of class I antigens with a number of diseases. In the case of HLA-B27, it is possible that an arthritogenic organism damages tissues and results in the formation of peptides not otherwise produced in that tissue. T cell activation might lead to cross-reactions with similar peptides present on cells elsewhere in the body, such as the enthesis, synovial membrane or uvea.

1.7.5.2 Epidemiology of HLA-B27

The strong association of HLA-B27 with AS explained the early studies noting familial aggregation of the disease. HLA-B27 is an autosomal dominant characteristic and is present in 50% of first degree relatives of HLA-B27-positive AS probands. Although the strength of the association varies with different ethnic groups, the association of AS with HLA-B27 is found worldwide. In blacks, 40-50% of AS patients are HLA-B27-positive compared with less than 1% in the control population. In Japan, 66% of AS patients are HLA-B27-positive compared with less than 1% in the control population (Sonozaki et al., 1975). In Mexico, the Mestizo population (a mixture of Spanish and American Indians) has a HLA-B27 gene frequency of 4%; HLA-B27 is found in 80% of Mexican AS patients (Arellano et al., 1984). In Hungary, the HLA-B27 antigen is found in 93% of AS patients compared with a prevalence of 13% in the general population (Gomor et al., 1977). There is no significant difference in the frequency of HLA-B27 between males and females with AS.

While HLA-B27 is an undisputed susceptibility factor for AS, it is clear that possession of this MHC class I molecule is not sufficient per se to cause the disease as healthy individuals also express this antigen. Conversely, HLA-B27 itself is not necessary for the development of the disease as 5% of Caucasians with AS and approximately 50% of black American AS patients are HLA-B27-negative. This suggests the possibility of some aetiological heterogeneity between HLA-B27-positive and HLA-B27-negative patients. Heterogeneity in clinical presentation is recognised. HLA-B27-negative cases have a later age of
onset, less severe disease, a lower incidence of affected relatives and are more likely to have inflammatory bowel disease or psoriasis than their HLA-B27-positive counterparts (Linssen, 1990). Acute anterior uveitis is more common in HLA-B27-positive AS patients than those who are HLA-B27-negative (Khan, 1977).

While over 70% of patients with Reiter's syndrome are HLA-B27-positive, most do not develop sacroiliitis. It is unexplained why two North American Indian tribes, the Pima (Lisse et al., 1982) and the Haida, both with a high frequency of HLA-B27 and sacroiliitis, develop Reiter's syndrome only rarely. In contrast, the Alaskan Inupiat Eskimos who have a very high population frequency of HLA-B27 of 25%, develop Reiter's syndrome more frequently than AS (Boyer et al., 1988). Both AS and Reiter's syndrome tend to 'breed true' within families (Calin et al., 1984).

Psoriatic spondyloarthropathy and spondylitis associated with inflammatory bowel disease are associated with an increased frequency of HLA-B27, 60% and 50% respectively (Brewerton et al., 1974). These associations with HLA-B27 are weaker than that with primary AS suggesting that genes involved in the aetiology of IBD and psoriasis might contribute to susceptibility for AS. Another possibility is that a critical part of the HLA-B27 gene is inserted into other alleles and expressed (gene conversion), but no evidence for this has been found (Pease et al., 1988).

Several studies performed to assess the risk of developing AS in HLA-B27-positive individuals have provided conflicting data. The prevalence of disease in HLA-B27-positive first degree relatives of HLA-B27-positive AS probands varies from 8% (Christiansen et al., 1977) to 35% (Kidd et al., 1977). Similarly, the prevalence of AS in HLA-B27-positive individuals who form part of the general population varies from 1.3% (van der Linden et al., 1984b) to 18% (Calin and Fries, 1975). The conflicting results are due to a number of factors. Differences in study design could have influenced the results; some surveys used non-selected population-based studies (van der Linden et al., 1984b) while others extrapolated data from blood donors and factory workers and applied these figures to the general population (Calin and Fries, 1975). Another factor lies in the criteria employed to diagnose AS. The diagnosis relies on the presence of radiological sacroiliitis. Interobserver variation in the radiographic interpretation of sacroiliitis contributes considerably to the discrepancies in the
prevalence estimates for AS (Hollingsworth et al., 1983; van der Linden et al., 1984b). This was minimised by van der Linden et al. (1984b) as all X rays were read by 5 rheumatologists and a majority decision made on diagnosing sacroiliitis.

The risk of a HLA-B27-positive relative of a HLA-B27-positive AS patient developing AS, is 20 times greater than for HLA-B27-positive relatives of HLA-B27-positive healthy individuals (Calin et al., 1983).

1.7.6 Theories to explain the association of HLA-B27 with AS

There have been two main models proposed to explain the association between HLA-B27 and AS: the two gene theory or linkage disequilibrium hypothesis, and; the one gene theory of which there are 4 variants.

1.7.6.1 The two gene theory

The two gene theory was proposed in 1974 by McDevitt and Bodmer who suggested that the HLA-B27 gene itself is not the causative gene for AS; but that it is in linkage disequilibrium with a second gene which is responsible for the disease. The association of AS with HLA-B27 is found worldwide in different ethnic groups. If 2 genes were responsible for the disease, this would imply an extremely marked degree of linkage disequilibrium maintained over generations and under different selective conditions. It would be implied that these genes (HLA-linked or non-HLA-linked) would be more prevalent in probands and relatives of probands than in HLA-B27-positive individuals without disease; and it would be expected that in some families these genes would occasionally become separated.

A search for restriction fragment length polymorphisms using different endonucleases and cDNA probes has failed to reveal either a HLA-B locus with a greater relative risk than HLA-B27, or evidence of an additional locus such as HLA-D or -DR genes contributing to susceptibility to AS (Dejelo et al., 1978). There has been no report of a family showing genetic recombination between HLA-B27 and a hypothetical closely-linked locus (Woodrow et al., 1983). Families were studied for co-segregation of alleles belonging to non-HLA-linked polymorphisms with AS but none was found (de Jongh et al., 1990). In addition, familial aggregation of HLA-B27-negative AS individuals does not appear to
occur. This suggests that the gene responsible for AS and HLA-B27 gene are the same.

If a second gene was responsible for the disease, there should be no difference between HLA-B27-positive and HLA-B27-negative AS patients. There is, however, clinical and genetic heterogeneity between the 2 groups.

In view of these considerations, the two gene theory is no longer accepted as a likely explanation of the HLA-B27-AS association.

1.7.6.2 The one gene theory

The one gene theory postulates that the HLA-B27 gene itself is directly involved in the development of AS. The variants of this theory are the molecular mimicry or cross-tolerance theory; the receptor theory; Geczy's plasmid theory and the chemotaxis theory.

Molecular mimicry theory

The molecular mimicry theory (Ebringer et al., 1976) suggests that the HLA-B27-positive host is colonised by microorganisms which express antigens that partially resemble HLA-B27, but which are sufficiently different from HLA-B27 to generate an immune response. The antibacterial antibodies produced cross-react with HLA-B27 molecules and activate complement with resultant inflammation. To explain the distribution of spinal involvement in AS, it is postulated (Ebringer, 1983) that if the bacteria are enteric organisms, they would induce a local gut mucosal antibody response; and, because of lymphatic drainage, the antibodies would 'home in' on the nearby sacroiliac joints and lumbar spine. This mechanism does not explain the involvement of peripheral joints or the uveal tract, or why most other tissues in the body which also express HLA-B27 molecules are unaffected by the inflammatory process.

Molecular mimicry between viral or bacterial antigens and vertebrate host self antigens is not uncommon (Oldstone, 1989). The classic example of a cross-reactive autoimmune disease is rheumatic fever in which the M protein from Group A β-haemolytic *Streptococcus pyogenes* and human cardiac myosin share antigenically similar epitopes (Dale and Beachey, 1986). The demonstration of antigenic similarity between host and organism, however, does not necessarily mean that this is of significance in disease pathogenesis.
Evidence for cross-reactivity between HLA-B27 and enteric bacteria does exist. Sera from rabbits which had been immunised with HLA-B27-positive lymphocytes were found to react with *Klebsiella* and *Yersinia* in immunodiffusion assays (Ebringer et al., 1976) and this was confirmed using haemagglutination and radiobinding assays by the same group. Rabbit anti-*Klebsiella* sera had cytotoxic activity against HLA-B27-positive lymphocytes from AS patients (Welsh et al., 1980). Polyclonal human tissue typing sera from gravid women which had anti-HLA-B27 activity bound to *Klebsiella* antigens (Avakian et al., 1980). A criticism of this work is that naturally occurring antibacterial antibodies are known to be present in allo-antisera; therefore, it is difficult to be certain that the reactions observed were due to the anti-HLA-B27 antibodies and not to these other antibodies (Ogasawara et al., 1986).

With the advent of monoclonal antibodies, binding of monoclonal anti-HLA-B27 antibodies (M1 and M2) was demonstrated to *Klebsiella pneumoniae* K21 and K43, and to antigens of *Shigella flexneri* (molecular weight 20,000 kD) and *Yersinia enterocolitica* (molecular weight 16,000) (van Bohemen et al., 1984). A monoclonal anti-*Yersinia* antibody (Ye-1) cross-reacted with all 12 HLA-B27-positive lymphoblastoid cell lines but only 4/31 HLA-B27-negative ones; 3/4 of the reactive HLA-B27-negative cell lines were HLA-B7-positive. A HLA-B27-positive cell line which lost its HLA-B27 expression because of experimentally-induced mutation, became unreactive with Ye-1 (Kono et al., 1985). Cross-reactivity between HLA-B27 and *K. pneumoniae* (but not other Gram-negative bacteria) was demonstrated with a monoclonal anti-HLA-B27 antibody (M2) in an ELISA using absorption experiments to ensure the validity of the results (Ogasawara et al., 1986). Chen et al. (1987) used a Western blot technique and a monoclonal anti-HLA-B27 antibody generated in their own laboratory to demonstrate cross-reactivity between a 19,000 kD outer membrane component of *Y. pseudotuberculosis* and HLA-B27. A rabbit antiserum prepared against this 19,000 kD protein was selectively reactive against HLA-B27-positive cells.

Molecular similarity between HLA-B27 and other proteins was sought by computer analysis using the Dayhoff Protein Sequence Database. An identical hexamer amino acid sequence was found which was shared by both HLA-B27.1 (residues 72-77) and *K. pneumoniae* nitrogen reductase enzyme (residues 188-193) (Schwimmbeck et al., 1987). The sequence is Glutamine-Threonine-Aspartate-Arginine-Glutamine-Aspartate and is known as QTDRED. To
investigate the significance of this shared sequence, synthetic peptides representing HLA-B27.1 (residues 69-84) and K. pneumoniae nitrogen reductase (residues 184-193) both containing the hexamer, were made and the sera of patients were tested by ELISA for antibodies to the peptides. Sera from 18/34 (53%) patients with Reiter's syndrome and 7/24 (29%) AS patients contained antibodies to the HLA-B27-derived peptide compared with none of the 22 controls. Over 40% of HLA-B27-positive patients with AS or Reiter's syndrome had antibodies to the Klebsiella-derived peptide while none of the controls did. This evidence appeared to support the cross-reactivity theory. The inability to detect antibodies in some AS and Reiter's syndrome patients was suggested to relate to poor immune responses or low affinity responses.

Other laboratories, however, using similar techniques have been unable to confirm these results. The sera of patients with AS and Reiter's syndrome from Norway and New Mexico were tested by ELISA for antibodies to synthetic peptides sharing the QTDRED hexamer (Tsuchiya et al., 1989). Among Norwegian males, 1/8 (12.5%) of Reiter's syndrome and 14/60 (23.3%) of AS patients had antibodies to the HLA-B27 peptide compared with 4% of male controls. None of 8 patients with Reiter's syndrome, only 2/26 (7.7%) of AS patients and 2/38 (5.3%) of male controls in New Mexico were positive. Antibodies to the HLA-B27.1 peptide were found in 20% of normal female controls with at least one previous pregnancy. Anti-Klebsiella peptide antibodies were neither significantly elevated in AS nor correlated with anti-HLA-B27.1 peptide antibodies either in Norwegian or in New Mexico populations. The difference between these results and those of Schwimmbeck et al. (1987) might be due to more stringent precautions taken to minimise non-specific serum binding, or to differences in HLA-B27 subtypes in the two populations.

In another study, failure to confirm Schwimmbeck's results was thought to be due to a high degree of non-specific serum binding in the sera of 93 patients with AS and 38 with Reiter's syndrome (de Vries et al., 1990). This group also reported the absence of cross-reactivity between the HLA-B27.5 peptide containing the mimicry epitope and antibodies raised in rabbits to the Klebsiella peptide containing the epitope. They concluded that the molecular mimicry between the two proteins does not have any biological significance and is not involved in the pathogenesis of HLA-B27-related rheumatic disorders.
Further evidence against cross-reactivity existing between HLA-B27 and arthritogenic enteric bacteria came from the finding of anti-HLA-B27 cross-reac-tive antibodies in only 2/63 patients recently infected with *S. flexneri*, *C. jejuni* or *Y. enterocolitica* who had significantly elevated antibody titres to the infecting bacterial species (Cavender and Ziff, 1986). This was supported by further studies in which lymphocytes from HLA-B27-positive patients with AS and Reiter's syndrome and matched controls were stimulated *in vitro* with polyclonal mitogens (Cavender and Ziff, 1988). There was no significant difference in the level of immunoglobulins produced between patients and controls and only negligible amounts of IgA, IgG or IgM antibodies to *Shigella*, *Campylobacter* and *Yersinia* were found. No anti-HLA-B27 antibodies were produced by any of the patients.

There is another piece of evidence against molecular similarity bearing any relationship to the pathogenesis of AS. The *K. pneumoniae* nitrogen reductase enzyme is present in only 10% of *K. pneumoniae* isolates and the gene is only expressed when the bacteria are in a nitrogen-free environment (Yu, 1988). In the human host, one must accept that this gene is not expressed. It is possible, though, that similar sequences are present in proteins of other (enteric) bacteria and are expressed under conditions that exist in the intestinal tract.

In summary, there is experimental evidence that molecular similarity does exist between the HLA-B27 antigen and some Gram-negative bacteria, but the pathological significance of this molecular mimicry is not clear.

The receptor theory

The receptor theory: This is the most favoured theory at present and is based on the suggestion that the HLA-B27 molecule itself presents specific polypeptides derived from bacteria or from a specific type of collagen, to CD8-positive T cells.

More sophisticated analytical techniques have identified subtypes of HLA-B27 that have slight modifications in their amino acid sequences. These techniques include one-way mixed lymphocyte cultures (Breuning et al., 1982); monoclonal antibodies (Grumet, 1983); and isoelectric focussing gel analysis of HLA-B27 immunoprecipitates (Choo et al., 1986).
There are 6 HLA-B27 subtypes (López de Castro, 1989) and a seventh variant (B27HS) already identified. The subtypes (B*2701-06) differ from each other in 1-6 amino acid residues and B27HS is identical to B*2705 in the α1 domain and to HLA-Bw61 in the α2 (Choo and Hansen, 1989). The positions that are polymorphic (59, 74, 77, 80, 81, 114, 116 and 152) are all located in the peptide binding groove of the molecule; and all except one (residue 59), are located in the right hand side of the groove. Most are located at the surface of the molecule potentially influencing interactions with processed antigen by being accessible to antibody and T cell receptor binding.

There are, however, 5 residues which are unique to HLA-B27 (Benjamin and Parham, 1990) and which are totally conserved in all subtypes. These residues (positions 24, 26, 35, 45 and 67) are spatially in close proximity near the mouth of the '45 pocket' in the α1 helix and situated at the left side of the peptide binding groove, clearly separated from the region where most of the subtype polymorphism occurs. One of these conserved residues is a cysteine at position 67 (Cys 67); and, although it is not part of the antigen binding groove, it has a strong influence on the conformation of the α1 helix. Cys 67 has a reactive sulphydryl side chain which points into the antigen binding groove.

The common subtypes (B*2702 and B*2705) are found in over 99% of white populations (Breur-Vriesendorp et al., 1987) and there is no difference in the relative frequencies of these subtypes in AS and in controls. This suggests that the structural differences between subtypes have little bearing on the disease mechanism (Parham, 1990; López de Castro et al., 1990); and it is not the variable but the conserved regions of the subtypes which are important in pathogenesis.

The unique characteristics of the HLA-B27 peptide binding groove indicate that HLA-B27 will bind antigenic peptides which cannot be bound by other HLA-A, -B or -C molecules. If peptides from joint-specific proteins were bound by HLA-B27, then these complexes could stimulate and be the target for autoimmune T cell responses. As most HLA-B27-positive individuals do not develop disease, additional factors must be involved. It is possible that certain joint-specific peptides not normally produced or only produced in low amounts, are produced in response to an environmental stimulus such as infection or trauma. It is postulated that the reactive sulphydryl group on Cys 67 could bind such joint-specific proteins (Parham, 1990), a cysteine-containing bacterial peptide (Stieglitz
et al., 1988), or even control the access of peptides to the HLA-B27 binding site (Benjamin and Parham, 1990).

An alternative explanation is that activated cytotoxic T cells, stimulated against a bacterial or other foreign peptide, recognise the joint peptide and HLA-B27 complex (Parham, 1990) and instigate a T cell-mediated autoimmune response.

Although HLA-B27 is a well-established serologic specificity, most human alloantisera do not distinguish between subtypes but only recognise the shared HLA-B27 determinant. Some alloantisera, however, can detect subtype polymorphism.

A study of alloreactive cytotoxic lymphocyte clones (López de Castro et al., 1990) demonstrated that polymorphism at residues 74-81 of the HLA-B27 molecule affected the pattern of reactivity of most of the T cell clones. Some HLA-B27 subtypes are recognised by T cells as being as different from each other as from any other MHC class II alloantigen; therefore, subtype differences have the potential to alter peptide binding and T cell recognition.

The chemotaxis model

This model suggests that an increased inflammatory response by HLA-B27-positive neutrophils to some arthritogenic bacteria could explain the increased proportion of Yersinia reactive arthritis in HLA-B27-positive hosts (Repo et al., 1983). By extrapolation, similar responses to other bacteria in HLA-B27-positive individuals could be involved in the pathogenesis of AS. An increased polymorph motility has been found in both HLA-B27-positive AS patients and HLA-B27-positive healthy controls (Pease et al., 1984). A HLA-B27-linked enhanced inflammatory response mediated by neutrophils is thus invoked to explain the association of the MHC class I antigen with AS. The corollary of this is that HLA-B27-positive individuals would be expected to show increased inflammatory responses to a number of infectious agents, but there is no evidence that this is the case.

The plasmid-associated model

The work performed by Dr A Geczy's group showed that rabbit antisera raised against a Klebsiella isolate (427) were cytotoxic for lymphocytes from HLA-B27-positive AS patients but not for lymphocytes from healthy individuals either
HLA-B27-positive or -negative (Seager et al., 1979). The cytotoxic activity was removed only by HLA-B27-positive lymphocytes from AS patients and not by HLA-B27-positive (or negative) lymphocytes from controls. This suggested that some *Klebsiella* antigens cross-react not with the HLA-B27 molecule *per se* but with a gene product closely associated with HLA-B27 or possibly a *Klebsiella*-modified HLA-B27 alloantigen complex. Incubation of HLA-B27-positive lymphocytes from healthy subjects with *Klebsiella* K43 culture supernatants resulted in 'transformation' of these lymphocytes. They were lysed by anti-*Klebsiella* K43 antiserum in a cytotoxicity assay (Geczy et al., 1980). None of the cells of HLA-B27-negative AS patients were lysed by the antisera which suggested that the HLA-B27 antigen was being modified by soluble factors from the bacteria.

One hypothesis to explain these findings was that a bacterial plasmid present in *Klebsiella* coded for a factor able to modify HLA molecules so that HLA-B27 expressed bacterial antigens (Cameron et al., 1983). *Klebsiella* antigens or fragments would be present on the cells of HLA-B27-positive AS patients and absent from HLA-B27-positive healthy controls. If the plasmid theory was correct, anti-*Klebsiella* antisera would bind more to HLA-B27-positive lymphocytes from patients than from healthy HLA-B27-positive controls or HLA-B27-negative cells. There was no difference in binding of anti-*Klebsiella* antisera to HLA-B27-positive lymphocytes regardless of their clinical source; but, HLA-B27-positive lymphocytes from patients or controls bound the antisera to a significantly greater extent than HLA-B27-negative lymphocytes, suggesting a recognition by anti-*Klebsiella* sera of some structure on HLA-B27-positive lymphocytes (Baines et al., 1990). These results support the molecular mimicry theory.

Several attempts in laboratories all over the world have failed to reproduce Geczy's results. The hypothesis of a plasmid-mediated modification of HLA-B27-positive cells is no longer an accepted hypothesis to explain the AS-B27 association.

1.7.6.3. Animal models (see Page 27a)

1.7.7 Faecal flora in AS

From Ebringer's work demonstrating cross-reactivity between rabbit antiserum raised to human HLA-B27-positive lymphocytes and *K. pneumoniae, Enterobacter aerogenes* and *Y. enterocolitica* (Ebringer, 1976), it was a logical step to examine for the presence of *Klebsiella* (a gut commensal organism) in faecal samples of AS patients.
1.7.6.3 Animal models

The importance of the HLA-B27 molecule in disease has been explored using animal models. HLA-B27 and human β2-microglobulin genes were injected into the male pronucleus of fertilised mouse eggs which were transferred to pseudopregnant foster mothers. This procedure gave rise to mice expressing the products of both genes on cell surfaces, so-called transgenic animals. Although the HLA-B27 gene product was functional in both hybrid and inbred mice (Taurog et al., 1988)*, no true similarity of features of HLA-B27-associated disease has been reported in transgenic mice. Nickerson et al. (1990)* reported that mice transgenic for the HLA-B2705 subtype when injected intravenously with Y. enterocolitica developed paravertebral abscesses more frequently and were more susceptible to the lethal effects of the pathogen than were nontransgenic controls. This work has not, however, been confirmed. These results suggested that susceptibility to spondyloarthropathies might not be related to the B27 gene.

By contrast, when HLA-B27 and human β2-microglobulin genes were introduced into rats, 2 transgenic lines spontaneously developed a number of features similar to those observed in the spondyloarthropathies (Hammer et al., 1990)*. Diarrhoea was invariable with chronic inflammation histologically particularly of the colon and mostly in the lamina propria. Arthritis of the tarsal joints occurred lasting a few days to several weeks and histologically the changes were similar to those seen in AS, with hyperplastic synovium and infiltration of large numbers of neutrophils, lymphocytes, and plasma cells. Inflammatory changes in the outer aspects of the annulous fibrosis and its attachments to vertebral endplates were identified. Hyperkeratosis of the tail, nail dystrophy, orchitis and epididymitis were common. Inflammatory lesions in the myocardium were identified in a few cases.

These results support a direct role for the HLA-B27 molecule in pathogenesis. Not all transgenic rat lines have developed such inflammatory disease and this may be due to quantitative or qualitative differences in expression of the transgenes or to differing effects of the transgene on the host genome. The disease in transgenic rats arose spontaneously in the apparent absence of infection, but it is possible that interaction occurred between HLA-B27 and commensal bacteria. Results on studies on germ-free transgenic rats are awaited with interest. * p.196
In their initial study, Ebringer and colleagues (1977) based disease activity on the presence of active peripheral manifestations, duration of early morning stiffness and anti-inflammatory and analgesic requirement. *Klebsiella* were isolated from the stools of 13/14 (93%) patients with active spondylitis, 10/21 (48%) patients with probably active disease, and in 1/28 (4%) of those with inactive spondylitis. This compared with an isolation rate of 47/124 (38%) in controls. It was concluded that carriage of *Klebsiella* was associated with active AS. If all AS patients, however, were taken as a group, the *Klebsiella* isolation rate was practically identical to that of controls. The interpretation of the findings depends on the criteria for defining 'active' disease, and there remains no consensus on the definition of disease activity in AS.

In a larger sequential study on 163 AS patients, the rate of *Klebsiella* isolation again correlated with disease activity and it was suggested that in those with inactive spondylitis the finding of *Klebsiella* in the stool was associated with a subsequent increase in inflammatory disease (Ebringer et al., 1978). In a survey of Navajo Indians in Arizona, 25 with AS and 18 with Reiter's syndrome (Kuberski et al., 1983), there was no significant difference in overall frequency of faecal *Klebsiella* in patients compared with controls. When the patients were divided on the basis of disease activity, however, significantly more *Klebsiella* were identified in the group with active AS or active Reiter's (54%) than in the inactive group (12%) or in the controls (13%). This applied to both AS and Reiter's patients. In this study active disease was determined by the presence of early morning stiffness with axial and/or peripheral joint involvement requiring non-steroidal anti-inflammatory drugs (NSAID).

Several other studies have shown no association of faecal *Klebsiella* with AS or any relationship between *Klebsiella* carriage and active spondylitis. These studies have been performed in many different laboratories including ones in London (Warren and Brewerton, 1980), Winnipeg (Hunter et al., 1981), Leeds (Eastmond et al., 1980) and the Netherlands (van Kregten et al., 1991). Recently in Brazil, a complete assessment of Gram-negative enteric bacteria in AS patients and healthy controls revealed no significant differences either qualitatively or quantitatively in *Klebsiella* isolation between the two populations. There was no association between the presence of *Klebsiella* and clinical or laboratory parameters of active disease (Ferraz et al., 1990).
An attempt to reduce *Klebsiella* carriage and so influence disease activity has been tried by dietary manipulation, but this met with no success (Shinebaum et al., 1984).

Increased carriage of *Klebsiella* has, however, been reported in the first week following acute anterior uveitis (AAU) especially in HLA-B27-positive or B7-CREG-positive patients (Ebringer, 1988). An earlier survey (Warren and Brewerton, 1979) did not show any association between *Klebsiella* and AAU, but in this case faecal samples were obtained up to 1 month after AAU started.

### 1.7.8 Humoral response to enteric bacteria

Another way of assessing the involvement of enterobacteria in the pathogenesis of AS has been to examine the humoral immune response to microorganisms. The evidence has been conflicting. Raised IgA antibodies to *Klebsiella* have been identified in patients with active spondylitis by a number of different techniques: enzyme linked immunosorbent assay (ELISA) (Trull et al., 1983; Trull et al., 1984; Khalifapour et al., 1988; Maki-Ikola et al., 1991); bacterial absorption (Ebringer et al., 1985); indirect Coombs' bacterial agglutination (Ebringer et al., 1985); and immunoblot (Shodjai-Moradi et al., 1992). Raised IgA, but not IgG, antibodies to *K. pneumoniae* K43 were similarly demonstrated by ELISA in AS patients compared with healthy controls (Cooper et al., 1988); but, they were also elevated in ulcerative colitis, Crohn's disease and rheumatoid arthritis. There was no correlation between disease activity (defined by a combination of clinical criteria and an elevated ESR) and titres of anti-K43 IgA antibodies. These results suggested that in AS there is a subclinical bowel inflammation which allows the penetration of bacteria across the mucosa to stimulate antibody production. The same group found that titres of IgM anti-K43 antibodies were significantly lower in AS and ulcerative colitis than in controls, but the reason for this was not clear (Cooper et al., 1988).

In contrast, a study using an ELISA to measure serum antibodies to sonicated (rather than whole) Gram-negative bacteria showed that there was no difference in IgA antibody titres to *K. oxytoca* K9 or *E. coli* in AS patients compared with healthy controls. There was, however, a slightly elevated mean anti-*Yersinia* titre in AS patients due to a few patients with raised levels. Salivary IgA antibodies to the three bacterial species were similar for patients and controls.
Another group failed to demonstrate specific IgA antibodies to *K. pneumoniae* K21, *S. flexneri* 2a, *Y. enterocolitica* 09 or *E. coli* in AS patients as a group, though it was observed in individual patients. These results, in conjunction with the elevated total IgA in these patients, suggested that a non-specific stimulation of the IgA system might occur in AS (van Bohemen et al., 1986).

The search for antibodies to other enterobacteria has, in general, been unrewarding. IgA antibodies to *Y. enterocolitica*, *S. typhimurium* or *P. aeruginosa* in active spondylitis were not elevated compared to controls (Trull et al., 1984). A wider survey by a Finnish group showed that the IgA level to *Campylobacter jejuni, Proteus mirabilis, Chlamydia trachomatis, Borrelia burgdorferi, Yersinia* and *Salmonella* did not differ in AS patients compared with controls (Maki-Ikola et al., 1991). Andreasen et al. (1991) agreed that there was no role for *C. jejuni* in the pathogenesis of AS.

Raised antibody levels to *B. burgdorferi* have been reported in AS patients and their relatives but the results were inconclusive (Blaauw et al., 1990). In contrast with the Finnish workers, a more frequent occurrence of specific *C. trachomatis* antibodies in AS compared with untyped healthy blood donor controls was reported suggesting a possible aetiological role for this intracellular organism (Csango et al., 1987). Streptococci have also been implicated in the pathogenesis of AS (Zilko et al., 1977).

Rather than measure antibodies to a particular bacterium, van Bohemen and colleagues (1988) measured serum antibodies to one of the major outer membrane proteins (h-momp) which constitutes a large part of the cell envelope structure. H-momp is cross-reactive among many *Enterobacteriaceae*; raised antibody titres to it would therefore reflect exposure to several different bacterial species. AS patients had over twice the level of IgA anti-h-momp antibodies than hospitalised controls who did not have arthritis or gastrointestinal disease. IgG and IgM anti-h-momp antibodies did not differ between the two populations.

Intestinal humoral immunity has been investigated by only one group (O'Mahony et al., 1992). High serum IgA antibodies to *K. pneumoniae* K43 in gut lavage fluid (measured by ELISA) were found in both AS patients with active disease and patients with Crohn's disease compared with controls. The
categorisation of active disease for AS patients was based on an ESR > 15 and serum IgA > 3g/l, similar to Ebringer's classification, but not accepted universally. The Crohn's group also had high serum IgG titres to K43. Intestinal IgA antibody levels to K43 were similar in the 3 study groups, but Crohn's patients had high intestinal IgM and IgG antibody titres to K43. The rises in intestinal IgA antibody levels to K43 were not, therefore, specific to AS and it was suggested that they probably related to the passage of bacterial antigens across inflamed gut mucosa. There was no evidence for an abnormal intestinal IgA response to K43 in AS patients.

Raised serum antibody levels to specific bacteria might indicate that the bacteria are involved in the pathogenesis of AS, but there is no direct evidence for an antibody- or complement-mediated disease process.

1.7.9 Total serum antibodies in AS

1.7.9.1 IgA

Elevated levels of total serum IgA in AS patients compared to controls have been reported in several studies (Veys and van Laere, 1973; Kinsella et al., 1975; Cowling et al., 1980b; Calguneri et al., 1981; Laurent and Panayi, 1983; Neumann et al., 1984; Trull et al., 1984; Franssen et al., 1985; van Bohemen et al., 1986; Pease et al., 1987; Sanders et al., 1987; Mackiewicz et al., 1989). This has been postulated to reflect stimulation by enterobacterial antigens via the gastrointestinal tract as IgA is the main immunoglobulin concerned with mucosal immunity.

In some studies no correlation of serum IgA with disease activity was made (Kinsella et al., 1975; Sanders et al., 1987). In others, the raised serum IgA was associated with active disease as determined by the laboratory parameters of an elevated ESR (Cowling et al., 1980b; Trull et al., 1983); an elevated CRP (Cowling et al., 1980b; Trull et al., 1984; Collado et al., 1987); or both an elevated ESR and CRP (Calguneri et al., 1981).

When disease activity is assessed by clinical criteria, some groups have shown no relationship between serum IgA and active disease (Laurent and Panayi, 1983; Pease et al., 1987). This contrasts with the results of a longitudinal study in which changes in serum IgA over time correlated with changes in disease activity assessed by a combination of subjective and objective clinical parameters.
(Franssen et al., 1985). The highest levels of IgA were present in those patients with the most extensive radiological changes.

van Bohemen and colleagues (1986) found elevated levels of serum IgA in active AS patients as determined by a combination of clinical criteria and raised CRP.

Measurement of total salivary IgA in AS patients has revealed conflicting results. Similar (Calguneri et al., 1981; Pease et al., 1987) and elevated (Neumann et al., 1984) levels of salivary IgA in AS patients compared with controls have been found.

The IgA subclasses produced in AS have been examined (van den Wall Bake et al., 1988). An increase in serum IgA restricted to IgA1 subclass was found in patients with AS compared with controls. An in vitro study of immunoglobulin synthesis by peripheral blood mononuclear cells (PBMC) in these patients established that there was no increase in IgA synthesis in either unstimulated or pokeweed mitogen-stimulated cultures. There was, however, an increase in the ratio of IgA1 to total IgA production in pokeweed mitogen (PWM)-stimulated PBMC cultures which was reflected in the raised serum IgA1/total IgA levels.

1.7.9.2 IgG

A mixed picture emerges for the total serum IgG in AS. No significant difference in total serum IgG was found between AS patients and controls (Kinsella et al., 1975; Cowling et al., 1980b). Calguneri and colleagues (1981) reported that the mean total IgG was similar for AS and control populations, but those patients with a raised ESR and CRP had significantly higher IgG levels than controls. Other groups have reported elevated total serum IgG in AS patients compared with controls (Veys and van Laere, 1973; Trull et al., 1984; Sanders et al., 1987). Only AS patients with iritis or peripheral arthritis had higher total IgG levels than controls (Laurent and Panayi, 1983). Total IgG correlated with disease activity as determined by a raised CRP (Trull et al., 1984), but not as determined by either a combination of clinical and laboratory parameters (Veys and van Laere, 1975) or by clinical criteria alone (Laurent and Panayi, 1983).
Most studies have shown no significant difference in total serum IgM between AS patients and controls (Kinsella et al., 1975; Cowling et al., 1980b; Calguneri et al., 1981; Laurent and Panayi, 1983; Trull et al., 1984). Elevated total IgM in AS patients compared with controls (Veys and van Laere, 1973) and compared with patients with rheumatoid arthritis (RA) and controls (Franssen et al., 1985) have been reported.

A significantly decreased production of IgM in unstimulated cultures of PMBC from AS patients has been described (van den Wall Bake et al., 1988). In a previous study (Vuento et al., 1984) a similar decrease in IgM production was evident in PWM-stimulated cultures; the addition of hydrocortisone to the cultures led to normalisation of IgM synthesis suggesting a role for suppressor cells.

### 1.7.10 Autoimmunity in AS

In view of the association of prostatic infection in AS and Reiter's syndrome (Mason et al., 1958), Grimble et al. (1964) searched for autoantibodies to prostate antigen and found them in most Reiter's syndrome patients and patients with AS but not in those with RA.

An antibody was identified in the sera of 39% AS patients which reacted with the 93kD heat shock puff of polytene chromosomes from larval salivary glands of *Drosophila melanogaster* (Lakomek et al., 1984). Heat shock proteins are produced by cells in response to physiological stress. Some act as molecular chaperones protecting immature proteins during the final stages of protein synthesis and transport. They may also be a source of antigenic target, modulate T cell function and might protect cells from free radicals at sites of inflammation.

Lakomek's group subsequently (1991) found that serum samples from 32/95 (34%) patients with AS and 15/34 (44%) from patients with either Crohn's or psoriatic spondyloarthropathy reacted with a 36kD drosophila antigen (not a heat shock protein). Antibodies purified from the 36kD antigen reacted specifically with a 69kD antigen present in separations of total protein preparation from human lymphocytes and HeLa cells. This antibody is in effect
an autoantibody. It was not found in 29 patients with RA or in 38 healthy controls and was found in similar proportions in HLA-B27-positive and -negative patients. The presence of anti-36kD antibodies did not correlate with the patients' age, sex or ESR and are distinct from those autoantibodies described by Schwimmbeck et al. (1987) which recognised 12kD and 45kD moieties of HLA-B27 antigen. It was postulated that there might be a defect in cell-mediated immunity resulting in an immunoreactive lymphocyte clone producing antibody to this 36kD antigen. The structure and function of the 69kD lymphocyte protein to which the 36kD antibody binds is not known.

Raised serum IgA immune complexes detected by PEG precipitation and pepsinogen binding have been associated with peripheral synovitis, but the correlation with other clinical and laboratory indicators of disease activity is poor (Maclean et al., 1992). There was no evidence for Klebsiella antigens in these complexes.

1.7.11 Cell-mediated immunity and B lymphocytes in AS

T lymphocyte numbers in AS have been studied by a few groups. Normal T cell counts (Hickling et al., 1982), a reduced T cell count (Fan et al., 1977) and an increase in the proportion but not the total numbers of helper T cells have all been reported (Veys et al., 1983).

Elevated levels of serum immunoglobulins have been considered an expression of B lymphocyte hyperreactivity. Raised B cell numbers (in association with raised serum IgG and IgA) have been found in active AS (Hickling et al., 1982). Increased proportions of peripheral B cells (Byrom et al., 1979) and immunoblasts (Eghtedari et al., 1976) have been noted. More recently, a normal proportion of circulating B cells was described in AS (Barbieri et al., 1990).

Tests of lymphocyte function have yielded conflicting results. Phytohaemagglutinin and aggregated human gammaglobulin caused increased transformation responses in PBMC from AS patients in a lymphocyte transformation (LT) test (Kinsella, 1979). An increased proliferative response and in vitro production of immunoglobulins from lymphocytes from AS patients to the Staphylococcus aureus strain Cowan I (a T cell independent B cell activator) was found (Barbieri et al., 1990). This hyperresponsiveness was associated with disease activity. No differences were found in the response to
pokeweed mitogen (PWM) and protein A (both T cell dependent polyclonal B cell activators). There was no difference in T cell responses to mitogens in patients and controls.

Following work by Nikbin and colleagues (1975) who showed a reduced LT response to a Yersinia immunogen (a formalinised mixture of Y. enterocolitica 03 and 09 and Y. pseudotuberculosis type 1), Sheldon et al. (1985) sought to determine whether AS lymphocytes showed fundamental differences in their response to enteric organisms, either commensals or pathogens compared with healthy individuals. PBMC from AS patients responded less well in a LT test to Y. enterocolitica 03, and 06 and K. pneumoniae antigens than did PBMC from patients with reactive arthritis, Reiter's syndrome or controls. There was a statistically significant hypo-responsiveness of AS PMBC to K. pneumoniae compared with controls. This agrees with the report that AS lymphocytes were hypo-reactive to 5 different Klebsiella species compared with those of HLA-B27-positive and HLA-B27-negative controls in a LT assay (Seager et al., 1979).

Impaired PBL transformation by Klebsiella from autogenous or heterogenous sources was not seen in AS patients or controls in a LT assay (Kinsella et al, 1984). No difference in reactivity in a LT assay to S. flexneri, S. typhi, Y. enterocolitica, K. pneumoniae or C. trachomatis was noted for cells from patients with AS or controls (Enlow et al., 1982). K. pneumoniae when added to mononuclear cell cultures with Y. enterocolitica had an adjuvant effect on transformation of the lymphocytes (Sheldon, 1985).

There was a poor proliferative response to a variety of cell preparations of Klebsiella K43 with peripheral blood lymphocytes from patients with AS, Yersinia reactive arthritis, those recovering from Klebsiella infection and controls (Gross et al., 1988). Compared with healthy controls, however, there was increased leucocyte migration inhibition factor (LIF) production from PBMCs from patients with yersiniosis and Yersinia arthritis in response to Yersinia, Klebsiella and protein I of the outer membrane. In contrast, enterobacterial common antigen did not induce LIF production in cells from patients or healthy individuals.

Peripheral blood T lymphocytes specific for cartilage proteoglycans (a major component of articular cartilage) have been reported (Mikecz et al., 1988). There was no significant difference in response, however, of either PBMC or synovial
fluid mononuclear cells to 5 preparations of human cartilage proteoglycans compared with that of normal and disease controls suggesting that autoimmune responses to cartilage proteoglycans are unlikely to be involved to a significant extent in the pathogenesis of AS (Jobanputra et al., 1992).

An investigation into the cellular mechanism of reduced rheumatoid factor (RF) production in AS showed that either spontaneous or PWM-induced total IgG and IgM production by PBMCs was similar for patients with AS, rheumatoid arthritis and controls (Yu et al., 1989). IgM-RF synthesis was significantly reduced in AS compared with controls and RA patients. Subsequent co-culture experiments adding T or B cells from normal subjects to B cells of AS patients revealed a diminished T cell helper function of AS lymphocytes. Suppressor T cell function and B cell function were normal. The deficiency of helper function was not due to a serum blocking factor.

When Epstein-Barr virus infects B lymphocytes in vitro proliferation and lymphoblastoid formation occurs. T cells normally control this and inhibit the proliferative response about day 12 of culture. With mononuclear cells from patients with AS, rheumatoid arthritis and multiple sclerosis no such regression occurs and cultures outgrow into lymphoblastoid cell lines at high frequency (Robinson and Panayi, 1986). In rheumatoid arthritis this defect has been identified to a deficiency in cytotoxic T cell response and it would not be unreasonable to predict a similar defect in AS.

1.8 SUSCEPTIBILITY FACTORS FOR DISEASE

Susceptibility to disease is dependent on both genetic and environmental factors. The HLA-B27 phenotype is a genetic factor and infection is possibly an environmental factor in the aetiology of AS. Susceptibility to certain diseases is associated with particular blood group phenotypes and in some cases to the ability of individuals to secrete ABO blood group antigens into their body fluids.

1.8.1 Blood groups in susceptibility to disease

Blood group phenotypes differ considerably in frequency between different populations but there has been no unifying hypothesis to explain this. Although about 20 independent blood group systems have been recognised, the physiological functions of blood group antigens remain to be discovered. Blood
group antigens are major transplantation antigens, they might be involved in tissue differentiation and may have evolved to play a role in conferring resistance to disease (reviewed by Marsh, 1990).

ABO blood group antigens are associated with a number of diseases, the most striking of which is that of diseases of the upper gastrointestinal tract. Duodenal ulcer is seen 40% times more frequently in individuals of blood group O than of other groups (Roberts, 1959). There is a marked association of blood group A and gastric carcinoma (Aird et al., 1953). ABO blood group antigens are also associated with thromboembolic disease (Mourant et al., 1978).

1.8.1.1 Historical review

In 1900 Landsteiner discovered the human ABO blood group system. Ten years later, von Dungren and Hirzfeld showed that these blood groups were inherited in a Mendelian pattern (reviewed by Mourant et al., 1978). The presence of A and B antigens in semen and saliva was first noted in 1926 by Yamakami, but it was not realised until 1930 when Lehrs and Putkonen (cited by Mourant et al., 1978) independently discovered that some individuals do, and others do not, secrete antigens corresponding to their ABO blood group into their saliva. This ability to secrete blood group antigens into body fluids was found to be inherited as a Mendelian dominant character (reviewed by Race and Sanger, 1975). In Europeans about 80% are secretors and 20% are non-secretors (Mourant et al., 1978). Different ethnic groups show different proportions of secretors and non-secretors (Mourant et al., 1978).

The Lewis blood group system was identified initially by the discovery of the antibody, anti-Lewis^a, by Mourant in 1946. This was complemented by the discovery by Andresen in 1948 of anti-Lewis^b. Secretors, in addition to the presence of ABH antigens, have either Lewis^b antigen or Lewis^b with variable amounts of Lewis^a antigen in their body fluids. Non-secretors have only Lewis^a in body fluids (Race and Sanger, 1975).

The ABH blood group antigens are more correctly termed histo-blood group antigens (Clausen and Hakomori, 1989) as they are found on most tissues of the body and indeed evolved earlier on epithelial cells than on blood cells.
1.8.1.2 Biochemistry of ABH antigens

ABH blood group determinants consist of carbohydrate chains bound to glycolipids and glycoproteins in cell membranes and predominantly to glycoproteins in secretions. They also exist in secretions as free oligosaccharides. They have a short basic precursor carbohydrate chain to which are added particular monosaccharides by specific glycosyltransferases, the primary products of the A, B and H genes.

There are 4 types of carrier precursor chains (Rege et al., 1963; Donald, 1981; Bremer et al., 1984) of which Type 1 and Type 2 precursors carry most of the ABH antigens in cells and in secretions. Type 1 chains have a D-galactose residue joined by a β1-3 linkage to N-acetyl-D-glucosamine, whereas in the Type 2 chain these two sugars are joined by a β1-4 linkage (Figure 1.1). Type 1 is the main carrier of ABH determinants in secretions and in endodermally-derived tissues such as lining epithelium; and Type 2 is the main carrier in mesodermally- and ectodermally-derived tissues, including red blood cells (Oriol et al., 1986). H Type 1 and 2 molecules are formed by the fucosylation of the respective precursor chains at the terminal D-galactose residue by the fucosyltransferase coded by the H gene (Figure 1.2). The H antigen is the substrate for glycosyltransferases coded by the A and B genes to form A or B determinants respectively. The biochemical pathways by which ABH antigens are formed is shown in Figure 1.3.

1.8.1.3 Genetic control of ABH antigens

The ABO locus was thought for years to be present on chromosome 9 (Westerveld et al., 1976), but it was not until recently that by cloning and sequencing of complementary DNAs for A and B glycosyltransferases, the expression of these enzymes has definitely been shown to be controlled by this locus (Yamamoto et al., 1990). The ABO locus has 4 alleles; A1 and A2 (the two subgroups of blood group A), and B which all code for a different glycosyltransferase; and, the O allele which does not give rise to a functional product.

The antigen known as H is present on red blood cells of practically all humans. It was previously thought to be the product of the O gene, but the discovery of the rare Bombay blood group (genotype hh) which lacks this antigen, has
Figure 1.1: Diagram of the two types of carbohydrate chain endings which form the basis of A, B and H determinants on cells and in secretions.
Present in secretions, and tissues of endodermal origin

Present in tissues of mesodermal and ectodermal origin including erythrocytes

**TYPE 1**

**H (O)**

β 1-3

**A**

β 1-3

**B**

β 1-3

**TYPE 2**

β 1-4

β 1-4

β 1-4

β 1-4

- L fucose
- N-Acetylgalactosamine
- D-galactose

Figure 1.2: Structure of H, A and B determinants based on Type 1 and Type 2 carbohydrate chains. The symbols for the constituents of precursor chains are as in Figure 1.1.
Figure 1.3: Biochemical pathway for the formation of H, A & B antigens. The symbols for the constituents of the precursor chains are the same as in Figures 1.1 and 1.2. H transferase (H) transfers fucose from guanosine diphosphate (GDP) L-fucose to the precursor chain. A transferase (A) transfers N-acetylgalactosamine from uridine diphosphate (UDP) N-acetylgalactosamine to the H structure. B transferase (B) transfers galactose from uridine diphosphate (UDP) D-galactose to the H structure.
clarified its nature (Bhende et al., 1952, as reviewed by Race and Sanger, 1975). The H gene has only recently been isolated (Ernst et al., 1989). It is located on chromosome 19 and is present in almost all individuals. It codes for a fucosyltransferase which produces the substrate (H) for the A and B transferases. The h gene is a rare silent allele of H. Individuals who have the Bombay phenotype can have A and/or B glycosyltransferases, but do not have A or B antigens as the absence of the H gene means that there is no substrate for the A and B transferases (Figure 1.4).

In AB individuals, competition between the glycosyltransferases coded by these genes for H Type 2 results in some molecules bearing the A determinant and some the B determinant (Watkins, 1980). The A and B determinants are found on different carrier molecules on human erythrocytes of blood group AB and approximately half of the blood group glycoproteins carry group A determinants and half carry group B determinants (Viitala et al., 1986). This contrasts with saliva where A and B specificities are carried on the same macromolecules (Morgan and Watkins, 1948). Individuals of group O express unmodified H on their cells as they have a non-functional gene at the ABO locus.

In secretors the Type 1 precursor chain is fucosylated by the fucosyltransferase encoded by the Se gene to form H Type 1 determinant (Watkins et al., 1988) (Figure 1.5). This acts as a substrate for A and B glycosyltransferases to form A and B determinants. In non-secretors the enzyme coded for by the Se gene is absent, and so the fucosylation of Type 1 precursor and subsequent modification to form A and B antigens does not take place in secretions.

1.8.1.4 Lewis antigens

Unlike ABH antigens, Lewis antigens on erythrocytes are not synthesised by the red blood cells, but are passively acquired from plasma (Sneath and Sneath, 1955) where they are present as glycosphingolipids (Marcus and Cass, 1969). In secretions they are carried on the same types of glycoprotein molecules as the ABH determinants (Watkins, 1974). Neither the site of synthesis of the Lewis antigens nor the type of attachment they have to cells is known. They might bind through specific receptors or by non-specific attachment. Lewis antigens are generated by the product of the Lewis (Le) gene which codes for a glycosyltransferase that adds fucose through an \( \alpha 1-2 \) linkage to the subterminal
Figure 1.4: Diagram showing the production of A, B and H determinants on Type 2 chains. Symbols as in Figures 1.1 and 1.2. The arrows represent the reactions possible in individuals possessing the genes indicated by the letters.
Figure 1.5: The pathway of production of H, A, B and Lewis determinants in secretions. Symbols as in Figures 1.1 and 1.2. The arrows represent the reactions possible in individuals possessing the genes indicated by the letters.
N-acetylglucosamine of H Type 1 precursor chains to form Lewis$^b$ (Le$^b$) and precursor Type 1 chains to form Lewis$^a$ (Le$^a$) (Figure 1.5).

1.8.1.5 Secretor and Lewis genes

Secretor status is controlled by the secretor gene (Se) on chromosome 19 and is inherited as a Mendelian dominant character (reviewed by Watkins, 1980). Secretors can be homozygous (SeSe) or heterozygous (SeSese) for the Se gene. Non-secretors are homozygous (SeSese) for the silent allele. The Se gene is closely linked to the H gene on the short arm of chromosome 19 (Oriol et al., 1981; Oriol et al., 1984) suggesting that it might have arisen by Se gene duplication during evolution (Le Pendu, 1985).

While both H and Se gene products fucosylate precursor chains to form the H determinant, the Se gene product has a higher affinity for the Type 1 precursor than for the Type 2 precursor. The H gene product preferentially fucosylates Type 2 precursor chains. Individuals with the genotype hh/SeSe or hh/Sese have an active Se gene but inactive H gene and will express Type 1 chain-based blood group antigens in their secretions but will have no ABH on their red cells. This is known as the para-Bombay phenotype. In Bombay individuals (hh/Sese genotype) both Se and H genes are inactive and ABH antigens are not expressed in cells or secretions (Oriol et al., 1986).

The Le gene is also located on chromosome 19 and is linked to the loci for H and Se genes (Elberg et al., 1983). The Le gene encodes a fucosyltransferase which acts on Type 1 precursor chains.

1.8.1.6 Interaction of the ABH, Se and Le genes

The assembly of blood group antigens is a complex process requiring the sequential action of a series of enzymes. This is shown in Figure 1.5. The fucosyltransferases coded for by the Se and Le genes compete for the same substrate (H Type 1). If the Se enzyme acts first, the resultant structure can be fucosylated by the Le enzyme to produce the Le$^b$ antigen. If, however, the precursor is first fucosylated by the Le enzyme, the resultant Le$^a$ antigen is unsuitable for further modification by the Se enzyme. Thus, both Le$^b$ and Le$^a$ are found in secretors, the relative amounts of each depend on the efficiency of the competing enzymes (Watkins et al., 1988). In non-secretors, if the Le gene is
present, it can act unopposed by the $Se$ gene on Type 1 precursors so that only Le$^a$ antigen is formed.

There is also competition between the A and B transferases and Lewis enzymes for H Type 1. Once the determinant is converted to Le$^b$, it cannot act as a substrate for the A and B enzymes. The A and B determinants, however, can act as substrates for the Lewis enzyme to form ALe$^b$ or BLe$^b$.

If $Se$ and $Le$ genes are both absent, secretions will contain unconverted Type 1 precursor chains.

In ectodermal tissues ABH antigens are expressed independently of the $Se$ gene although the $Se$ gene can quantitatively influence their expression (Dabelsteen et al., 1982). Endodermal tissues express ABH and Lewis antigens under the control of the products of the both Le and $Se$ genes (Oriol, 1987). The differences in the expression of the blood group antigens between secretors and non-secretors are summarised in Table 1.5.

**Table 1.5**

Blood group antigens found on cells and in body fluids of secretors and non-secretors

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Secretors</th>
<th>Non-secretors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Secretions</td>
</tr>
<tr>
<td>H (A/B)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lewisa</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Lewisb</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Present
- Absent
+/- Present in small amounts
1.8.2 Secretor status and susceptibility to disease

Epidemiological studies have found associations between the inability to secrete blood group antigens into body fluids (non-secretion) and various diseases. There is a striking association between duodenal (but not gastric) ulcers and non-secretion (Clarke et al., 1959). Non-secretors are more susceptible to certain bacterial and superficial yeast infections and also to those autoimmune diseases which have a proposed infectious trigger (Blackwell, 1989). Non-secretors are over-represented in patients with infections due to Streptococcus pyogenes in rheumatic fever and rheumatic heart disease (Havercorn and Goslings, 1969); Escherichia coli urinary tract infections (Kinane et al., 1982); oral and genital infections due to Candida albicans (Thom et al., 1989); invasive disease due to Haemophilus influenzae (Blackwell et al., 1986b), Streptococcus pneumoniae and Neisseria meningitidis (Blackwell et al., 1986a). There are two recent reports of associations between secretor status and patients with viral infections, with secretors rather than non-secretors being over-represented. This has been seen with respiratory viral disease (Raza et al., 1991) and among individuals who have acquired the human immunodeficiency virus through heterosexual intercourse (Blackwell et al., 1991).

Non-secretors are more common among patients with insulin-dependent diabetes (Blackwell et al., 1987a), a disease in which viruses are implicated in the aetiology and Graves' disease (Collier et al., 1988) in which Yersinia infection is suggested to have a role. There is no association between secretor status and autoimmune diseases which epidemiologically are most unlikely to have an infective aetiology.

A marked preponderance of non-secretors was reported in ankylosing spondylitis (49%) and in Reiter's syndrome (45%) compared to the control population (27%) (Shinebaum et al., 1987b). This study gave support to the hypothesis of an infective aetiology in this disease.

1.8.2.1 Blood group antigens as receptors for microorganisms

One possible mechanism to explain the association of secretor status with infection is that the ABH or Lewis determinants serve as receptors for microorganisms. Blood group antigens are found on the surface of many tissues, including the surface of epithelial cells which form the site of
colonisation for many microorganisms. The secretor gene controls the expression of blood group antigens in secretions and some of these secreted antigens are passively adsorbed onto the cell surface. Carbohydrates which are present in abundance on cell surfaces act as receptors for bacteria and their products (Lark, 1986) and a number have been identified; several bacterial species bind specifically to mannose (reviewed by Bock et al., 1988); the ganglioside GM1 acts as a specific receptor for Vibrio cholera toxin (Eidels et al., 1983). Fucose, which is the immunodominant sugar of the H and Lewis blood group antigens, might be a receptor for some Candida species (May et al., 1989).

Although blood group antigens are expressed in high density on cell surfaces, are distributed widely in the body and are postulated to act as receptors for microorganisms, there is little evidence for this. The Duffy blood group determinants Fya or Fyb are the erythrocyte receptors for Plasmodium knowlesi merozoites (Miller et al., 1975); H. influenzae binds to the Anton blood group antigen on oropharyngeal epithelial cells (van Alphen et al., 1986); the P antigen (Kallenius et al., 1980) and the M antigen (Vaisanen et al., 1982) act as receptors for uropathogenic strains of E. coli. It has been suggested that Lea antigen acts as a receptor for C. albicans (May et al., 1989) and also for Staphylococcus aureus (Saadi et al., in press).

1.8.2.2 Humoral immune responses of secretors and non-secretors

A second mechanism proposed to explain the increased susceptibility of non-secretors to infection is that non-secretors have an impaired ability to generate a humoral immune response to bacteria. This followed from the observations of increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease which occurs following a streptococcal throat infection (Clarke et al., 1960). Lower serum immunoglobulin levels were found in non-secretors compared with secretors. It was postulated that if a humoral response is important in the defence against streptococci, the lower immunoglobulins of non-secretors could result in their being more susceptible to streptococcal infection and consequently developing rheumatic heart disease. Although the low immunoglobulin levels in non-secretors were demonstrated in serum, it was deemed reasonable by these authors that similar findings were likely in the throat secretions which form the first line of mucosal defence against the streptococcus (Clarke et al., 1960).
Another group reported lower levels of total serum IgG and IgG anti-B isohaemagglutinin in non-secretors than secretors (Grundbacher and Shreffler, 1970). Total serum IgA (in blacks and whites) and total serum IgG (in whites only) were similarly lower in non-secretors than in secretors when a population of children > 5 years of age and young adults were studied (Grundbacher, 1972).

IgA (as secretory IgA) is the main immunoglobulin in exocrine body fluids and is protective against a variety of foreign antigens including bacteria. Total salivary IgA was significantly lower in non-secretors than secretors (Waissbluth and Langman, 1971). Among adolescents, total salivary IgM and salivary IgM antibodies to N. lactamica and N. meningitidis were significantly lower in non-secretors compared with secretors (Zorgani et al., in press). In infants secretory IgM is necessary to protect mucosal surfaces as very little IgA is present (Mellander et al., 1984). This might explain the increased susceptibility of non-secretors to colonisation and disease by N. meningitidis (Blackwell et al., 1990).

Other researchers have shown different results regarding immunoglobulin levels in secretors and non-secretors. Total serum IgA, M and G were similar in both secretors and non-secretors (Zorgani et al., in press). In another survey two groups of women with recurrent urinary tract infection (UTI) were studied; one group had recently been referred to the clinic and the other had been monitored for 20 years. The proportion of non-secretors was increased in both groups compared with controls (Blackwell et al., 1987b). There was no difference in total serum IgA or IgG between secretors and non-secretors in the recently-referred group. In women who had been monitored long-term, however, non-secretors had significantly higher levels of total serum IgA and IgG than secretors. It was postulated that if the lower serum immunoglobulins in non-secretors reported in previous studies (Clarke et al., 1960; Grundbacher and Shreffler, 1970; Grundbacher, 1972) predispose to initial infections, then non-secretors would be more prone to UTI. Repeated UTI would result in high immunoglobulin levels as found in women in the long-term group. Although there was no difference in the total IgG and IgA between secretors and non-secretors in the recently-referred group, it is suggested that the increased UTI rate in this group results in a total immunoglobulin level comparable to that in secretors.

In a study at Stonehouse, Gloucestershire, on the carriage of N. meningitidis, total salivary IgA in non-carriers was similar in secretors and non-secretors,
while among carriers, non-secretors had higher levels of salivary IgA than secretors (Blackwell et al., 1989a). In non-carriers the serum IgA, however, was significantly lower in non-secretors than in secretors. There was no significant difference in serum IgA of secretors carrying serogroupable or non-serogroupable strains when compared with secretor controls. Non-secretor carriers of serogroupable strains had a significantly higher serum IgA than did non-secretor controls. It was postulated that in carriers, either the meningococci caused polyclonal B cell activation, or the increased total immunoglobulin levels reflected an increased specific response.

At present, therefore, there is no clear evidence relating serum or secretory immunoglobulin levels with secretor status.

1.9 AIMS OF THE STUDY

The aetiology of AS remains unknown. Interaction of genetic and environmental factors are involved. The most likely environmental trigger is infection. There is no hard evidence to implicate a particular organism although many have suggested Klebsiella species as the most likely candidate. There is no agreement on this hypothesis and it is more likely that a number of bacteria could be involved.

The aims of this work were:

1. to re-assess the association of non-secretion of ABO blood group substances in a cohort of patients with spondyloarthropathy;
2. to correlate the disease activity as assessed clinically, of non-secretors and secretors with the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) as laboratory parameters of inflammation.
3. to study the faecal flora in a cohort of patients with spondyloarthropathy, with particular reference to known arthritogenic bacteria and to compare it with that of healthy controls;
4. to determine the prevalence of bacteria expressing molecules with structural similarities to the HLA-B27 antigen;
5. to examine the humoral responses of patients and controls to their own faecal flora.
2.1 INTRODUCTION

Non-secretion of ABO blood group substances has been identified as a susceptibility factor for infection of mucosal surfaces (Blackwell, 1989). One hypothesis to explain this is that non-secretors are more easily colonised by some pathogens. Microbial adhesins preferentially bind to carbohydrates rather than to peptides (Lark, 1986). ABO and Lewis antigens are well-characterised carbohydrates and it was suggested that the different distribution of Lewis antigens between secretors and non-secretors might be involved in susceptibility to colonisation. The H type I structure in the body fluids of secretors might bind to adhesins on the surfaces of microorganisms that bind to H antigen on epithelial cells and interfere with their attachment; colonisation and consequently infection would be reduced. As Le\(^a\) antigen found on the cells of non-secretors appears to be a receptor for some strains of *Candida albicans* (May et al., 1989) and *Staphylococcus aureus* (Saadi et al., in press), it might also be one of the receptors for some arthritogenic bacteria. The report by Shinebaum et al. (1987b) of an association between non-secretion and AS provided circumstantial evidence in support of the hypothesis that there is an infective aetiology in AS.

One possible explanation for the increased susceptibility of non-secretors to AS could be that these individuals are colonised more readily by arthritogenic bacteria. A patient and a control population were therefore recruited to investigate the prevalence of potential pathogens with reference to secretor status. If a difference was found, then the role (in bacterial attachment) of those blood group antigens which vary between secretors and non-secretors would be investigated. Since a large patient and control population was available for investigation, the prevalence of secretors and non-secretors in those groups was re-examined. The secretor status was determined by the standard haemagglutination inhibition assay (HAI) and an enzyme-linked immunosorbent assay (ELISA) for Lewis antigens.
A study of 86 girls with urinary tract infections reported elevated inflammatory responses (temperature, ESR, urinary leucocyte count and especially CRP) in non-secretors compared with secretors. It was postulated that an over-reactive acute phase response could predispose to the later development of renal scarring (Lomberg et al., 1989). Accordingly, CRP and ESR were measured in all patients with spondyloarthropathy and correlated with disease activity.

Disease activity in AS is well-recognised to be difficult to assess (Scott et al., 1981; Anonymous, 1987; Zukovskis et al., 1991). The difficulties have arisen partly because of the heterogeneity of the disease (Calin, 1989). Some individuals have disease confined to the sacroiliac joints, while others have extra-pelvic spinal involvement with or without peripheral joint involvement or systemic complications. In general, disease activity is easier to define in those with extra-articular features than those with predominantly spinal disease. Back pain in AS patients might relate to acute inflammation or be of a mechanical nature due to damage incurred as a result of repeated bouts of inflammation.

The methods used to determine disease activity have included clinical and laboratory criteria either singly or in combination. In some studies disease activity assessment has been based solely on clinical features such as duration of morning stiffness and pain, the use of non-steroidal anti-inflammatory drugs (NSAID), the presence of uveitis or synovitis or worsening back pain (Ebringer et al., 1977; Ebringer et al., 1978; Cowling et al., 1980a; Warren and Brewerton, 1980; Hunter et al., 1981; Trull et al., 1983; Nashel et al., 1986; Pease et al., 1987). Visual analogue scales have been employed for pain measurement.

Some studies (notably by Ebringer's group) have used solely laboratory criteria. Patients were divided on the basis of an elevated ESR and total IgA (Ebringer and Ghuloom, 1986; Ghuloom and Ebringer, 1987; Shodjai-Moradi et al., 1992); on an elevated ESR and CRP (Khalafpour et al., 1988); or, on a raised CRP (Trull et al., 1984). Maki-Ikola et al. (1991) also graded patients on the basis of both an elevated ESR and CRP.

Other workers have used a combination of clinical and laboratory assessments; clinical parameters with an elevated ESR (Cooper et al., 1988), or clinical criteria with a raised CRP (van Bohemen et al., 1986).
Radioisotope scanning has been used as an aid to diagnosis in early sacroiliitis but not in the routine assessment of disease activity (Spencer et al., 1979).

In an attempt to measure disease activity at the enthesis which is the site of pathological damage in AS, an enthesis index (EI) was devised in Newcastle by Mander and colleagues (1987). This is a 3 min evaluation of the degree of tenderness on over 70 sites of tendon or ligament insertion. The EI was recorded by one observer on 18 AS patients and it correlated with the severity of pain and stiffness (as determined by visual analogue scales) but not with the ESR. The EI was then measured on 18 AS patients by 3 observers on 3 visits in a single 'observer blind' crossover study in which patients were on/off NSAID therapy and vice versa. The EI scores improved with NSAID treatment. Unfortunately, no controls were measured in this study for validation of the EI, nor was there, as pointed out previously (Anonymous, 1987), a placebo group included in the NSAID crossover part of the assessment; therefore, the observed fall in EI with treatment might simply reflect expectation. It is also possible that patients with other inflammatory joint disorders would score highly even though they have synovial rather than enthesopathic disease. As with most subjective methods of assessment of inflammatory joint disease such as the Ritchie index (Ritchie et al., 1968), interobserver variation is inevitable and it is difficult to standardise the amount of pressure applied. Mander and colleagues found interobserver variation in the EI only on the first visit.

A subsequent longitudinal study over 1 year by the Newcastle group showed that each AS patient's profile of disease activity was practically unique (Goodacre et al., 1991). Dawes et al. (1987) independently developed a similar EI assessed on 52 AS patients and also found a positive correlation between their index and pain and severity of morning stiffness but not with ESR, CRP or measurements of spinal movement.

None of these assessments of disease activity takes into account functional capabilities. This has been addressed by means of a self-administered questionnaire which is reproducible and which correlates with radiological and metrological evaluation (Nemeth et al., 1987).

For the purpose of this present study, clinical disease activity was based on clinical parameters, and functional assessments were not formally addressed.
The aims of this part of the study were:

1. to re-assess the association of non-secretion of ABO blood group substances in a large cohort of patients with spondyloarthropathy. An ELISA for Lewis antigens was used as a control for secretor status which was determined by the standard haemagglutination inhibition assay;

2. to compare secretors and non-secretors of ABO blood group substances with regard to clinical disease activity and with reference to ESR and CRP as laboratory indicators of inflammation;

3. to correlate disease activity with ESR and CRP irrespective of secretor status;

4. to correlate HLA-B27 antigen status in AS patients with disease activity, ESR and CRP.
2.2 MATERIALS AND METHODS

2.2.1 Subjects

The clinical and laboratory information for all subjects was coded for confidentiality and stored in a Database 3 plus database.

2.2.1.1 Characteristics of controls

Controls (103) were healthy university staff and students and hospital nursing, medical, administrative, secretarial and portering staff. The age and sex of the controls are shown in Table 2.1. The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were not measured in controls.

<table>
<thead>
<tr>
<th>Table 2.1</th>
<th>Characteristics of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Number</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
</tr>
<tr>
<td>Female (F)</td>
<td>54</td>
</tr>
<tr>
<td>Male (M)</td>
<td>49</td>
</tr>
<tr>
<td>Ratio M:F</td>
<td>0.9:1</td>
</tr>
</tbody>
</table>

2.2.1.2 Characteristics of patients

Patients with spondyloarthropathies who were currently attending or who had attended rheumatology outpatient clinics within the past 5 years were invited by letter to take part in this study. Of patients on the Rheumatic Disease Unit AS register compiled in 1987, 43 agreed to take part; the remainder either refused, had moved address, failed to respond to a second invitation or were deceased. A further 69 patients who had been referred to the Unit since 1987 were recruited. The patients were classified into four categories: ankylosing spondylitis (n = 92) all of whom satisfied the New York criteria (Bennett and Burch, 1967) and the modified criteria (van der Linden et al., 1984a); spondylitis associated with inflammatory bowel disease (IBD) (n = 6); post-infective reactive spondyloarthopathy (ReA) (n = 8); and psoriatic spondyloarthropathy (PSA) (n = 6). The age and sex of the patients are shown in Table 2.2.
Patients were seen on 2 occasions. At the initial visit (visit 1) a full history and examination were undertaken, clinical disease activity was assessed and a sample of saliva was obtained for secretor status analysis. Blood samples were taken for ESR, CRP, ABO blood group testing and serum was stored for antibody determination. Patients were assessed 6-8 months later (visit 2) as part of a longitudinal study to note any changes in clinical disease activity and to see if these changes related to changes in ESR, CRP or immunoglobulin levels (Chapter 5).

2.2.2 HLA-B27 antigen status

Several of the patients had previously been typed for the HLA-B27 antigen. The HLA-B27 status was determined in a number of the others by the standard microlymphocytotoxicity assay of Terasaki and Park (1976) by the Scottish National Blood Transfusion Service at the Royal Infirmary, Edinburgh. The analysis was incomplete in 20 patients due to technical factors.

2.2.3 Assessment of clinical disease activity

Patients were assessed for the duration of early morning stiffness (EMS), their requirement for a NSAID, the presence of worsening back pain over the previous 2-3 weeks and the presence of iritis or synovitis. Using a classification of disease activity similar to that used in previous studies (Ebringer et al., 1977;
Warren and Brewerton, 1980; Cowling et al., 1980a; Kuberski et al., 1983) patients were classified into one of three categories:

a) Inactive disease - little or no back pain or EMS (< 30min), and requiring no or infrequent use of a NSAID;

b) Probably active - significant EMS (30-60min) and requiring a regular NSAID for control of symptoms;

c) Active disease - prolonged EMS (> 60min), or worsening back pain over the previous 2-3 weeks, or the presence of iritis, synovitis or significant pain on sacroiliac compression.

2.2.4 Determination of ABO blood group

ABO blood groups were determined by a slide agglutination technique with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). To one volume of antiserum on a slide an equal volume of 10% red blood cell suspension from a test sample was added. Appropriate controls of known ABO blood groups were included. After rocking the slide gently to mix well, the slide was examined within 2-5min for agglutination.

2.2.5 Determination of secretor status

Secretor status was determined on saliva samples by HAI and confirmed by an ELISA for Lewis^a and Lewis^b antigens on a sample-blind basis. Analysis was complete in 109 patients; the specimens from 2 patients with AS and 1 with ReA were insufficient for testing.

2.2.5.1 Processing of saliva samples

Fresh unstimulated saliva specimens (5-10ml) were collected and kept at 4°C prior to centrifugation for 15min at 1000g. Supernatants were transferred to glass universal containers and placed in a boiling water bath for 25min to inactivate enzymes and bacteria. They were stored at -20°C prior to testing. The time from collection of saliva sample to storage did not exceed 5hr.
2.2.5.2 Haemagglutination inhibition assay (HAI)

All procedures were carried out at room temperature. Three dilutions (25 μl) in phosphate buffered saline (PBS) (pH 7.2) of monoclonal anti-A and anti-B antisera (Scottish National Blood Transfusion Service) (previously titred against known A and B red blood cells) and two dilutions (25 μl) in PBS (pH 7.2) of *Ulex europaeus* lectin (Sigma, Poole, Dorset) were added to separate wells in microtitre plates. Boiled saliva (25 μl) from each subject was added to wells containing the above reagents for 30 min. Standard red blood cells (25 μl) (Scottish National Blood Transfusion Service) of the appropriate group diluted in saline (1/5) were then added to the wells. The plates were allowed to stand for 2 hr and were examined for agglutination of red blood cells. The pattern of haemagglutination expected is given in Table 2.3.

<table>
<thead>
<tr>
<th>Category</th>
<th>Anti-A</th>
<th>Anti-B</th>
<th>Ulex</th>
</tr>
</thead>
<tbody>
<tr>
<td>A secretor</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B secretor</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AB secretor</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O secretor</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non-secretor (all ABO groups)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Agglutination is represented by + and non-agglutination by -.

2.2.5.3 ELISA for the detection of *Lewis*\(^a\) and *Lewis*\(^b\) antigens

**Materials:**

Coating buffer

This buffer (pH 9.6) contained 15 mM sodium carbonate, 35 mM sodium bicarbonate and 3 mM sodium azide.

Washing buffer

For washing procedures, 0.01 M PBS (pH 7.2) containing 0.1% (w/v) bovine serum albumen (BSA) and 0.05% (v/v) Tween 20 was used.
Blocking buffer

Blocking buffer (pH 7.2) is composed of 0.01M PBS containing 1% (w/v) BSA.

Phosphate citrate buffer (PCB)

PCB was composed of 0.1M sodium hydrogen phosphate and 0.1M citric acid and the pH was adjusted to pH 5.

Substrate solution

The substrate solution used to detect horseradish peroxidase (HRP) contained 40mg O-phenylenediamine (Sigma, Poole, Dorset) in 100ml of 0.1M PCB and was activated immediately prior to use by adding 40μl H₂O₂ (30%,v/v).

Method:

The wells of polystyrene plates (Dynatech, Billinghamurst, Sussex, UK) were coated overnight at 4°C with 100μl of affinity-purified monoclonal anti-Lewis\(^a\) antibody diluted in coating buffer (1/50) or affinity-purified monoclonal anti-Lewis\(^b\) antibody diluted in coating buffer (1/20) (Dr R. Fraser, Glasgow and West of Scotland Blood Transfusion Service, Carluke, Lanarkshire). All subsequent procedures were carried out at room temperature. The wells were washed 3 times with washing buffer and sites unoccupied by anti-Lewis antisera were blocked with blocking buffer (100μl) for 30min. One hundred microlitres of each test saliva sample diluted in blocking buffer (1/100 for Lewis\(^a\); 1/20 for Lewis\(^b\)) were added to the appropriate wells. Dilutions of saliva from known secretors and non-secretors were used in each plate as controls. The plates were incubated for 2hr, the saliva removed and the wells washed 3 times with washing buffer. Goat polyclonal anti-Lewis\(^a\) antibody (100μl) diluted in blocking buffer (1/500) or goat polyclonal anti-Lewis\(^b\) antibody (100μl) diluted in blocking buffer (1/250) (Behring Diagnostics, Hounslow, Middlesex) was added for 30min. After washing 3 times with washing buffer, 100μl of HRP-conjugated polyclonal donkey anti-sheep/goat IgG (Scottish Antibody Production Unit, Carluke, Lanarkshire) diluted 1/250 in blocking buffer was added to the appropriate wells. The plates were incubated for 2hr and washed 3 times. Activated substrate (100μl) was added to each well. The orange/yellow colour reaction was allowed to develop in the dark for 10-15min and stopped by adding 50μl sulphuric acid (12.5%, w/v) to each well. The absorbance at 490nm was measured with a Dynatech MR700 plate reader.
2.2.6 The effect of storage conditions on the HAI assay

An experiment was set up to determine whether the results of the HAI assay would be affected by (i) boiling saliva samples at different times after collection e.g., within a few hours of collection or 24 hours later, and (ii) subjecting the samples to different temperatures. Saliva (10ml) from known secretors was collected over 1-2 hr and centrifuged at 1000g for 15min. Each supernatant was divided into 2 equal volumes of 4-5ml. One volume was boiled at 100°C for 20min; half was stored at 4°C overnight and the other half frozen at -20°C. The unboiled 4-5ml was divided into 3 equal volumes, each stored overnight at a different temperature: 4°C, 22°C and 37°C. The HAI assay was performed the day after processing specimens.

2.2.7 Determination of ESR and CRP

ESR and CRP values were determined by clinical laboratories from venous blood (4ml) added to a tube containing EDTA for ESR measurement (Westergren) and clotted blood (4ml) for serum CRP measurement (fluorescence polarisation immunoassay using TDx System, Abbott Laboratories).

2.2.8 Statistical methods

Patient groups were compared for prevalence of non-secretors by chi-squared tests with Yates' correction. For 2 x 2 tables, associations between disease activity, ESR and CRP were tested by Spearman rank correlations. Correlations between ESR and CRP were determined by the Kendall correlation coefficient. Differences between secretors and non-secretors with regard to disease activity, ESR and CRP were determined by Mann-Whitney tests. Comparison of the HLA-B27 antigen prevalence between disease categories was analysed by the Kruskel-Wallis test. Correlations between the HLA-B27 antigen status and ESR, CRP and disease activity were done by Wilcoxon rank sum test.
2.3 RESULTS

2.3.1 ABO blood group distribution

The ABO blood groups of the patients and controls are shown in Table 2.4. There was no significant difference in the distribution of ABO blood groups between the total patient group and controls ($X^2 = 2.61$ with 3 DF) or between AS patients and controls ($X^2 = 2.08$ with 3DF). The number of patients in each subgroup of AS (in association with other disease) was too small to make separate comparisons with controls.

Table 2.4
Distribution of ABO blood groups in patients and controls

<table>
<thead>
<tr>
<th>Subject</th>
<th>O</th>
<th>A</th>
<th>B</th>
<th>AB</th>
<th>NK*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>42</td>
<td>26</td>
<td>17</td>
<td>2</td>
<td>16</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>(48.3)**</td>
<td>(29.9)</td>
<td>(19.5)</td>
<td>(2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>38</td>
<td>11</td>
<td>3</td>
<td>7***</td>
<td>112</td>
</tr>
<tr>
<td>AS</td>
<td>45</td>
<td>29</td>
<td>10</td>
<td>2</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>IBD</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>ReA</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>PSA</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

* Not known
** Figures in parentheses represent the percentage of the 'known' total.
*** In the controls ABO blood group was determined by HAI of boiled saliva and confirmed by slide agglutination of red blood cells. For 16 individuals (all non-secretors by HAI and ELISA) blood samples were not obtained.
**** Of the 7 patients whose ABO blood group was unknown, 5 were non-secretors by HAI and ELISA but a blood sample had not been obtained for ABO blood group testing. In the other 2 patients, the secretor status in addition to the ABO blood group was not known (the saliva sample was insufficient for analysis and a blood sample was not collected for ABO testing).

2.3.2 Secretor status

The secretor status of patients and controls was determined by HAI and confirmed by ELISA. There were 4 Lewis negative individuals (1 AS patient and 3 controls) who were secretors by HAI. There were no discrepancies between the two methods for the other 208 specimens tested. There was no significant
difference in the proportion of non-secretors in either the total patient population, or in any of the disease subgroups, compared with that of the control population (Table 2.5). 5 of the 80 patients who were secretors and 8 of the 72 controls who were secretors had small amounts of Lewis\textsuperscript{a} in addition to Lewis\textsuperscript{b} in the ELISA.

### Table 2.5

<table>
<thead>
<tr>
<th>Subject</th>
<th>No. tested</th>
<th>No. non-secretors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>103</td>
<td>29 (28)</td>
</tr>
<tr>
<td>Total patient group</td>
<td>109</td>
<td>29 (26.6)</td>
</tr>
<tr>
<td>AS</td>
<td>90*</td>
<td>25 (27.7)</td>
</tr>
<tr>
<td>IBD</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ReA</td>
<td>7*</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>PSA</td>
<td>6</td>
<td>2 (33.3)</td>
</tr>
</tbody>
</table>

* 2 patients with AS and 1 with ReA were unable to provide a satisfactory saliva sample for analysis of secretor status.

### 2.3.3 The effect of different storage conditions on the HAI

The aliquots of the samples which had been boiled and frozen were taken as the reference, as all secretor status typing in the present study was done on samples processed in this way. These aliquots were confirmed to be from secretors on HAI. Storing the samples overnight at 4°C after boiling, or storing the unboiled fractions at 4°C or 22°C did not alter the HAI result. 5 of the 13 aliquots incubated overnight at 37°C, however, were interpreted as non-secretors in the HAI assay.

### 2.3.4 Prevalence of the HLA-B27 antigen

The distribution of the HLA-B27 antigen in the patient population is shown in Table 2.6. There was no significant difference in the HLA-B27 antigen prevalence between any of the disease categories (P = 0.19).
Table 2.6
Prevalence of HLA-B27 antigen in the patient population

<table>
<thead>
<tr>
<th>HLA-B27</th>
<th>AS No.(%)</th>
<th>IBD No.(%)</th>
<th>ReA No.(%)</th>
<th>PSA No.(%)</th>
<th>Total No.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>61 (85)*</td>
<td>5 (83)</td>
<td>8 (100)</td>
<td>5 (83)</td>
<td>79 (86)</td>
</tr>
<tr>
<td>Negative</td>
<td>11 (15)</td>
<td>1 (17)</td>
<td>0</td>
<td>1 (17)</td>
<td>13 (14)</td>
</tr>
<tr>
<td>Not known</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>112</td>
</tr>
</tbody>
</table>

* The figures in parentheses represent percentages within each disease category of the 'known' status.

2.3.5 Comparison of ESR and CRP in different disease categories

The ESR and serum CRP levels of patients were determined at the first visit and at a second visit 6-7 months later. At the first visit, comparison of the mean ESR and mean CRP in patients with primary AS or secondary AS showed that there was no significant difference in ESR or CRP between any of the disease categories (Table 2.7). Similar results were found at visit 2 for ESR and CRP and no overall change in values was found between both visits.

Table 2.7
Mean ESR and CRP for each disease category

<table>
<thead>
<tr>
<th>Visit</th>
<th>Mean value</th>
<th>AS</th>
<th>IBD</th>
<th>ReA</th>
<th>PSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ESR</td>
<td>25.9*</td>
<td>23.7</td>
<td>25.6</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>ESR</td>
<td>23.2</td>
<td>26</td>
<td>14.7</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>CRP</td>
<td>2.33</td>
<td>2.05</td>
<td>2.55</td>
<td>1.82</td>
</tr>
<tr>
<td>2</td>
<td>CRP</td>
<td>2.16</td>
<td>1.67</td>
<td>1.93</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* 4 blood samples were clotted and therefore unsuitable for ESR analysis.

2.3.6 Correlation of disease activity with ESR and CRP

There was a significant correlation between disease activity and ESR for both the total patient group ($P < 0.001$, $r = 0.47$) (Table 2.8) and the group with only AS ($P < 0.001$, $r = 0.48$) (Table 2.9), and there was also a significant correlation between
disease activity and CRP for both the total patient group ($P < 0.001$, $r = 0.36$) (Table 2.8) and the group with only AS ($P < 0.01$, $r = 0.28$) (Table 2.9). When this was further analysed, the difference between the inactive and the probably active AS patients is significant for ESR ($P < 0.001$) but not for CRP. The ESR and CRP correlated significantly with each other ($P < 0.001$, $r = 0.51$) in the total group and the AS patients. The two parameters correlated significantly with each other within all three levels of disease activity: inactive ($P < 0.001$, $r = 0.62$); probably active ($P < 0.01$, $r = 0.45$); and active ($P < 0.01$, $r = 0.75$) categories.

Table 2.8
Disease activity, ESR and CRP in total patient group (visit 1)

<table>
<thead>
<tr>
<th>Disease activity</th>
<th>No.</th>
<th>Mean ESR (95%CI)*</th>
<th>No.</th>
<th>Mean CRP (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
<td>108**</td>
<td></td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>Inactive</td>
<td>49</td>
<td>17.3 (12.3-22.3)</td>
<td>51</td>
<td>1.8 (1.4-2.2)</td>
</tr>
<tr>
<td>Probably active</td>
<td>46</td>
<td>26.0 (20.8-31.2)</td>
<td>48</td>
<td>1.9 (1.5-2.3)</td>
</tr>
<tr>
<td>Active</td>
<td>13</td>
<td>54.5 (37.3-71.7)</td>
<td>13</td>
<td>5.9 (2.5-9.3)</td>
</tr>
</tbody>
</table>

* CI, confidence interval  
** 4 blood samples clotted

Table 2.9
Disease activity, ESR and CRP in AS patients (visit 1)

<table>
<thead>
<tr>
<th>Disease activity</th>
<th>No.</th>
<th>Mean ESR (95%CI)*</th>
<th>No.</th>
<th>Mean CRP (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
<td>88**</td>
<td>26.0 (21.2-30.8)</td>
<td>92</td>
<td>2.3 (1.7-2.9)</td>
</tr>
<tr>
<td>Inactive</td>
<td>43</td>
<td>18.4 (12.8-24)</td>
<td>45</td>
<td>1.8 (1.4-2.2)</td>
</tr>
<tr>
<td>Probably active</td>
<td>36</td>
<td>27.8 (21.4-34.2)</td>
<td>38</td>
<td>1.9 (1.5-2.3)</td>
</tr>
<tr>
<td>Active</td>
<td>9</td>
<td>54.7 (33.9-75.5)</td>
<td>9</td>
<td>6.5 (1.7-11.3)</td>
</tr>
</tbody>
</table>

* CI, confidence interval  
** 4 blood samples clotted

The disease activity for each AS patient at visit 1 was compared with that at visit 2 (Table 2.10). 53 patients remained within the same disease activity classification: 26 inactive; 25 probably active; 2 active. There were 13 patients who improved: 8 probably active and 2 active at the first visit who all became
inactive at the second; and 3 active at the first visit who were probably active at the second. 6 patients deteriorated from being inactive at the first visit to probably active at the second.

Table 2.10
Disease activity in AS patients in visits 1 and 2

<table>
<thead>
<tr>
<th>VISIT 1</th>
<th>Inactive</th>
<th>Probably active</th>
<th>Active</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive</td>
<td>26</td>
<td>6</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>VISIT 1</td>
<td>8</td>
<td>25</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Active</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>34</td>
<td>2</td>
<td>72</td>
</tr>
</tbody>
</table>

The changes in ESR correlated with changes in disease activity between both visits \( (P = 0.002, r = 0.29) \), and changes in CRP correlated with changes in disease activity \( (P = 0.017, r = 0.24) \). The change in ESR correlated with the change in CRP \( (P = 0.002, r = 0.27) \).

Significant correlations between both changes in ESR and changes in CRP with changes in disease activity were also found with the total group of spondyloarthropathies.

Correlations of disease activity, ESR and CRP were examined in patients < 40 and ≥ 40 years of age as it had previously been suggested that younger patients who have had the disease for a shorter duration were more likely to have active disease than older patients in whom the disease is likely to have 'burnt out' (Ebringer et al., 1977). Correlations of disease activity, ESR and CRP with disease duration was examined independently. Equal numbers of AS patients were < 40 years and ≥ 40 years of age and equal numbers of patients had the disease for < 10 years and ≥ 10 years (Table 2.11). As expected, a greater proportion of AS patients < 40 years old had had the disease for < 10 years compared with those ≥ 40 years old. Conversely, a higher proportion of those ≥ 40 years old had had the disease for ≥ 10 years than those < 40 years old.
There was a significant correlation between disease activity and ESR regardless of the patients' age and regardless of the duration of disease (Table 2.12). A significant correlation between disease activity and CRP was present in those ≥ 40 years old (but not in those < 40 years old) and in those with disease duration ≥ 10 years (Table 2.13).

Table 2.12
Correlations of disease activity and ESR with respect to age and disease duration in AS

<table>
<thead>
<tr>
<th>Age/disease duration (years)</th>
<th>No.*</th>
<th>Mean ESR</th>
<th>P</th>
<th>r**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 40</td>
<td>43</td>
<td>29.2</td>
<td>&lt;0.001</td>
<td>0.48</td>
</tr>
<tr>
<td>Age ≥ 40</td>
<td>45</td>
<td>23.0</td>
<td>&lt;0.01</td>
<td>0.39</td>
</tr>
<tr>
<td>Disease duration &lt; 10</td>
<td>44</td>
<td>25.4</td>
<td>&lt;0.05</td>
<td>0.36</td>
</tr>
<tr>
<td>Disease duration ≥ 10</td>
<td>44</td>
<td>26.6</td>
<td>&lt;0.001</td>
<td>0.49</td>
</tr>
</tbody>
</table>

* Although total no. of AS patients was 92, 4 blood samples for ESR were clotted.
** Correlation coefficient
Table 2.13
Correlations of disease activity and CRP with respect to age and disease duration in AS

<table>
<thead>
<tr>
<th>Age/disease duration (years)</th>
<th>No.</th>
<th>Mean CRP</th>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 40</td>
<td>45</td>
<td>2.24</td>
<td>NS*</td>
<td>0.20</td>
</tr>
<tr>
<td>Age ≥ 40</td>
<td>47</td>
<td>2.41</td>
<td>&lt;0.01</td>
<td>0.46</td>
</tr>
<tr>
<td>Disease duration &lt; 10</td>
<td>46</td>
<td>1.79</td>
<td>NS</td>
<td>0.28</td>
</tr>
<tr>
<td>Disease duration &gt; 10</td>
<td>46</td>
<td>2.8</td>
<td>&lt;0.05</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Not significant

2.3.7 Correlation of secretor status with ESR, CRP and clinical disease activity

Although the proportion of non-secretors in the patient group did not differ from that in the controls, the results were analysed to determine whether non-secretors had more active disease than secretors as assessed clinically or as reflected in the ESR and CRP. For both the total patient group and AS patients alone, no significant difference was found between non-secretors and secretors with regard to disease activity, ESR, or CRP (P < 0.3 for each variable). (Tables 2.14 and 2.15 for results pertaining to the total patient group).

Table 2.14
Secretor status and disease activity in total patient group

<table>
<thead>
<tr>
<th>Secretor status</th>
<th>Inactive</th>
<th>Probably active</th>
<th>Active</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.(%)</td>
<td>No.(%)</td>
<td>No.(%)</td>
<td>No.(%)</td>
</tr>
<tr>
<td>Secretors</td>
<td>39 (49)*</td>
<td>35 (44)</td>
<td>6 (7)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Non-secretors</td>
<td>13 (45)</td>
<td>10 (34)</td>
<td>6 (20)</td>
<td>29 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>45</td>
<td>12</td>
<td>109</td>
</tr>
</tbody>
</table>
Table 2.15
Secretor status and ESR and CRP in total patient group

<table>
<thead>
<tr>
<th>Secretor status</th>
<th>Number</th>
<th>Mean CRP</th>
<th>Mean ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretors</td>
<td>80</td>
<td>2.04 (1.63)*</td>
<td>24.2 (22.7)**</td>
</tr>
<tr>
<td>Non-secretors</td>
<td>29</td>
<td>3.08 (4.62)</td>
<td>28.1 (23.4)</td>
</tr>
<tr>
<td>Total no.</td>
<td>109</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses represent the standard deviation from the mean
** 4 samples from secretors were clotted

2.3.8 Correlation of HLA-B27 antigen status and ESR, CRP and clinical disease activity in AS patients

There was no correlation between the HLA-B27 antigen status and the ESR or CRP; neither did patients who were HLA-B27-positive have more clinically active disease than those who were HLA-B27-negative.
2.4 DISCUSSION

2.4.1 Secretor status

There are equivalent numbers of individuals in the control and patient populations. There is a male preponderance in the patient population, whereas the sex ratio is equal in controls. The mean age is higher in patients than in controls with the exception of the few males with ReA. There is no association between adult age or sex and either ABO blood group or secretor status (Mourant et al., 1978), therefore valid comparisons can be made between the two groups. The distribution of ABO blood groups was similar for patients and controls and agrees with the findings of Shinebaum et al. (1987b). A normal distribution of MN, Rhesus and I blood groups in AS patients has been reported in Norway, but this study did not examine for ABO blood group antigens (Kornstad et al., 1968).

The results of the present study show that there is no increase in the proportion of non-secretors of ABO blood group substances in this population of patients with ankylosing spondylitis. This agrees with the results with the results of Moller (1990) in Norway. The secretor status of 104 HLA-B27-positive first degree relatives of AS patients was determined by Lewis red blood cell agglutination. Of the relatives who also had AS, 4/18 (22%) were non-secretors (Lewis\textsuperscript{a} positive) compared with 19/86 (22%) of the relatives without AS (Moller, 1990).

The current findings are, however, in contrast to the earlier UK report in which 49% of AS patients were non-secretors in comparison to 27% of controls (Shinebaum et al., 1987b). The results of both studies are tabulated for comparison (Table 2.16). The main differences between the 2 studies are in the types of control population, the method of collecting the saliva sample and the techniques used to determine secretor status.
## Table 2.16
Results of two studies on secretor status in spondyloarthropathies

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. tested</th>
<th>No. (%) non-secretors</th>
<th>No. tested</th>
<th>No. (%) non-secretors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>334</td>
<td>84 (27)</td>
<td>103</td>
<td>29 (28)</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>114</td>
<td>54 (47)</td>
<td>109</td>
<td>29 (27)</td>
</tr>
<tr>
<td>sacroiliitis</td>
<td>97</td>
<td>46 (47)</td>
<td>109</td>
<td>29 (27)</td>
</tr>
<tr>
<td>AS</td>
<td>84</td>
<td>41 (49)</td>
<td>90</td>
<td>25 (28)</td>
</tr>
<tr>
<td>IBD</td>
<td>3</td>
<td>1 (3)</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ReA</td>
<td>20</td>
<td>9 (45)</td>
<td>7</td>
<td>2 (29)</td>
</tr>
<tr>
<td>PSA</td>
<td>7</td>
<td>3 (43)</td>
<td>6</td>
<td>2 (33)</td>
</tr>
</tbody>
</table>

The number of controls (historical) in the original study was over 3 times the number of patients in that study and also over 3 times the number of controls in the current work. The proportion of non-secretors in controls, however, is virtually the same in both surveys. With the exception of the ReA subgroup, the numbers of patients in each disease category are similar. There is a significant difference in the numbers of non-secretors in the total patient groups between the two studies ($X^2 = 9.41, P < 0.01$). This is also found comparing the AS patients alone ($X^2 = 9.08, P < 0.01$).

The reason for this discrepancy was explored. Of 112 patients in the present study, 43 had been included in the previous investigation and could be considered as an 'overlap' group; 22 of these 43 patients had previously been typed as non-secretors and 21 as secretors. On re-testing the secretor status of these patients in the present study, 6 of the 22 patients typed originally as non-secretors were revealed to be secretors. All 21 patients originally typed as secretors were confirmed as such in the present analysis. The secretor status in patients in the 'overlap' group as determined in both studies is shown in Table 2.17.
Table 2.17
Secretor status of patients in overlap group as assigned in the present study and original study (in parentheses)

<table>
<thead>
<tr>
<th>Disease Category</th>
<th>Secretor status</th>
<th>AS</th>
<th>IBD</th>
<th>ReA</th>
<th>PSA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-secretor</td>
<td>13</td>
<td>(16)</td>
<td>1</td>
<td>(2)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Secretor</td>
<td>19</td>
<td>(16)</td>
<td>3</td>
<td>(2)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>32</td>
<td>(32)</td>
<td>3</td>
<td>(3)</td>
<td>4</td>
</tr>
</tbody>
</table>

In no instance was a non-secretor wrongly typed as a secretor in the earlier study. This indicates that 6/22 (27%) of non-secretors in the sample of 43 patients from the first study were really secretors. If this error rate is extrapolated to the remainder of the patient group, it suggests that the true proportion of non-secretors in all patients with spondyloarthropathy studied was 35%. There is no way, however, of determining the correct value for the original patient group. The mistyping suggests that in the earlier study blood group antigen activity was lost in a proportion of saliva specimens prior to testing for secretor status.

The control samples in Shinebaum's study were historical controls and therefore not subjected to the same conditions as the patients' samples. One can speculate that if they had been treated in the same way, some of the control specimens would also have been mistyped and perhaps no significant difference would have been found between the two groups. In that event, an unusually high proportion of non-secretors in controls would have alerted the investigators and resulted in checking of samples.

Review of the procedures used in sample collection in Shinebaum's study (Dr R Shinebaum, personal communication) suggests that enzymatic degradation of blood group antigens in some saliva specimens might have resulted from inadequate cooling prior to processing and storage. In that survey, saliva samples were collected by several clinicians at the hospital and were not always refrigerated. They were then sent across the city to the University. If the samples arrived 'out of hours' they were left overnight unrefrigerated. The
transit time, in addition to delays at the clinic and the University, might have been sufficient for enzymatic degradation of the carbohydrate blood group antigens in the saliva; therefore, a secretor would be typed as a non-secretor. In the present survey samples were collected by only one person and refrigerated promptly prior to boiling thereby minimising such enzyme activity.

The work of Raza et al. (1991) showed that Lewis antigens were not detected in a significant number (192/584; 33%) of upper respiratory tract secretions which had been subject to transport delays. In the current work, a proportion (5/13) of secretor saliva samples incubated overnight at 37°C, but not at 4°C or 22°C, lost detectable blood group antigen activity (as measured by HAI). This emphasises that for reliable determination of secretor status from saliva, rapid processing of specimens or inhibition of degradative enzymes prior to storage and testing is essential. It also emphasises the importance of collecting samples from controls and patients under the same conditions and in the same time period and not relying on historical controls for comparison with new data.

In the original paper (Shinebaum et al., 1987b) secretor status was determined by HAI alone; the ELISA method for Lewis antigens had not been devised. In contrast, in the current work saliva specimens were tested by HAI, confirmed by Lewis antigen ELISA and repeated by both methods in a sample-blind manner. 4/212 (1.9%) of subjects were Lewis negative by ELISA, therefore the secretor status could not be determined by this method in these subjects. 100% concordance was found between the HAI and ELISA in the other 208 saliva specimens. This compares with 97% concordance for both techniques performed on 1089 saliva samples (Raza et al., 1991). These workers reported that most of the mismatched pairs (27/31) were 'false secretors', of Le^a phenotype but secretors by HAI. The other 4/31 of their mismatched pairs were 'false non-secretors', of Le^b phenotype by ELISA, but non-secretors by HAI (Raza et al., 1991).

The finding of 'false secretors' in the HAI has been suggested to be due to blood contamination of saliva from poor oral hygiene (Blackwell et al., 1989b; Raza et al., 1991). This hypothesis has been tested formally by heavily contaminating known non-secretor saliva specimens with blood; non-secretors did not 'become' secretors (Dr J Stewart, personal communication). The presence of 'false non-secretors' in the HAI assay has been attributed to the dilution of small samples (Raza et al., 1991).
The HAI assay is quicker to perform than the ELISA and is reliable. Experience is needed to determine the presence of agglutination in the microtitre wells. An advantage is that in secretors the ABO blood group is also determined, whereas the same is not true for non-secretors. The ELISA for Lewis antigens determines the secretor status by Lewis phenotype unless the patient is Lewis negative (normally approximately 4% of the north European population (Mourant et al., 1978)). The Lewis antigen ELISA, of course, does not identify the ABO blood group. Less than 2% of patients and controls were Lewis negative in the present study.

In view of the absence of association of non-secretion of ABO blood group antigens with AS, there was no longer any indication to investigate the binding of arthritogenic bacteria to blood group antigens. These negative results do not provide evidence to support an infective aetiology in AS.

2.4.2 ESR and CRP

In the current study laboratory measurements such as ESR, CRP and serum IgA were not included in the assessment of disease activity, although other groups, in particular that at the Middlesex Hospital, have used levels of acute phase reactants with (Ghuloom and Ebringer, 1987; Khalafpour et al., 1988; Shodjai-Moradi et al., 1992) or without (Trull et al., 1984; van Bohemen et al., 1986) serum IgA as sole criteria for assessment of disease activity. In this study clinical parameters alone were used to assess disease activity and a high ESR did not preclude a classification of inactive disease.

There has been no uniform consensus regarding the value of ESR and CRP as markers of clinically assessed disease activity in AS. This present report of a positive correlation between both ESR and CRP with clinical disease activity agrees with some authors (Cowling et al., 1980a) but disagrees with others (Laurent and Panayi, 1983; Sheehan et al., 1986) who have all used essentially the same criteria to classify patients as in the present study. In other surveys, no correlation between ESR and clinically-assessed disease activity was found (Kendall et al., 1973; Hunter et al., 1981). Kendall et al. (1973) employed only a pain scale and Hunter et al. (1981) used a pain scale with EMS duration and NSAID/analgesic requirement, to determine disease activity. In the latter study, numbers were small (17), with only 6 patients having active disease. CRP, but
not ESR, correlated strongly with disease activity in an American survey in which a median CRP value of 1.8 mg/ml (measured by rate nephelometry) was found in the active group and a median value of 0.65 mg/ml in the inactive group (Nashel et al., 1986). This compares with a mean value of 6.5 mg/ml for AS patients with active disease and 1.8 mg/ml for those with inactive disease in the present report. The different results between separate groups can partly be explained by differences in criteria used to assess disease activity. Technical factors such as different methods of CRP determination could contribute to the disparity. CRP has, however, shown a significant correlation with clinical disease activity in patients with juvenile spondyloarthropathies (Hussein et al., 1987).

The significant correlation of ESR with disease activity was present irrespective of the patients' age or duration of disease. The positive correlation of CRP and disease activity was found only in those ≥ 40 years of age and only in those with disease duration ≥ 10 years.

It has been suggested that there is no correlation between either CRP levels or ESR and AS activity except in those individuals with peripheral arthritis (Scott et al., 1981; Laurent and Panayi, 1983). In the present work, of the AS patients with clinically active disease, similar numbers of those with peripheral synovitis (4) and those with only spinal symptoms (3) were found (Table 2.18). Those with peripheral synovitis had a higher mean ESR (74.5) than those with only spinal symptoms (47). The converse was true for the CRP. The patient with iritis had clinically quiescent spinal disease and no peripheral arthropathy. The patient who had marked unilateral sacroiliac joint tenderness had had no exacerbation of low back pain or peripheral arthritis. There were only 2 patients with active disease at visit 2; therefore, no conclusions can be drawn.

<p>| Table 2.18 |</p>
<table>
<thead>
<tr>
<th>AS patients with active disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
</tr>
<tr>
<td>Knee synovitis</td>
</tr>
<tr>
<td>Spinal</td>
</tr>
<tr>
<td>Iritis</td>
</tr>
<tr>
<td>Sacroiliitis*</td>
</tr>
</tbody>
</table>

* This patient had marked unilateral sacroiliac joint tenderness.
From the present results, there is no evidence in AS patients that non-secretors have a more intense acute phase response than secretors; there was no significant difference in ESR or CRP values in non-secretors compared with secretors. This is in contrast to the previous report of elevated ESR and CRP levels in non-secretors with urinary tract infections (Lomberg et al., 1989).

There was no correlation in AS patients between HLA-B27-positivity and ESR, CRP or clinical disease activity. HLA-B27-positive patients were not more likely to have active disease than HLA-B27-negative patients. This is in contrast to the report of a correlation of disease activity and CRP only in HLA-B27-positive, and not in HLA-B27-negative AS patients (Reynolds et al., 1991). The clinical criteria used to assess disease activity in the latter survey were not dissimilar to those employed in the present report, so the reason for the disparity in the results is not clear.

The main conclusions that can be drawn are:

1. there is no association between non-secretion of ABO blood group substances and ankylosing spondylitis;
2. for reliable determination of secretor status from saliva, rapid processing of specimens or inhibition of degenerative enzymes prior to storage and testing is required;
3. there is no significant difference between non-secretors and secretors with respect to ESR, CRP or clinically-assessed disease activity;
4. in patients with spondyloarthropathy, ESR and CRP both correlate with clinical disease activity;
5. HLA-B27 antigen status has no bearing on ESR, CRP or clinical disease activity.
3.1 INTRODUCTION

AS and reactive arthritis have several features in common both in clinical expression of disease (enthesitis, sacroiliitis, spondyloarthropathy, peripheral arthritis and uveitis) and in the strong association with HLA-B27. The observation that reactive arthritis often followed an enteric infection, led to speculation that AS might also be triggered by a gut infection.

Serological cross-reactivity between HLA-B27 and *K. pneumoniae* (Ebringer et al., 1976; Avakian et al., 1980; Welsh et al., 1980) prompted several groups to study the faecal flora of individuals with spondyloarthropathy. Increased prevalence of faecal *Klebsiella* in AS patients with active disease (Ebringer et al., 1977; Ebringer et al., 1978; Kuberski, 1983) was noted, but if the patients were not categorised by disease activity, the isolation of *Klebsiella* in patients was similar to that of controls. Several investigators worldwide have not confirmed this association of *Klebsiella* with AS (Eastmond et al., 1980; Warren and Brewerton, 1980; Hunter et al., 1981; Ferraz et al., 1990; McLellan, 1990; van Kregten et al., 1991).

Ebringer et al. (1978) reported that the isolation of *Klebsiella* from the stools of patients with inactive disease was associated with a flare-up of the disease 2 to 3 months later and suggested that *K. pneumoniae* may be an initiating agent in AS. The interpretation of the results of this survey are controversial but nevertheless excited considerable interest.

The disparity in results between surveys of faecal flora might be due to a combination of several factors: differences in stool collection and culture methods between studies; different types of controls used for comparison with patient groups eg., healthy individuals, hospital in-patients or out-patients; different regions within the UK; different countries. Most studies after Ebringer's original findings, concentrated on isolating *Klebsiella* and paid little attention to other enterobacteria. Only a Brazilian study (Ferraz et al., 1990) has
extensively examined the range of enterobacteria from AS patients and found essentially no difference in the type of organisms isolated compared with that of normal controls.

Although *Klebsiella* are gut commensal organisms, they have been implicated as pathogens in cases of neonatal enterocolitis (Reisner and Garty, 1977). They have also assumed increasing importance (with other Gram-negative bacilli) as causes of infection in hospital patients (Steinhauer et al., 1966) including intensive care patients (Casewell and Phillips, 1978). Such hospital-acquired *Klebsiella* infections are common; 16% of coliform infections of urine, wound, respiratory or other infection were identified as *Klebsiella* (Cooke et al., 1979). In approximately 50% of patients, *Klebsiella* of the same serotype was identified in the bowel as in the infection. Spread of *Klebsiella* to patients can arise from the hospital environment or from personnel (Gardner et al., 1969), but the gastrointestinal tract is usually assumed to be the source of these bacteria and the source of *Klebsiella* in the gut is likely to be from food.

Only patients and controls who had not taken antibiotics for the previous 3 months were included in the present study; antibiotic therapy is well-known to suppress normal faecal flora and allow the emergence of resistant or abnormal organisms. *Proteus, Pseudomonas, Klebsiella, Candida* and *Staphylococci* are the most frequently observed organisms emerging on broad-spectrum antibiotic treatment (Finegold et al., 1966).

Antibiotic therapy in patients who underwent haemodialysis followed by renal transplantation was associated with an increase in numbers of *Klebsiella* in faecal flora (Montgomerie et al., 1970). This suggested that bacteria sensitive to the antibiotics were replaced by *Klebsiella*.

The aims in the present survey were:

1. to examine in a semi-quantitative manner the faecal flora of patients with spondyloarthropathy and compare it with that of healthy controls;

2. to determine any association between a particular organism and disease activity.
3.2 MATERIALS AND METHODS

3.2.1 Culture media

All plates used for culture media were Sterilin 90mm diameter triple-vented disposable Petri dishes.

Inositol agar
This was prepared by the Department of Medical Microbiology, University of Edinburgh, and consisted of 1% inositol in a nutrient agar base (Oxoid, Basingstoke, Hampshire) and neutral red as an indicator. This agar detects inositol fermenters such as *Klebsiella* species, some bacteria belonging to the *Enterobacter*, *Serratia*, *Yersinia* and *Citrobacter* species and some members of uncommon species such as *Cedecea* and *Providentia*. Formal identification of inositol fermenters was done by Analytical Profile Index (API) 20E identification kits (API Laboratories Ltd., Basingstoke, Hampshire).

Beta glucuronidase agar (BGA)
This agar consisted of a solution 0.03% p-nitrophenyl β-D-glucuronide (Sigma, Poole, Dorset) in a proteose peptone No.2 agar base (Difco Laboratories, East Molesley, Surrey) and was used for the detection of *E. coli*.

MacConkey agar
MacConkey agar (without salt) was prepared as per manufacturer's instructions (Oxoid, Basingstoke, Hampshire) for the cultivation of enterobacteria. This agar contains a bile salt to inhibit non-intestinal bacteria, and lactose with neutral red to distinguish the lactose-fermenting coliforms from the lactose non-fermenting salmonella and dysentery groups.

Yersinia selective agar
This was prepared as per manufacturer's instructions (Oxoid, Basingstoke, Hampshire) for the isolation of *Yersinia* species.

Bacterial diluent
This consisted of 20% (v/v) nutrient broth No.2 (Oxoid, Basingstoke, Hampshire) in physiological saline (0.85%).
3.2.2 Collection of faecal sample:

Each person was provided with a sterile universal container, a sterile disposable wooden spatula, a sealable plastic bag and a padded envelope with a Biohazard label. The instructions were to sit forward on the toilet seat so that the stool specimen landed on the downward slope of the toilet bowl. Faeces (3-4 scoopfuls from the surface of the sample) was placed in the universal container, sealed in the bag and posted first-class mail by patients. Controls delivered their sample to the laboratory and were received within 3 hr of defecation.

3.2.3 Preparation of stool sample

Faeces (1g) was weighed in a sterile universal container and thoroughly mixed with 10ml of bacterial diluent using a Vortex mixer. Ten-fold dilutions to $10^6$ were made in the diluent using a sterile plastic pipette. Each dilution was mixed by rolling the bottle several times on the bench top to avoid undue aeration.

3.2.4 Inoculation of culture media

From the original undiluted suspension, 20µl were plated onto inositol agar and onto Yersinia selective agar. The inositol plate was incubated under standard conditions, and the Yersinia plate incubated at 37°C.

3.2.5 Incubation conditions

Standard incubation conditions, unless otherwise stated, were overnight at 37°C in an aerobic CO2-enriched (10%) humidified atmosphere.

3.2.6 Identification of colonies on inositol plates

Plates were examined for colony types. Gram stains were made of each type and examined microscopically under oil immersion x 100 (Leitz Laborlux).

Any colonies with a mucoid appearance and consisting of Gram-negative bacilli were subcultured onto MacConkey agar and then formally identified by API 20E strips. Non-mucoid colonies consisting of Gram-negative bacilli were subcultured onto BGA, incubated overnight and the plate examined for colour. A yellow-green colour indicated the presence of beta glucuronidase and that the
bacterium was almost certainly *E. coli*. If no colour appeared, formal identification was done by API 20E strips.

### 3.2.7 Identification of colonies on Yersinia selective agar

These plates (incubated at 35°C) were examined daily for 7 days for the appearance of red bullseye colonies. Gram stains were made of suspect colonies and Gram-negative bacilli identified by API 20E.

### 3.2.8 Testing for urinary tract infection

A midstream specimen of urine (MSU) was obtained from each patient and cultured by the hospital microbiology laboratory using standard procedures. MSUs were not obtained from controls.
3.3 RESULTS

3.3.1 Characterisation of patients and controls

a) Patients

Stool samples were collected from 82 patients; 66 males and 16 females (sex ratio; male: female, 5.1: 1). The disease category and age of these individuals are shown in Table 3.1. The mean age of all the patients was 41.8 years, range 21-63 years.

b) Controls

Stool samples were obtained from 36 healthy individuals with no present or past history of rheumatic disease; 29 males and 7 females (sex ratio; male: female, 5.1: 1). The mean age of the group was 36.3 years, range 22-62 years.

None of the patients or controls had received antibiotics within the previous 2 months.

Table 3.1
Characterisation of patients providing faecal sample

<table>
<thead>
<tr>
<th>Disease category</th>
<th>Patients</th>
<th>No.</th>
<th>AS</th>
<th>IBD</th>
<th>ReA</th>
<th>PSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>82</td>
<td>67</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Male (M)</td>
<td>66</td>
<td>54</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Female (F)</td>
<td>16</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>42.3</td>
<td>37.1</td>
<td>39.5</td>
<td>40.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 Diet

Of the 36 controls, all except 1 vegetarian ate a mixed diet. Hospital food was eaten 1 to 3 times per week by 5 controls. None of the patients, all of whom were out-patients, had eaten hospital food.
3.3.3 Growth on inositol agar

a) Patients
Stool samples from 82 patients were analysed. Of these, 8 samples had no growth on inositol (< 500 organisms per gram of faeces) and 74 samples grew at least 1 organism. A total of 93 different organisms were identified from the 74 stool samples; 58 samples grew 1 species of bacteria, 13 samples grew 2 species and 3 samples grew 3 species. The range of organisms identified is shown in Table 3.2.

b) Controls
All 36 samples produced colonies on the inositol plates. A total of 57 different bacteria were isolated from these stool samples. 1 species was isolated from each of 18 stool samples. 2 species grew from each of 15 stools and from the remaining 3 samples, 3 species per sample were isolated. The range of organisms identified is shown in Table 3.2.

7/82 (8.5%) stool samples from patients grew Klebsiella compared with 7/36 (19.4%) of samples from controls. Of the 7 controls from whose faeces Klebsiella were isolated, 2 ate hospital food once or twice per week.

3.3.4 Growth on Yersinia selective agar

Yersinia were not isolated from any of the patients' stool samples but Y. enterocolitica was isolated from the stool of a control individual. This individual had experienced some abdominal discomfort which he felt was insignificant 2 weeks prior to providing a stool specimen. The discomfort became more severe and he then presented to his general practitioner. A stool sample sent by the general practitioner to the routine microbiology laboratory also grew Y. enterocolitica.

3.3.5 Urine culture

None of the patients had a urinary tract infection.
Table 3.2
Bacteria isolated from patients' and controls' stools

<table>
<thead>
<tr>
<th>Species</th>
<th>Patients (n = 82)</th>
<th>Controls (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>69</td>
<td>30</td>
</tr>
<tr>
<td>Enterococci</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia e</em></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Citrobacter diversus</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Kluyvera</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Aeromonas hydrophilia</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella group C</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Yersinia</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>93</strong></td>
<td><strong>57</strong></td>
</tr>
</tbody>
</table>

* 2 weeks prior to providing a stool sample for this study, this control had vomiting and diarrhoea for 1-2 days. He was asymptomatic at the time of stool collection. The Public Health Department was notified and the likely source of the Salmonella gastro-enteritis traced to a contaminated hamburger.
3.4 DISCUSSION

3.4.1 Influence of diet on Klebsiella carriage

All types of hospital food, particularly salads and cold meat, may become contaminated with Klebsiella organisms. In one study, Klebsiella were widely distributed in the hospital kitchen environment which was considered at least in part to be the source of the bacteria in hospital food (Cooke et al., 1980). From the present data, 5/36 controls ate hospital food at least once per week in contrast to the patients, none of whom had eaten hospital food. Klebsiella was isolated from the stool samples of 2 of these 5 controls.

One might expect that when patients are admitted to hospital the carriage of faecal Klebsiella would increase. Datta (1969), however, found no increased rate of faecal carriage of Enterobacter, Klebsiella, Citrobacter, Proteus or Pseudomonas in patients admitted to hospital.

The incidence of faecal Klebsiella was examined in renal patients who later received a transplant (Montgomerie et al., 1970). There was no significant difference in the incidence of Klebsiella whether these patients were admitted to hospital or seen as out-patients. Transplantation with immunosuppressive therapy also did not affect the appearance of Klebsiella in the faeces.

3.4.2 Klebsiella isolation

There was no increased isolation of Klebsiella from the faecal samples of AS patients than from controls. This supports the findings of many others (Eastmond et al., 1980; Warren and Brewerton, 1980; Hunter et al., 1981; Ferraz et al., 1990; McLellan, 1990; van Kregten et al., 1991). Indeed, Klebsiella were isolated significantly less frequently from patients' stools (8.5%) than controls' samples (19.4%). The method of collection in both groups was the same. Because of the wide geographical area of patient recruitment, for practical reasons, stool specimens were sent by post and therefore were 24-48hr old at the time of processing. By contrast, delivery of control specimens was within 3hr of defecation. The delay in arrival and possible lower temperature exposure of samples in the post might explain why; 8 patients' samples did not grow any bacteria on inositol; fewer species of bacteria were isolated from patients' stools;
and, the isolation rate for *Klebsiella* in patients' specimens was lower than that of healthy individuals.

The *Klebsiella* isolation rate of 19.4% in control samples in this study is comparable with those of other studies eg. 13% in Arizona (Kuberski, 1983) and 22.2% in Brazil (Ferraz et al., 1990); is lower than that in two English surveys, 38% (Ebringer et al., 1977) and 37% (Ebringer et al., 1978); and considerably lower than in a Dutch investigation (60%) (van Kregten et al., 1991).

Apart from possible geographical variations in *Klebsiella* prevalence and differences in culture methods, these control populations differed widely from each other. In the two studies of Ebringer's (Ebringer et al., 1977; Ebringer et al., 1978) the control groups were not a homogeneous population but included as well as healthy individuals, convalescent hospital in-patients. These in-patients, while they did not have arthritis, were in a hospital environment and thus were not strictly comparable with the patient group who were all out-patients. Hospital out-patients without arthritis or sacroiliitis formed the control group in another survey (Kuberski, 1983) and Hunter and colleagues (1981) used individuals with mechanical back pain for comparison with AS patients. Healthy relatives of patients attending general practitioner clinics have also been used as controls (Ferraz et al., 1990). van Kregten et al. (1991), by contrast, used rheumatology out-patients with a variety of inflammatory arthritides and gout as controls. It could be argued that this control group consisted of a group of diseased individuals who were not really comparable to another diseased population (although the authors state that the controls were chosen to anticipate a possible effect of a hospital visit on faecal carriage of *Klebsiella*).

A much higher *Klebsiella* isolation rate in controls (60%) was noted by a Dutch group (van Kregten et al., 1991), but this did not differ significantly from that in the patient population. The culture method employed a liquid and a solid phase enrichment medium to potentiate the growth of *Klebsiella* organisms.

The *Klebsiella* isolation rate in AS patients shows variation between different studies: 8.5% in the current work; 27% (Eastmond et al., 1980); 27% (Warren and Brewerton, 1980; 32% (Ferraz et al., 1990); 38% (Ebringer et al., 1977; 40% (Kuberski et al., 1983)); 54% (van Kregten et al., 1991). The only other study of faecal flora in AS patients which has been performed in Scotland was in
Glasgow and an isolation rate of 17.6% was found (McLellan, 1990). No controls were done in this latter survey.

3.4.3 Faecal sampling and culture methods

The method of faecal sampling has differed between previous studies. Fresh faeces delivered or posted by subjects, or collected by investigators have been most commonly employed (Ebringer et al., 1977; Ebringer et al., 1978; Ferraz et al., 1990; van Kregten et al., 1991). Rectal swabs (Kuberski, 1983; Hunter et al., 1981; McLellan, 1990) which can be done by the investigators or by the subjects themselves, are less messy to collect than faecal samples and are probably more acceptable to subjects. The ability of subjects, however, to accurately and comparably use a rectal swab is open to question.

In the present study a faecal specimen was necessary as it was required to be processed in a number of ways and the amount of material on a swab would have been insufficient. Analysis of stool samples of patients and controls was done during the same period to avoid any seasonal variation in faecal flora which might have biased the results.

Different culture methods have been used. MacConkey-inositol-carbenicillin (MIC) agar was developed in 1970 (Thom) as a nutrient medium selective for the isolation of Klebsiella from faeces. This was based on MacConkey agar in which lactose was replaced as a carbon source by 1% inositol and 100mg/ml of carbenicillin was added. About 97-99% of Klebsiella strains and only 0-1% of E. coli strains are able to ferment inositol and appear as pink colonies. Most strains of E. coli are susceptible to carbenicillin, so the medium is selective for Klebsiella. This concentration of carbenicillin will, however, prevent the growth of 10-15% of Klebsiella strains (depending on the geographical area) so some investigators have reduced the concentration of carbenicillin to 10mg/ml. van Kregten and colleagues (1991) (who found particularly high Klebsiella isolation rates in patients and controls) used Simmons citrate agar (SCA) with 1% inositol but without antibiotics, because of the risk of missing susceptible strains. An advantage of using the medium SCA with inositol, is that the two carbon sources, citrate and inositol, can be utilised by nearly all K. pneumoniae and K. oxytoca strains but not by E. coli.
A comparative study of 15 methods to isolate *Klebsiella* from faeces was made using different types and combinations of media (Cooke et al., 1979). The solid media producing the highest isolation rates were MIC and SCA and the highest isolation rates overall were obtained by subculturing citrate broth to MIC agar and SCA. There was no difference in isolation rate by inoculation of the media with a suspension of faeces or by inoculation with a swab (Cooke et al., 1979).

van Kregten et al. (1984) also compared the ability of different media and media combinations to grow *Klebsiella* from faecal samples. They found that the selectivity of the SCA with inositol for *Klebsiella* equalled that of MIC and SCA combined, but SCA with inositol was more efficient than the latter combination.

Most culture techniques used in the studies of faecal flora in AS have employed a differential medium containing inositol (Ebringer et al., 1977; Ebringer et al., 1978) and standard identification methods including API 20E strips. Alternatives have been; to culture specimens on MacConkey and Hektonen enteric media prior to growing in Gram-negative enrichment broth (Kuberski, 1983); to simply inoculate faeces onto either MacConkey agar (Ferraz et al., 1990) or both blood agar and MacConkey agar plates (Hunter et al., 1981) and identify organisms by standard methods. van Kregten’s group (1991) refrigerated faecal specimens at 4°C prior to processing but do not comment on the duration of refrigeration. 1g faeces was added to a liquid enrichment medium for isolation of *Klebsiella*. An aliquot of this was inoculated onto a solid phase (SCA with 1% inositol), incubated at 37°C and examined 48hr later. After overnight incubation, a second aliquot of the faecal suspension was subcultured onto a SCA with inositol plate and examined 48hr later for *Klebsiella*.

In the present work the objectives were to examine stool specimens of patients and controls not only for *Klebsiella* but also to determine if any other organism was more prevalent in the faecal flora in AS patients compared with healthy individuals. For this reason a medium selective for the growth of *Klebsiella* was not chosen. The medium chosen was inositol-containing nutrient agar with a neutral red indicator. This is not selective *per se* for *Klebsiella* as it allows the growth of many other bacterial species, but it allows the easy identification of inositol fermenters such as *Klebsiella*. By making serial dilutions of faecal samples, it was calculated that the detection rate was 500
organisms/g of faeces. Carbenicillin was not incorporated to avoid missing carbenicillin-sensitive Klebsiella strains.

By these methods, no significant difference between the faecal flora of patients with AS and healthy subjects was found.

3.4.4 *Yersinia* isolation

The absence of *Yersinia* in the patient group in the present study is in agreement with others (Ebringer et al., 1977; Hunter et al., 1981; Kuberski, 1983). The optimum conditions for isolation of *Yersinia* are considered to be incubation at 35°C for 24-48hr and enrichment culture using phosphate buffered saline pH 7 at 4°C for 3 weeks (Pai et al., 1979). In our hospital microbiology department faecal specimens suspected of harbouring *Y. enterocolitica* are routinely subcultured onto *Yersinia* selective agar and incubated at 37°C for 48hr. If no growth occurs, the sample is regarded as being negative for *Yersinia* organisms. Prolonged cold incubation is not performed as it is considered that any growth significant to cause disease will grow under the conditions employed. This practice agrees with the conclusions by Pai et al. (1979) that cold enrichment is not indicated for the culture of *Y. enterocolitica* from diarrhoeal stools. The most common diarrhoeal strain is serotype 03 and as samples are submitted during the acute phase of the illness, direct plating is sufficient to recover the 03 serotype. Cold enrichment methods, however, increase considerably the sensitivity of *Y. enterocolitica* isolation in convalescent and asymptomatic subjects but only minimally in patients with diarrhoea caused by serotype 03. Recovery of non-03 serotypes of *Y. enterocolitica* is increased by cold enrichment but the clinical significance of these isolates is uncertain.

A criticism of the present study would be that cold enrichment methods were not used for the isolation of *Yersinia*. The culture conditions employed however did succeed in isolating *Y. enterocolitica* from the stool sample of a control who had abdominal pain 2 weeks prior to providing a faecal sample.

3.4.5 Urinary tract infection

A link between chronic genitourinary infection and the development of sacroiliitis has been speculated from the observation of an increased prevalence of sacroiliitis in Reiter's disease (Ford, 1953; Wright, 1963). This was suggested to be due to venous drainage from the prostate and seminal vesicles which pass
directly over the sacroiliac joints (Oates and Young, 1959). The finding of an increased incidence of chronic prostatitis in AS patients (Mason et al., 1958) suggested that AS might be due to chronic genitourinary infection. Another piece of evidence that initially suggested continued antigenic challenge from the urinary tract might in some way initiate inflammatory change in the sacroiliac joints, was the identification of sacroiliac joint changes (similar to those in AS) in paraplegics (Abramson and Kamberg, 1949; Abel, 1950). Paraplegics have a high incidence of urinary tract infections. Wright et al. (1965), however, made a detailed study of 36 paraplegics and concluded that the sacroiliac joint changes observed were distinct from those of AS and Reiter's syndrome.

None of the patients in the present study, however, had a urinary tract infection which is in agreement with a earlier report (Ebringer, 1977).

The conclusions that can be drawn are:

1. the faecal flora (as determined by the media employed) of the patient population and the healthy controls was essentially similar;

2. there was no increase in the isolation rate of *Klebsiella* in this population of patients with spondyloarthropathy compared with healthy controls;

3. of the patients in whom *Klebsiella* was identified there was no relationship with its presence and disease activity;

4. no proposed arthritogenic organism or any other organism was found in increased numbers in patients with spondyloarthropathy.
Chapter 4

Cross-reactivity Studies on Enteric Bacteria in Ankylosing Spondylitis

4.1 Introduction

Much work has been done on the molecular mimicry hypothesis of disease since cross-reactivity of antibodies to HLA antigens and bacterial components was demonstrated (Hirata et al., 1973). Integral to the molecular mimicry theory of AS is the existence of antigenic similarity between HLA-B27 and bacteria. Cross-reactivity between Klebsiella and HLA-B27 has been reported by several groups. Rabbits injected with HLA-B27-positive cells developed antibodies reactive against K. pneumoniae and Y. enterocolitica (Welsh et al., 1980). Beukelman et al. (1988) immunised several rabbits with vaccines prepared from enterobacteria isolated from the stools of AS patients and tested the antisera in a cytotoxicity assay with mononuclear cells of HLA-B27-positive AS patients and controls. 3/12 rabbit sera were lytic for the cells of HLA-B27-positive patients. At least 1 of these discriminated between the cells of patients and controls.

The observation that faecal carriage of Klebsiella was higher in AS patients with active disease (Ebringer et al., 1977) or who were about to become active (Ebringer et al., 1978), than it was in non-spondylitic controls or in patients with inactive disease, suggested a possible pathogenic role for antigenic similarity between HLA-B27 (carried by the majority of AS patients) and enteric bacterial antigens in the development of AS.

A number of workers (Eastmond et al., 1980; Warren and Brewerton, 1980; Hunter et al., 1981; Ferraz et al., 1990; van Kregten et al., 1991), however, have been unable to confirm the findings of Ebringer (1977) that Klebsiella is found more frequently in the stools of AS patients than in healthy individuals. This led to speculation that if enteric bacteria are involved in the pathogenesis of AS, it might be that there is a factor(s) common to several bacterial species which is important rather than a factor(s) present on members of a single species. Serological cross-reactivity among members of the Enterobacteriaceae is well-established (Pease et al., 1988).
This study investigated the presence of bacteria showing antigenic similarity to HLA-B27 in the faecal flora of patients with spondyloarthropathy and healthy controls. It was of interest to determine if such bacteria were only found in patients with spondyloarthropathy, or if they were also found in healthy individuals. If such bacteria were found in HLA-B27-positive AS patients, it is possible that because of the antigenic similarity, the host might show a degree of tolerance to these organisms rather than mounting an immune response against them. This would allow the bacteria to colonise the gut mucosa leading to inflammation. Identification of the bacterial species expressing these antigens was of interest to determine whether these antigens were present only on known arthritogenic organisms such as *Klebsiella, Yersinia* and *Shigella* or whether they were present on gut commensal organisms.

In previous studies of enteric bacteria in AS, the role of anaerobic organisms (which comprise the majority of faecal flora) has been neglected. This is probably because of time factors involved as anaerobes take considerably longer to grow in the laboratory, and also because anaerobes have more fastidious growth requirements than aerobes. It seemed appropriate in this study to investigate for cross-reacting epitopes on anaerobes as well as aerobes.

HLA-B27 cross-reactive epitopes have previously been identified on *Klebsiella* and *Shigella* (van Bohemen et al., 1984) and *Yersinia* (van Bohemen et al., 1984; Chen et al., 1987). In this study, once the organisms were isolated and identified, the epitope that the anti-HLA-B27 antibody bound to would be characterised. Possible sites could be on the outer membrane component of the bacterial cell wall, or indeed the epitope could even be internal and secreted, or only released on cell death.

The immunoblotting procedure employed to identify colonies was the same as that devised by a Dutch group (Groeneweld et al., 1990) and used to identify colonies of *H. influenzae* (van Alphen et al., 1991). The immunoassay was modified in the present study by using a monoclonal anti-HLA-B27 antibody as a probe instead of monoclonal antibodies directed against epitopes on the outer membrane of *H. influenzae*. (There were no commercially-available polyclonal anti-HLA-B27 antibodies).
The principle of the technique is as follows:

MC27 (an IgG antibody) is added to bacteria (or outer membrane peptidoglycan). If a suitable receptor is present, MC27 will bind to it. The enzyme amplification step involves the addition of protein A conjugated with horseradish peroxidase (HRP). Protein A (a cell wall component of certain strains of staphylococci) binds immunoglobulin molecules, especially IgG, with high affinity. Therefore, the protein A-HRP complex will bind to immunoglobulins already bound to bacteria. When the hydrogen peroxide-activated substrate for HRP is added, the reaction results in a colour change: a blue/green colour indicates that the protein A-HRP complex has bound.

The aims of this part of the study were:

1. to examine the faecal flora of healthy individuals and patients with spondyloarthropathy for bacteria (aerobes and anaerobes) with epitopes cross-reactive with the HLA-B27 antigen;
2. to identify such bacteria by standard bacteriological methods;
3. to isolate and characterise these epitopes if found.
4.2 MATERIALS AND METHODS

4.2.1 Immunoblotting of bacterial colonies

4.2.1.1 Materials

Blood agar
The blood agar was composed of a Columbia agar base prepared as per manufacturer's instructions (Oxoid, Basingstoke, Hampshire) with the addition of 6% horse blood.

Phosphate buffered saline (PBS) (pH 7.2)
PBS contained 0.01M phosphate buffer pH 7.2 with 0.15M NaCl.

High salt buffer (HSB)
HSB was composed of PBS containing 1M NaCl and Tween 20 (0.5%, v/v) (BDH, Poole, Dorset).

Barbitone complement fixation test diluent (BCFD)
This was prepared as per manufacturer's instructions (Oxoid, Basingstoke, Hampshire).

Mouse monoclonal anti-human HLA-B27 antibody (MC27)
The monoclonal IgG2a anti-human HLA-B27 antibody (Code no. MCA 116 from Serotec, Kidlington, Oxford) was diluted 1/250 in HSB. It is a cytotoxic antibody directed against an epitope common to HLA-B27 molecules of healthy controls and patients with spondyloarthropathy (Trapant et al., 1983).

Protein A-horseradish peroxidase (protein A-HRP)
Protein A-HRP (Sigma, Poole, Dorset) was diluted 1/5 in BCFD and stored at -20°C. Prior to use, the protein A-HRP was diluted a further 100 times in HSB to make an overall dilution of 1/500.

Anti-mouse gammaglobulin conjugated with horseradish peroxidase
This was obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire) and diluted 1/100 in PBS.
ELISA buffer
ELISA buffer contained 24.3ml 0.1M citric acid (BDH, Poole, Dorset), 25.7ml 0.2M Na$_2$HPO$_4$ (BDH, Poole, Dorset) and 50ml H$_2$O.

Substrate
The substrate solution used to detect protein A-HRP activity contained 160mg dioctyl sulfo-succinate (DONs) (Sigma, Poole, Dorset) and 49mg tetramethyl benzidine (TMB) (Sigma, Poole, Dorset) in 92ml of:

- 12ml ELISA buffer
- 20ml Ethanol
- 60ml H$_2$O

The mixture was placed in a water-bath at 60°C for about 30min to solubilise the DONs and TMB. The substrate was activated prior to use by adding 60µl hydrogen peroxide (H$_2$O$_2$) (0.3%, v/v).

4.2.1.2 Methods

Bacterial culture

A 20µl sample from the 10$^{-2}$ and the 10$^{-3}$ dilutions of each faecal suspension (see chapter 3) was spread over the surface of separate blood agar plates using a sterile glass rod and incubated overnight under standard conditions. In the same way, 20µl from the 10$^{-5}$ and the 10$^{-6}$ dilutions were plated out onto two blood agar plates and incubated anaerobically at 37°C for 7 days.

Immunoblotting

For each individual, one plate incubated aerobically and one incubated anaerobically were selected for immunoblotting with Biotrace NT nitrocellulose (NC) membrane (Gelman Sciences, Northampton). The plates selected were those in which the colonies were separate from each other and not too numerous to facilitate identification of positive colonies.

The NC membrane was cut into circles (90mm diameter) and two small V-shaped incisions made in the membrane for orientation purposes. The circle was carefully placed on top of the colonies with alcohol-flamed (and cooled) forceps. An indelible mark was made on the side of the Petri dish corresponding
to the notches on the NC membrane to allow later identification of positive colonies.

After 3min, the NC membrane was lifted carefully off the plate with clean forceps and transferred to a clean Petri dish with the adherent colonies facing upwards. The membrane was immersed in HSB for 1 to 2min.

Excess bacteria were gently wiped off into the HSB. The membrane was washed again in HSB and the fluid discarded. The membrane was submerged in HSB and incubated for 30min at 37°C. It was then rinsed twice in HSB and the fluid discarded.

MC27 (1ml) diluted 1/250 in HSB was added to each membrane and incubated at 37°C for 1hr. The antiserum was washed off by immersing the membrane in HSB for 15min and then running it under tap water for 1min. The water was discarded and the membrane placed in HSB immediately.

Protein A-HRP (1ml) diluted 1/250 in HSB was added to each membrane and incubated for 1hr at 37°C. After another washing step with HSB, 3 to 4ml of activated substrate was poured over each NC blot and left at room temperature for 5min.

Positively-stained colonies appeared as green-blue dots on the NC (Plate 4.1). The reaction was stopped by washing with tap water. Positive colonies were identified on the original blood agar plate, a mirror image of the NC blot. These colonies were subcultured and identified by routine bacteriological methods (section 4.2.3).

4.2.2 Dot-immunoblotting

4.2.2.1 Preparation of bacterial suspensions

A heavy bacterial suspension was made by aseptically transferring several colonies with a wire loop from a subculture plate to a tube containing 200μl PBS and thoroughly mixing with a rotary mixer.
4.2.2.2 Materials

*Staphylococcus epidermidis*
A stock culture of *S. epidermidis* was stored on blood agar at 4°C.

Anti-staphylococcal antibody
Hyperimmune mouse serum obtained after 6 weekly injections of *S. epidermidis* (2 x 10^9/ml) was kindly donated by Dr J Stewart and diluted 1/10 in PBS.

Bovine serum albumen (BSA)
A 1% (w/v) solution of BSA in distilled water was used.

*Klebsiella* K43 and K30 strains
These were laboratory stock strains grown on egg slopes and stored at room temperature.

Protein A
Staphylococcal protein A (Sigma, Poole, Dorset) was diluted 1/250 in HSB.

4.2.2.3 Method

The NC membrane was cut into rectangles to fit into split plates and a notch was made at the top for orientation purposes. Circles (diameter 0.5cm) were drawn in pencil on the shiny side of the membrane. These circles marked the areas or wells to which the bacteria were added.

A sample (10μl) of each bacterial suspension was added to similarly-positioned circles on each rectangular strip of NC membrane which was due to receive different treatments. Controls (BSA or *S. epidermidis* suspensions) were added to the strips in a similar manner. The strips were incubated for 10-15min at 37°C until nearly dry. Antibody (10μl) or HSB as appropriate was added to particular circles and incubated at 37°C for 1hr. A washing stage in HSB followed for 15min and the fluid was then discarded. The strips were immersed in tap water for 1-2min and placed in HSB immediately. HSB was discarded and protein A-HRP (0.5ml) was added and incubated at 37°C for 1hr. After a final wash in HSB, 2ml of substrate were added and left at room temperature for 5-10min for colour to develop. The reaction was stopped by washing with water.
In some experiments anti-mouse gamma-globulin-HRP (1/100) was used as the enzyme amplification system as an alternative to protein A-HRP.

4.2.2.4 Blocking experiments to investigate the non-specific binding of protein A-HRP

The non-specific binding of protein A-HRP to whole bacteria and outer membrane peptidoglycan (OMPG) complexes was investigated initially by attempting to block possible binding sites on bacteria for protein A-HRP (1/500) with protein A (1/250). This was repeated with protein A-HRP at the same concentration and protein A (1/5). A dot-blot technique was used. Test samples included whole bacteria (E. coli MS and Acinetobacter calco. var. anitratus), the prepared OMPG from these bacteria and Klebsiella K43 and K30 strains). BSA and mouse anti-BSA antibody (1/10 dilution in PBS) were controls (Table 4.1).

Table 4.1

<table>
<thead>
<tr>
<th>Scheme to test protein A as a blocking agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria/OMPG/BSA</td>
</tr>
<tr>
<td>STEP 1</td>
</tr>
<tr>
<td>STEP 2</td>
</tr>
<tr>
<td>STEP 4</td>
</tr>
</tbody>
</table>

4.2.2.5 The effect of different concentrations of protein A-HRP

It was possible that at the concentration of protein A-HRP used (1/500), the protein A-HRP could still bind to sites on the bacteria not blocked by protein A. A dot-blot experiment was set up adding a standard concentration of MC27 (1/250) and different concentrations of protein A-HRP (1/250, 1/500, 1/1000, 1/2000, 1/4000) to whole bacteria or OMPG. BSA and S. epidermidis and their respective antibodies were used as controls (Table 4.2).
Table 4.2
Dot-blot scheme for testing effect of different concentrations of protein A-HRP

<table>
<thead>
<tr>
<th>Test</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP 1</td>
<td>bacteria/OMPG</td>
<td>bacteria/OMPG</td>
<td>BSA</td>
</tr>
<tr>
<td>STEP 2</td>
<td>MC27</td>
<td>HSB</td>
<td>anti-BSA</td>
</tr>
<tr>
<td></td>
<td>dilution</td>
<td>dilution</td>
<td>dilution</td>
</tr>
<tr>
<td>STEP 4</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
</tr>
</tbody>
</table>

4.2.2.6 To test for the presence of peroxidase

The presence of peroxidase was investigated in 11 bacteria isolated from patients' stools and in 7 laboratory strains (N. meningitidis type B:4; N. meningitidis type C:4; H. influenzae type b; Salmonella enteritidis; Klebsiella K30; Klebsiella K43; E. coli MS) which were stored on egg slopes at room temperature. The dot-blot technique was used.

4.2.2.7 Inactivation of peroxidase

An attempt was made to inactivate peroxidase by treating the bacteria impregnated in the nitrocellulose paper with 0.3% H₂O₂ in methanol (Table 4.3).

Table 4.3
Scheme for inactivation of peroxidase with 0.3% H₂O₂ in methanol

<table>
<thead>
<tr>
<th>Test</th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
</tr>
<tr>
<td>0.3% H₂O₂ in methanol</td>
<td>HSB</td>
<td>HSB</td>
</tr>
<tr>
<td>substrate</td>
<td>substrate</td>
<td>HSB</td>
</tr>
</tbody>
</table>

This was repeated, but this time the bacteria were fixed in coating buffer to an ELISA plate instead of to nitrocellulose paper. The bacteria were treated with 0.3% H₂O₂ in methanol and TMB/DONS was used as substrate.
Further attempts were made to inactivate bacterial peroxidase by buffered paraformaldehyde (PFD). This chemical impairs movement of proteins within the bacterial cell wall by cross-linking them. If the peroxidase enzyme requires such movement to be biologically active, then the paraformaldehyde would prevent this. When substrate is added to bacteria so-treated, no colour change would be seen.

**Materials**

Tris-hydrochloric acid (Tris-HCl)
This was composed of Tris 0.605g, sodium chloride 0.85g, 0.1N hydrochloric acid (HCl) (4ml) and distilled water (96ml).

Diaminobenzidine
Stock Tris-HCl (5ml) was added to 1 bottle of diaminobenzidine (20mg) and the mixture added to 20ml Tris-HCl. This was activated prior to use by the addition of 1 drop of neat H₂O₂.

Acetate buffer
This consisted of 1M sodium acetate (50ml), 1M HCl (10ml) and distilled water (200ml).

Amino ethyl carbazole
This consisted of amino ethyl carbazole (0.4%) in dimethyl formamide made up to 1/10 solution in acetate buffer and activated just prior to use by the addition of 1 drop of neat H₂O₂.

**Method**

A dot-blot assay was used (Table 4.4). Bacterial suspension (10μl) was added to NC dots and incubated for 30min at 37°C. Buffered paraformaldehyde (3ml) was added to test NC strips and HSB to control NC strips. After 20min at room temperature, NC membranes were washed twice with HSB and substrate added. 3 different chemicals which are known peroxidase substrates were tested to determine if the reaction was more pronounced with one substrate than another; TMB/DONS; diaminobenzidine; amino ethyl carbazole.
Table 4.4
Scheme for inactivation of peroxidase with buffered paraformaldehyde

<table>
<thead>
<tr>
<th></th>
<th>(a)</th>
<th></th>
<th>(b)</th>
<th></th>
<th>(c)</th>
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<tbody>
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<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
</tr>
<tr>
<td>PFD</td>
<td>HSB</td>
<td>PFD</td>
<td>HSB</td>
<td>PFD</td>
<td>HSB</td>
</tr>
<tr>
<td>TMB/DONS</td>
<td>diaminobenzidine</td>
<td></td>
<td>amino ethyl carbazole</td>
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<td></td>
</tr>
</tbody>
</table>

4.2.3 Identification of colonies that were positive in the immunoblot assay

4.2.3.1 Aerobic bacteria

Catalase test

This is performed to distinguish staphylococci from streptococci. A few colonies from a pure culture of an organism are transferred by a flamed (and cooled) wire loop to a drop of distilled water on a clean slide. 1-2 drops of H$_2$O$_2$ are added and a cover slip placed on top. If bubbles appear these are due to hydrogen released from the breakdown of H$_2$O$_2$ by catalase (peroxidase) in staphylococci. Streptococci do not have this enzyme and so no bubbles materialise.

Method

Bacteria from positively-staining colonies were subcultured onto blood agar plates and a Gram film made. Gram-negative bacilli were further subcultured onto BGA plates to determine if the isolate was *E. coli*. If the Gram-negative bacilli were not *E. coli*, the organisms were identified by API 20E. If Gram-positive cocci were found, a catalase test was performed to differentiate staphylococci from streptococci. The organism was then identified by either API Staph or API Strep identification kits (API Laboratories Ltd., Basingstoke, Hampshire).
4.2.3.2 Anaerobic bacteria

Anaerobic organisms were identified to genus level by examination of a combination of cellular morphology, Gram stain reaction and short-chain fatty acid end-products of glucose metabolism (Brown et al., 1989). Blood agar plates were inoculated from frozen stock cultures of the test strains and incubated anaerobically at 37°C in an atmosphere containing 10% hydrogen, 10% carbon dioxide and 80% nitrogen in an anaerobic cabinet (Forma Systems). Single colonies were subcultured from the plates into 5ml volumes of prereduced Proteose Peptone Yeast extract broth containing 1% glucose and incubated anaerobically for 48 or 96hr. These cultures were examined in Gram-stained films and the pH was recorded before they were processed for gas chromatographic examination. Ether extractions were made by the method of Thomann and Hill (1986), and the chromatography was performed on a Perkin Elmer model 8410 gas chromatograph fitted with a flame ionisation detector, AS8300 autosampler and integrator computer. The column was 2m x 4mm glass, containing 15% SP1220 + 1% HPO4 on 100/120 mesh Chromosorb WAW. Operating conditions were 120°C for 1.5min, rising at 4°C/min to 160°C and held for 2.5min. Injection and detector temperatures were 160°C and 300°C respectively. Flow rates were 35ml/min for the oxygen-free nitrogen carrier gas and 500ml/min for air. The hydrogen flow rate was adjusted to give the most sensitive response. The injection volume was 0.5µl. Identification and quantification of the separated components was done by comparison of their retention times and peak areas with those of known standards.

4.2.4 Storage of bacteria which stained positively in the immunoblot assay

4.2.4.1 Cooked meat broth (CMB)

The modification of 'Robertson's bullock-heart' medium by Lepper and Martin as detailed by Collee and Marr (1989) was used.

4.2.4.2 Method

Bacteria from positively-staining colonies on immunoblot assay were subcultured onto a blood agar plate. A few of these colonies were transferred aseptically to a glass universal containing CMB and stored at room temperature until required.
4.2.5 Preparation of outer membrane peptidoglycan (OMPG) complexes

In order to identify the component of the bacteria that was responsible for the positive reaction in the immunoblot assay, the protein component parts of the outer membrane peptidoglycan were separated by polyacrylamide gel electrophoresis (PAGE). The bands that bound MC27 were identified by Western blotting using protein A-HRP as the enzyme amplification step. The two bacteria chosen were those which had produced the most vivid reaction in the immunoassay; *E. coli* MS and *Acinetobacter calco.* var. *anitratus.*

Nutrient broth No.2 (500 ml) (Oxoid, Basingstoke, Hampshire) was inoculated with a few colonies of bacteria from a blood agar plate and incubated at 37°C in a Gallenkamp orbital incubator with continuous gentle shaking. The bacteria were harvested at 48hr by centrifugation at 3000g for 20min and washed twice in PBS at 4°C. The bacteria were resuspended in 20ml PBS (pre-cooled to 4°C) and transferred to a sterile glass universal container which was placed in a beaker of ice. The resuspended bacteria were broken up by sonication at an amplification of 6 microns for five 1min periods with 30sec intervals between each sonication period to allow cooling. Unbroken cells were removed by centrifugation at 5000g and the supernatant mixed with 5ml of 22% sodium N-lauryol sarcosinate (Sigma, Poole, Dorset) for 30min at room temperature to solubilise the cytoplasmic membranes. The OMPG complexes were pelleted by centrifugation at 40,000g for 45min, resuspended in 0.5ml PBS and stored at -70°C prior to analysis.

4.2.6 Preparation of the polyacrylamide gels

4.2.6.1 Materials

Acrylamide stock solution
This contained 30g acrylamide and 0.8g methylene bisacrylamide (both from FSA Laboratory Supplies, Loughborough, Leicestershire) in 100ml distilled water.

Resolving gel buffer
This consisted of 3.0M Tris-HCl (BDH) at pH 8.8.
Stacking gel buffer
This consisted of 0.5M Tris-HCl (BDH) at pH 6.8.

Sodium dodecyl sulphate (SDS) stock solution
This consisted of a 10% (w/v) solution of SDS (Sigma, Poole, Dorset) in distilled water.

Ammonium persulphate stock solution
A 1.5% ammonium persulphate solution (Fisons Fine Chemicals, Loughborough, Leicestershire) was made in distilled water.

4.2.6.2 Method

The resolving gel and the stacking gel were prepared by mixing the constituents listed in Table 4.5.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Resolving gel (ml)</th>
<th>Stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10% acrylamide)</td>
<td>(4% acrylamide)</td>
</tr>
<tr>
<td>Acrylamide stock solution</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.75</td>
<td>---</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>---</td>
<td>5.0</td>
</tr>
<tr>
<td>SDS stock solution</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Ammonium persulphate solution</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>Distilled water</td>
<td>14.45</td>
<td>11.3</td>
</tr>
</tbody>
</table>

4.2.7 Preparation of samples for polyacrylamide gel electrophoresis

4.2.7.1 Materials

Samples
There were 5 molecular weight markers varying from 14,300 to 71,500kDa (BDH, Poole, Dorset).
Bovine serum albumen (BSA)
A 10% (w/v) solution of BSA (Sigma, Poole, Dorset) was prepared in PBS.

Test samples
Outer membrane peptidoglycan prepared from E. coli MS and A. calco. var. anitratus.

Sample buffer
Sample buffer (pH 6.8) consisted of 0.125M Tris-HCl (BDH) at pH 6.8 containing 4% (w/v) SDS (BDH Specially Pure), 20% (v/v) glycerol (BDH), 2% (v/v) 2-mercaptoethanol (BDH) and 0.002% (v/v) bromophenol blue (BDH).

4.2.7.2 Method

Equal volumes of bacterial OMPG and sample buffer were mixed and placed in a boiling water bath for 3min. Boiling SDS (a detergent) inactivates bacterial autolytic enzymes which otherwise would degrade peptidoglycan.

4.2.8 Polyacrylamide gel electrophoresis (PAGE) and Western blotting

4.2.8.1 Materials

Electrode buffer
Electrode buffer (pH 8.3) consisted of 0.025M Tris (BDH) in which 0.192M glycine (BDH Chromatographically Pure) and 0.1% (w/v) SDS (BDH) were added.

4.2.8.2 Method

Proteins were separated by SDS-PAGE using the continuous buffer system of Laemmli (1970) on a mini-protein II cell (Biometra, Maidstone, Kent). The resolving gel solution was poured between alcohol-treated glass plates of the slab gel electrophoresis equipment. The gel was overlayed with water-saturated butan-2-ol (BDH) and allowed to polymerise for 30min. The butan-2-ol was removed and the stacking gel poured onto the resolving gel. A comb was introduced and the stacking gel allowed to set. After removing the comb, the gel was fitted into an electrophoresis tank and the electrode buffer added. Samples were loaded into the wells of the stacking gel and electrophoresis carried out at a constant voltage of 60V. When the blue dye front had reached the bottom of the stacking gel, the voltage was increased to 150V to allow
electrophoresis through the resolving gel. The gel was removed from the PAGE apparatus and cut in 2 parts. One part was stained for proteins and the other part used for Western blotting.

4.2.9 Staining of polyacrylamide gels for protein

4.2.9.1 Materials

The following solutions were made up in distilled water:

1. 25\% (v/v) propan-2-ol (BDH General Purpose), 10\% (v/v) glacial acetic acid (BDH General Purpose) and 0.05\% (w/v) Coomassie brilliant blue (BDH).
2. 10\% (v/v) propan-2-ol, 10\% (v/v) glacial acetic acid and 0.005\% (w/v) Coomassie blue.
3. 10\% (v/v) glacial acetic acid and 0.0025\% (w/v) Coomassie blue.
4. 40\% (v/v) methanol (BDH General Purpose) and 10\% (v/v) glacial acetic acid.
5. 10\% (v/v) glacial acetic acid.

4.2.9.2 Method

The gel was placed in solution 1 overnight and then sequentially through stains 2-5, each for 45-60min at room temperature with gentle shaking throughout.

4.2.10 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose

4.2.10.1 Materials

Transfer buffer
This buffer (pH 9.2) contained 39mM glycine, 48mM Tris, 0.0375\% (v/v) SDS and 20\% (v/v) methanol in distilled water.

4.2.10.2 Method

The second part of the gel was laid on top of a square of nitrocellulose membrane which was on 4 sheets of Whatman No.3 filter paper, all of which had been presoaked in transfer buffer. An additional 4 sheets of filter paper were laid on top of the gel and the 'sandwich' trimmed with an alcohol-treated scalpel blade. The sandwich was then placed in an Ancos Semi-Dry
Electroblotter. A constant current of 0.8mA/cm² was applied for 1hr at room temperature.

4.2.11 Visualisation of antibody-antigen reactions

4.2.11.1 Materials

Mouse anti-BSA antibody
Mouse hyperimmune serum (kindly donated by Dr J Stewart) was produced by injection of 0.2ml BSA (1mg/ml) intraperitoneally each week for 6 weeks. The blood was collected 1 week after the last injection, allowed to clot and the serum stored at -20°C.

Blocking buffer (i)
This consisted of 4% (w/v) ovalbumen (Sigma, Poole, Dorset) in PBS.

Blocking buffer (ii)
This contained 0.05% (v/v) Tween in PBS.

4.2.11.2 Method

After electrophoretic transfer of proteins, the NC membrane was cut into 3 strips and all were soaked in blocking buffer (i) for 15min. The strips received the following treatments:

Strip 1: Mouse anti-BSA antibody (1/40 in PBS)

Strip 2: MC27 (1/250 in PBS)

Strip 3: Blocking buffer (ii).

The strips were incubated overnight at room temperature, washed in blocking buffer (ii) for 15min and rinsed in tap water for 1-2min. They were incubated with protein A-HRP (1/250 in HSB) for 30-60min at 37°C. Activated substrate was added and colour change noted after 10-15min. The reaction was stopped by the addition of tap water.
4.2.12 Flow cytometry to investigate the presence of a HLA-B27 cross-reactive epitope on bacteria

The presence of a HLA-B27 cross-reactive epitope on the bacteria was investigated by flow cytometry.

4.2.12.1 Materials

Anti-N. meningitidis antibody
Polyclonal rabbit antiserum to group C polysaccharide of N. meningitidis was obtained from Wellcome Diagnostics, Beckenham, Kent.

Anti-rabbit immunoglobulin FITC (Fab fragment)
This was obtained from the Scottish Antibody Production Unit (SAPU) (Carluke, Lanarkshire) and diluted 1/10 in HSB.

Anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC) (Fab fragment).
This was obtained from Sigma (Poole, Dorset) and diluted 1/50 in HSB.

Bacteria
Aerobic (32) and anaerobic (34) isolates, strains from both patients and controls which had stained positively in the original immunoblot assay, were tested.

4.2.12.2 Method

A heavy suspension of each bacterial strain was made in PBS. Equal volumes (50μl) of test bacterial suspension and either HSB or MC27 (1/250 in HSB) were incubated together for 1hr (Table 4.6).
Table 4.6
Scheme for flow cytometry experiment

<table>
<thead>
<tr>
<th>Test (a)</th>
<th>Test (b)</th>
<th>Control (c)</th>
<th>Control (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
</tr>
<tr>
<td>MC27 (20µl)*</td>
<td>MC27 (40µl)*</td>
<td>HSB</td>
<td>HSB</td>
</tr>
<tr>
<td>anti-mouse Ig</td>
<td>anti-mouse Ig</td>
<td>anti-mouse Ig</td>
<td>anti-mouse Ig</td>
</tr>
<tr>
<td>FITC**</td>
<td>FITC*</td>
<td>FITC*</td>
<td>HSB</td>
</tr>
</tbody>
</table>

* or anti-neisseria antibody or anti-staphylococcal antibody
** or anti-rabbit immunoglobulin FITC

Positive controls were (i) *N. meningitidis* serogroup C serotype 4 and antibody for the C capsular polysaccharide, and (ii) *S. epidermidis* with its corresponding antibody.

The samples were washed twice (1500g for 15min) in HSB and 50µl of FITC-conjugated antibody added for 1hr at 37°C. After washing twice (1500g for 15min) in HSB, 0.5ml of buffered paraformaldehyde was added to each bacterial pellet and the samples stored at 4°C prior to analysis by flow cytometry.

4.2.13 Analysis of bacteria by flow cytometry

Analysis was done on an Electronically Programmable Immunofluorescent Cell Sorter (EPICS) ‘C’ flow cytometer (Coulter Electronics, Luton, UK) equipped with a 5 watt laser using a power output of 300mw at 488nm. The bacteria were selected from a display of forward angle light scatter by means of a cursor set to eliminate debris. The percentage of cells showing fluorescence greater than the background level was recorded on a one parameter histogram measuring fluorescence on a logarithmic scale. The results were analysed by the immunoanalysis program (Coulter).
4.3 Results

4.3.1 Analysis of faecal flora of patients and controls for bacteria with antigens cross-reactive with HLA-B27

Stool samples were obtained from 82 patients and 36 controls. The details of these individuals are recorded in Table 4.7. The plates tested in the immunoassay were those with 50-100 colonies per plate.

Table 4.7
Characteristics of patients and controls providing faecal sample

<table>
<thead>
<tr>
<th>Patients</th>
<th>No.</th>
<th>AS</th>
<th>IBD</th>
<th>ReA</th>
<th>PSA</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>82</td>
<td>67</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>Male</td>
<td>66</td>
<td>54</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>41.8</td>
<td>42.8</td>
<td>37.1</td>
<td>39.5</td>
<td>40.8</td>
<td>36.3</td>
</tr>
</tbody>
</table>

4.3.1.1 Aerobic bacteria

27/36 (75%) stool samples from controls and 42/82 (51%) stool samples from patients contained aerobic colonies which were positive in the immunoblot assay. One of the patients' stools contained 2 species that were positive.

4.3.1.2 Anaerobic bacteria

Of 36 stool samples from controls, 18 (50%) grew anaerobes which reacted in the immunoblot assay. A total of 23 different bacteria were grown from these 18 samples. 23/82 (28%) of the patients' samples produced positive colonies on anaerobically-incubated plates. One sample grew 2 different positively-staining organisms.

All bacteria which were positive on the immunoblot assay were purified after initial identification and stored in cooked meat broth (CMB) at room temperature for 5-6 months until screening was complete.
4.3.2 Identification of organisms that were positive in immunoblot assay

4.3.2.1 Aerobic bacteria

The range of aerobic bacterial species which were identified are shown in Table 4.8. Two organisms isolated from controls' samples died before identification could be made. The most commonly identified aerobe in both populations was E. coli. The range of species was similar for the two groups and included a number of types of Streptococcus and Enterobacter species as well as a few other species. Two species of Klebsiella were identified in the assay from the patient population.

Table 4.8
Aerobic bacteria that were positive in the screening assay with anti-HLA-B27 antibody

<table>
<thead>
<tr>
<th>Organism</th>
<th>Controls (n = 27)</th>
<th>Patients (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus bovis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus lactis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus sanguis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Enterococcus durans</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus intermedium</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus avium</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Escherichia aderboxylata</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A. calco. var. anitratus</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
<td><strong>43</strong></td>
</tr>
</tbody>
</table>

* 2 organisms died before identification could be made.
4.3.2.2 Anaerobic bacteria

The range of anaerobic bacteria identified are listed in Table 4.9. The most commonly identified anaerobe in the assay from both patients and controls, was *Bacteroides* spp. followed by *Lactobacillus* spp. which are the two most common anaerobes in gut flora.

Table 4.9

Anaerobic bacteria that were positive in the immunoblot assay

<table>
<thead>
<tr>
<th>Genus/organism</th>
<th>Controls (n = 18)</th>
<th>Patients (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides</em></td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Fusobacterium</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Propionobacterium</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Eubacterium lentae</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Actinomyces</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

4.3.3 Ability of bacteria to remain positive on immunoblot assay on subculture

Bacteria from the positively-staining colonies were subcultured and re-tested with monoclonal anti-HLA-B27 antibody (MC27) to see if they retained the ability to bind the antibody.

4.3.3.1 Aerobic bacteria

On initial subculture, 14/25 bacteria from control samples were able to bind MC27; 11 were not. These 14 were repeatedly subcultured and the binding ability tested on each subculture (Table 4.10). After the second subculture, 12/14 had retained the ability to react positively in the assay; on third subculture, only 1 of these 12 isolates retained the ability and this was lost on the next subculture.
24/43 bacteria from patients' samples were able to bind MC27 on initial subculture; 19 were not. The results of repeat blotting on subculture of the 24 bacteria are shown in Table 4.10. Of these isolates 12/24 reacted positively after the second subculture; only 4 of these 12 retained the ability to react positively on third subculture; on fourth subculture 2/4 stained positively; 1 organism reacted in the assay after a total of 7 subcultures.

### 4.3.3.2 Anaerobic bacteria

Of the 23 anaerobic bacteria isolated from controls' stools which stained positively in the immunoblot assay, 9 retained the stain on initial subculture. These were further subcultured and re-tested (Table 4.11). Of these anaerobic isolates, 3/9 retained the ability to react positively in the assay after the second subculture; 1 of these 3 stained positively on third subculture but did not stain on further subculture and testing.

### Table 4.10

Reactivity of aerobic isolates in the immunoblot assay following subculture

<table>
<thead>
<tr>
<th>No. of subcultures</th>
<th>No. of isolates</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table 4.11

Reactivity of anaerobic isolates in the immunoblot assay following subculture

<table>
<thead>
<tr>
<th>No. of subcultures</th>
<th>No. of isolates</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Only 3/24 anaerobic bacteria isolated from patients' stools which stained positively in the immunoblot assay retained the ability to react in the assay on initial subculture. Of these, 2/3 isolates retained the ability to react in the assay after a second subculture; 1/2 still stained on a third subculture but not on a fourth.

When the screening was completed after 5 months, 10 stored isolates from patients' stools and 10 stored isolates from controls' stools were subcultured from CMB onto blood agar plates and tested in the immunoassay. Of the 20 isolates only 16 remained viable and of these 5 retained the ability to stain positively. All the viable bacteria were passaged several times on both blood agar and MacConkey agar at room temperature and 37°C. Both treatments failed to induce a positive reaction in those bacteria which were negative on initial re-testing after storage; the other 5 bacteria continued to display reactivity in the assay.

4.3.4 Identification of the bacterial component responsible for the positive reaction in the immunoblot assay

4.3.4.1 Protein staining after PAGE

There were 5 bands corresponding to the molecular weight markers: 1 thick band with BSA; 3 bands corresponding to the separated OMPG of A. calco. var. anitratus and several bands with E. coli MS (Plate 4.2).
Plate 4.2 Polyacrylamide gel electrophoresis of: Lane A - 5 molecular weight markers from 14,300 to 71,500 kDa; Lane B - Bovine serum albumin, molecular weight 66,000 kDa; Lane C - separated OMPG of *A. calco. var. anitratus*; Lane D - separated OMPG of *E. coli* MS.
4.3.4.2 Western blotting

There was no binding of MC27 to the nitrocellulose membrane after transfer of the separated proteins, but binding of anti-BSA to BSA was identified.

It was important to determine whether intact OMPG complexes i.e., unseparated by PAGE, bound MC27 and whether this ability was destroyed during the extraction process. The complexes under test were also boiled in sample buffer to mimic the conditions of the sample prior to PAGE. The various preparations were tested in a dot-blot assay.

The reaction of the unboiled samples of the OMPG complexes was the same regardless of the presence of antibody (Table 4.12). This suggested that protein A-HRP was binding non-specifically to OMPG complexes. A reaction with BSA was seen only in the presence of anti-BSA antibody. Boiling in sample buffer, however, destroyed the reactivity of the OMPG complexes and weakened the reactivity of BSA and anti-BSA antibody. The possibility that protein A was binding non-specifically to bacteria or OMPG was investigated using anti-mouse gammaglobulin-HRP as an enzyme detection system instead of protein A-HRP. Anti-mouse gammaglobulin-HRP also appeared to bind to whole bacteria and to the OMPG of *E. coli* and *A. calco. var. anitratus* in the absence of MC27.

**Table 4.12**

Staining pattern of bacterial preparations in the dot-blot assay

<table>
<thead>
<tr>
<th>Preparation</th>
<th>OMPG MC27***</th>
<th>protein A-HRP substrate</th>
<th>OMPG HSB</th>
<th>protein A-HRP substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMPG-AC*</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>OMPG-EC**</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>OMPG-AC (boiled)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>OMPG-EC (boiled)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>BSA (boiled)</td>
<td>+/-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* OMPG from *A. calco. var. anitratus*

** OMPG from *E. coli* MS

*** or anti-BSA antibody
4.3.5 Investigation of non-specific binding of protein A-HRP

Various experiments were performed to try to eliminate the apparent non-specific binding of protein A-HRP to bacteria.

4.3.5.1 Blocking experiments with protein A

Unconjugated protein A was used to try to block the binding of protein A-HRP in dot-blot experiments. Colour reaction with BSA was observed where expected i.e., in those wells in which anti-BSA was present; protein A at either concentration used (1/250 or 1/500 dilution) did not interfere with this reaction (Table 4.13). Positive colour reactions were seen in all test conditions with both bacterial OMPG indicating that there was non-specific binding by protein A-HRP to the bacterial OMPG which was not blocked by protein A. Positive reaction was present also in all test conditions with the whole bacterium of E. coli MS and to a lesser extent with the whole bacterium of A. calco. var. anitratus; this reaction was non-specific and not blocked by protein A. Other whole bacteria, Klebsiella K43 and K30 did not show any reaction in any of the test conditions.

**Table 4.13**

The effect of protein A as a blocking agent

<table>
<thead>
<tr>
<th>Preparation</th>
<th>bacteria/OMPG /BSA</th>
<th>TEST SCHEME</th>
<th>bacteria/OMPG /BSA</th>
<th>bacteria/OMPG /BSA</th>
<th>bacteria/OMPG /BSA</th>
<th>bacteria/OMPG /BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>protein A</td>
<td>HSB</td>
<td>protein A-HRP</td>
<td>HSB</td>
<td>protein A-HRP</td>
<td>HSB</td>
</tr>
<tr>
<td></td>
<td>MC27*</td>
<td>MC27*</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
</tr>
<tr>
<td>E. coli MS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OMPG-EC**</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. calco. var.</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>anitratus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMPG-AC***</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella K43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella K30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* or anti-BSA antibody
** E. coli MS OMPG
*** A. calco. var. anitratus OMPG
+ colour reaction
- no colour reaction
4.3.5.2 The effect of different concentrations of protein A-HRP

It was possible that at the concentration of protein A-HRP (1/500), it could still bind to sites on bacteria not blocked by protein A. A range of dilutions of protein A-HRP (doubling dilutions 1/250-1/4000) were tried in the assay to avoid this problem.

The colour reactions were equally intense with all dilutions of protein A-HRP and developed quickly for all bacteria and for the BSA and S. epidermidis positive controls (Table 4.14). This strongly suggested either that protein A-HRP was reacting with a substance on the bacteria or that the substrate itself was reacting with a bacterial enzyme.

Table 4.14
The effect of protein A-HRP concentration in dot-blot assay

<table>
<thead>
<tr>
<th>Bacteria/OMPG/BSA</th>
<th>Bacteria/OMPG/BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>protein A-HRP dilution</td>
</tr>
<tr>
<td></td>
<td>substrate</td>
</tr>
<tr>
<td>MC27*</td>
<td></td>
</tr>
<tr>
<td>protein A-HRP</td>
<td></td>
</tr>
<tr>
<td>dilution</td>
<td></td>
</tr>
<tr>
<td>substrate</td>
<td></td>
</tr>
<tr>
<td>HSB</td>
<td></td>
</tr>
<tr>
<td>protein A-HRP</td>
<td></td>
</tr>
<tr>
<td>dilution</td>
<td></td>
</tr>
<tr>
<td>substrate</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>MS</th>
<th>E. coli</th>
<th>MS-OMPG</th>
<th>A. calco. var. anitatus</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. calco.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>var. anitatus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. pneumonias</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* or anti-BSA- or anti-S. epidermidis antibody.
+ colour reaction
- no colour reaction
4.3.5.3 Detection of a peroxidase enzyme

To investigate this further, the scheme in Table 4.15 was employed. A concentration of protein A-HRP (1/500) was used and bacteria from 8 patients' stools were tested.

Table 4.15

Scheme to test for peroxidase in a dot-blot assay

<table>
<thead>
<tr>
<th>STEP 1</th>
<th>Test</th>
<th>(b) Control</th>
<th>(c) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP 2</td>
<td>bacteria</td>
<td>bacteria</td>
<td>bacteria</td>
</tr>
<tr>
<td>STEP 3</td>
<td>protein A-HRP</td>
<td>protein A-HRP</td>
<td>HSB</td>
</tr>
<tr>
<td>STEP 4</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
</tr>
</tbody>
</table>

The colour reactions were equally intense for all 3 treatments for each bacterium. The treatment (c) indicates that the substrate was reacting directly with the bacteria to produce a colour change. This implies that the bacteria have a peroxidase enzyme.

4.3.6 Attempts to inactivate peroxidase

Attempts were made to inactivate bacterial peroxidase to see if a colour reaction then occurred with protein-A-HRP and MC27 which would suggest specific binding of MC27.

The attempt to inactivate peroxidase by treating the bacteria impregnated in the NC paper with 0.3% H₂O₂ in methanol was unsuccessful due to dissolution of the nitrocellulose by the methanol.

When bacteria were fixed to an ELISA plate and treated with 0.3% H₂O₂ in methanol and substrate added, no colour reaction developed in either plate. Possibilities were that too few bacteria were added, or that the bacteria were washed off in the washing process, both possibilities being unlikely. More likely is that the enzyme was inactivated by the high pH (9.6) of the coating buffer.
Some inhibition of peroxidase activity by paraformaldehyde occurred with some bacteria (regardless of substrate, either TMB/DONS, diaminobenzidine or amino ethyl carbazole) but paraformaldehyde did not totally inactivate the enzyme.

4.3.7 Testing for the presence of peroxidase in aerobic bacteria

All the bacteria found to be positive in the initial screening assay were tested definitively by dot-blot for the presence of peroxidase. TMB/DONS was employed as substrate; the scheme is outlined in Table 4.16 and the results of some of the bacteria are shown.

Table 4.16
Scheme to test definitively for the presence of peroxidase

<table>
<thead>
<tr>
<th>(a) bacteria protein substrate</th>
<th>(b) bacteria protein substrate</th>
<th>(c) bacteria substrate</th>
<th>(d) HSB substrate</th>
<th>(e) HSB protein substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MC27</em></td>
<td>HSB</td>
<td>MC27</td>
<td>HSB</td>
<td>HSB</td>
</tr>
<tr>
<td>A-HRP</td>
<td>A-HRP</td>
<td>substrate</td>
<td>HSB</td>
<td>protein</td>
</tr>
</tbody>
</table>

| K. pneumoniae                 | +                              | +                     | +                | -                          |
| A. calco. var. anitratus      | +                              | +                     | +                | -                          |
| E. coli MS                    | +                              | +                     | +                | -                          |
| E. coli                       | +                              | +                     | +                | -                          |
| C. freundii                   | +                              | +                     | +                | -                          |
| BSA                           | +                              | -                     | -                | -                          |

* or anti-BSA antibody
+ colour reaction
- no colour reaction

Of 23 patients' aerobic isolates which had previously stained positively and had been stored in CMB, 6 showed mixed growth on subculture and were thus unsuitable for testing; 1 organism failed to grow; 2 isolates showed no staining; 14 showed equal reaction in both the test and the control demonstrating the presence of a peroxidase enzyme. MC27 and protein A-HRP were superfluous to the colour reaction.
For 27 isolates from controls tested, 8 bacteria failed to grow; 5 isolates showed no staining; 14 showed equal reaction in test and control assays demonstrating the presence of peroxidase.

### 4.3.8 Testing for the presence of peroxidase in anaerobic bacteria

Anaerobic bacteria which had been stored at -70°C were also tested for their ability to react with substrate (TMB/DONS) in a dot-blot assay (scheme same as outlined in Table 4.16).

Of 34 anaerobes, 15 showed colour reaction in (a) but no colour in (b). These bacteria included *Bacteroides* (10), *Lactobacilli* (4) and *Eubacterium lentae* (1). This suggested that these bacteria do not have a peroxidase enzyme and that the colour in (a) is due to specific binding of MC27 to bacteria.

There were 16/34 anaerobes that showed equal reaction in (a) and (b) indicating that these bacteria have catalase. These bacteria included *Bacteroides* (13), *Lactobacilli* (2) and *Bifidobacterium* (1). The remaining 3/34 samples grew but there was no colour reaction in (a) or (b).

### 4.3.9 Flow cytometry to investigate the presence of a HLA-B27 cross-reactive epitope

Although there was a positive reaction in control dots, the possibility of the presence of a bacterial HLA-B27 cross-reactive epitope in addition to peroxidase activity, could not be excluded by the above experiments. To determine definitively whether or not aerobic bacteria bind MC27 specifically, all previously isolated organisms which had stained positively on nitrocellulose were tested for the presence of the epitope by flow cytometry. Controls of *S. epidermidis* with anti-staphylococcal antibody and *N. meningitidis* and Group C *Neisseria* capsular polysaccharide antibody were used.

#### 4.3.9.1 Controls for assay system for aerobic bacteria

*N. meningitidis* reacted with antibody to Group C *Neisseria* capsular polysaccharide to produce a value of 44% compared with 4% for *Neisseria* without its corresponding antibody. For *S. epidermidis*, the higher reading of 9% with its corresponding antibody, contrasted with 0.9% without antibody. 19% of the staphylococci were positively fluorescent after immunoanalysis.
Although this reading was not as high as expected, it was sufficiently high to show that the assay was working.

The lower readings of staphylococci compared with *Neisseria* are probably a reflection of the age and loss of avidity of the staphylococcal antiserum in comparison with the meningococcal antiserum. There was no significant difference in readings between any of the tubes containing *Neisseria* and MC27 and those containing *Neisseria* without MC27 indicating the absence of a HLA-B27 cross-reactive epitope on the surface of the *Neisseria*.

### 4.3.9.2 Aerobes

The level of fluorescence in the test and control tubes for each bacterium was almost identical for all bacteria under investigation. There is no evidence for specific binding of MC27 to any of the enteric bacteria previously tested. There is no masking of a HLA-B27 cross-reactive epitope on the bacteria by the colour observed in the peroxidase/substrate reaction.

### 4.3.9.3 Anaerobes

The presence of a HLA-B27 cross-reactive epitope on anaerobes (n = 34) was assessed by the flow cytometry assay.

The staphylococcal control with antibody had a higher reading of 7% compared with a background reading of 0.7% without antibody. 18% of staphylococci were positively fluorescent after immunoanalysis.

The readings of all of the test samples showed little difference regardless of the presence of MC27. There was no evidence for specific binding of MC27 to the anaerobic bacteria isolated previously.

The above experiment was repeated using a higher concentration of MC27 (1/10) and a dilute suspension of bacteria (1/10 of a heavy suspension). There was no significant difference in readings between those tubes with and without MC27.

### 4.3.10 Testing for peroxidase and a HLA-B27 cross-reactive epitope on bacteria isolated from fresh faecal samples

Although the experiments were originally performed on bacteria from a fresh stool sample, all of the above experiments were done on stored bacteria. It is
possible that if a HLA-B27 cross-reactive epitope had been present initially, during storage in CMB structural changes could have occurred in the bacteria which might have obliterated the presence of this epitope. A further collection of stool samples was obtained from healthy individuals, dilutions and subcultures made and immunoblot screening performed. Of 10 stool samples, 4 (incubated aerobically) and 2 (incubated anaerobically) showed positively-staining colonies on immunoassay. The anaerobic subcultures did not grow, therefore no further investigation could be done. The aerobic bacteria were subcultured, identified as *E. coli* and tested for the presence of peroxidase; all were positive. All 4 isolates were tested by flow cytometry for the presence of a HLA-B27 cross-reactive epitope; all were negative.
4.4 DISCUSSION

Much controversy and confusion exists in the literature concerning the antigenic similarities between HLA-B27 and micro-organisms.

Antisera raised in rabbits to certain enteric bacteria lysed lymphocytes of HLA-B27-positive patients with AS but not the lymphocytes of HLA-B27-positive or HLA-B27-negative healthy individuals (Geczy et al., 1983). Further work (Prendergast et al., 1984) showed that a variety of organisms (Gram-positive as well as Gram-negative) from rectal swabs of 20 HLA-B27-positive AS patients were able to absorb the lymphocytotoxic activity of these antisera. Only 1 bacterial sample from 35 HLA-B27-negative controls showed a similar finding; isolates from 11 HLA-B27-positive healthy controls were unable to do this. These organisms persisted in the bowel flora of 5 patients for at least 9 months. The authors suggested that transmission of a plasmid could explain the cross-reactivity between these diverse bacteria. These findings were supported by a study on a larger series of 52 AS patients (McGuigan et al., 1986). Enteric organisms with determinants cross-reactive with HLA-B27-positive lymphocytes from AS patients were found in 100% of HLA-B27-positive AS patients but only in 2% of HLA-B27-positive controls.

Antibodies produced against one enteric bacterial species (E. coli, Y. enterocolitica, K. pneumoniae, S. flexneri or P. maltophilia) have shown cross-reactivity with the other members of this selection of Enterobacteriaceae (Pease et al., 1988). No evidence of cross-reactivity was shown, however, between HLA-B27 and K. pneumoniae by Archer and colleagues (1981) or between HLA-B27 and Y. enterocolitica by Pease et al. (1980). The latter authors suggested that if bacterial antigens were involved in the pathogenesis of AS, no specific bacterium was involved. The latter group subsequently used a formalin-treated preparation of Y. enterocolitica (to avoid destruction of heat-labile antigens) to prepare antisera in rabbits. They showed cytotoxicity against HLA-B27-positive lymphocytes from AS patients in some of the pre-immunisation sera as well as the post-immunisation sera, suggesting a previous encounter with certain organisms (MacIntosh et al., 1982).

In a lymphoproliferative assay, HLA-B27-positive cells from AS patients had a lower in vitro responsiveness to Klebsiella (isolates 411, 427, 433, 462 and 468)
compared with cells from HLA-B27-positive and HLA-B27-negative healthy controls. This could suggest either a cross-reactivity between *Klebsiella* antigens and HLA-B27, impaired antigen processing by macrophages or the production of suppressor cells and/or suppressor factors by lymphocytes of AS patients (Seager et al., 1979). Antisera to 1/4 strains of *Klebsiella* (427) produced lymphocytotoxicity.

In the present study, faecal samples were initially screened for aerobic bacteria with antigens cross-reactive with MC27 using the technique of van Alphen et al. (1991). One unusual feature of the procedure is the use of a high salt buffer, an environment which often interferes with antigen/antibody reactions; but, when the assay was tested in the current work and a high salt buffer compared with a low salt buffer, the high salt buffer was found to reduce non-specific background staining while allowing antigen/antibody interaction to take place.

A significantly higher proportion (75%) of samples from controls were positive compared with patients (51%). Similarly, on anaerobic culture a higher proportion of controls' samples were positive on immunoblot (50%) than patients' (28%). This is probably not be due to intrinsic differences in the flora of the two populations, but is more likely to reflect the longer period taken for the patients' specimens to arrive at the laboratory.

The range of aerobic and anaerobic bacteria identified in the immunoblot assay was diverse but similar in both populations. These results suggested that the HLA-B27-like epitope was present on a wide range of enteric organisms. *E. coli*, the most common aerobe in gut flora, was the most frequently identified aerobe while *Bacteroides* and *Lactobacilli* spp., the most common anaerobes, were the most frequently identified anaerobes in the assay.

In order to characterise this bacterial epitope, its stability on repeated subculture of organisms was assessed. Most of the aerobes (whether from patients' or controls' samples) remained positive in the immunoblot assay for one subculture. After that there was a low positivity on repeat subculture. Far fewer anaerobes remained positive on immunoassay on repeat subculture (9/23 from controls; 3/24 from patients) compared with aerobes (14/25 from controls; 24/43 from patients).
In order to identify the location of the cross-reactive epitope on the bacteria, the first step was to determine whether it was present on the outer surface of the bacterial cell wall. The 2 bacteria which showed the strongest reaction in the immunoassay were *E. coli MS* and *A. calcoaceticus* var. *anitratus*. The latter organism is one variant of *Acinetobacter calcoaceticus* spp which is a frequent commerial in man. The outer membrane peptidoglycan of the 2 bacteria were obtained by sonication and separated by polyacrylamide electrophoresis. Western blotting was performed. The absence of binding of MC27 to the separated OMPG led to testing the intact OMPG for this ability. The results suggested non-specific binding of protein A-HRP to the OMPG. A further series of experiments to investigate this non-specific binding included (unsuccessful) attempts to block binding with protein A. Different dilutions of protein A-HRP were used and equally intense reactions were found with the most dilute and the most concentrated sample of protein A-HRP. A reaction also occurred between bacteria and substrate alone. This suggested that the colour reaction in the assay was due to the presence of peroxidase on the bacteria. Further experiments confirmed this; MC27 and protein A-HRP were superfluous to the reaction.

Hydrogen peroxide is a strong oxidising agent and is toxic to most living organisms. Peroxidase enzymes (including catalase) degrade H$_2$O$_2$ and are present in the majority of aerobes and anaerobes. In this study several aerobic bacteria were shown to have peroxidase. Some bacteroides and other anaerobes were also shown to have the enzyme while other strains of bacteroides did not.

It is of interest that the Dutch workers have not had non-specific staining problems with this assay except with very old bacterial samples and have used protein A conjugates to detect antibodies to a variety of organisms including *H. influenzae*, *Neisseria*, *E. coli* and streptococci, all of which have peroxidase (Dr L van Alphen, personal communication). The reason for this is not clear as the same procedure was used in this work. There is no detail in their methodology of steps taken to inactivate bacterial peroxidase.

These results did not refute the possibility that in addition to a peroxidase enzyme, a HLA-B27-like epitope was also present. This was examined by flow cytometry. The EPICS allows thousands of cells to be counted and is thus much less subjective and time-consuming than microscopic assays.
In this case, it allowed the use of a detection system which did not include an activity possessed by the bacterium. In all aerobes and anaerobes tested there was no evidence of such an epitope.

The original experiments were performed on bacteria from a fresh stool sample, but the subsequent experiments were done on stored bacteria. In the event that a HLA-B27-like epitope had been present initially, but that during storage changes occurred which removed the presence of this epitope, a further collection of stool samples was obtained (from healthy individuals) and bacteria tested by flow cytometry for such an epitope. None was found.

There is no evidence for cross-reactivity between a mouse monoclonal anti-HLA-B27 antibody and a range of aerobic and anaerobic enteric bacteria isolated from patients with spondyloarthropathy (and healthy individuals) as determined by flow cytometry. This does not exclude the presence of a HLA-B27-like epitope which might be detected by other monoclonal antibodies to the HLA-B27 antigen. The majority of previous studies on bacterial antigens that cross-react with HLA-B27 were carried out with polyclonal sera. It is possible that in the present study, a polyclonal anti-HLA-B27 antiserum would have had a greater chance of detecting such determinants on bacterial surfaces cross-reactive with HLA-B27, but none was available at the time of the study.

The main conclusion is that there is no evidence, as detected by a mouse monoclonal anti-HLA-B27 antibody, for the presence of a HLA-B27-like epitope on a range of enteric bacteria isolated from patients with spondyloarthropathy and from healthy individuals.
CHAPTER 5

HUMORAL STUDIES IN ANKYLOSING SPONDYLITIS

5.1 INTRODUCTION

In some studies of the humoral immune response of patients with AS, total levels of immunoglobulins, particularly IgA, have been determined. In others, the specific antibody response to a variety of enterobacteria implicated in the disease pathogenesis has been measured. These studies have concentrated on the humoral response to laboratory strains of bacteria, in particular to Klebsiella. Interest has also been directed at the antibody response in AS to pathogenic bacteria associated with reactive arthritis such as Shigella, Yersinia and Salmonella. Antibody levels have sometimes been assessed with reference to disease activity, while other researchers have chosen to ignore disease activity.

There have been conflicting results for a number of reasons. There have been technical differences in the method employed to measure antibody levels; radial immunodiffusion (Kinsella et al., 1975; Cowling et al., 1980b; Calguneri et al., 1981; Laurent and Panayi, 1983; Franssen et al., 1985; van Bohemen et al., 1986; Collado et al., 1987; Sanders et al., 1987; Mackiewicz et al., 1989), linear plate method (Veyss and van Laere, 1973), rate nephelometry (Pease et al., 1987) and ELISA (Trull et al., 1983; Trull et al., 1984) for total antibody levels; radioimmunoassay (Ebringer and Ghuloom, 1986), radiobinding assay (Ghuloom and Ebringer, 1987), immunoblot (Shodjai-Moradi et al., 1992) and ELISA (Trull et al., 1983; Trull et al., 1984; Pease et al., 1987; Cooper et al., 1988; Khalafpour et al., 1988; van Bohemen et al., 1988; Makikola et al., 1991) for specific antibody measurement.

Different types of bacterial preparations have been used to measure specific antibody levels; intact bacteria (Trull et al., 1983; Trull et al., 1984; Ghuloom and Ebringer, 1987; Cooper et al., 1988; Khalafpour et al., 1988; van Bohemen et al., 1988), sonicated preparations (Pease et al., 1987); sonicated preparations separated by PAGE (Shodjai-Moradi et al., 1992) and outer membrane preparations (Makikola et al., 1991). These different preparations represent different 'targets' for antibodies in serum samples to bind to. For example, assays using formalin-killed bacteria are thought to measure antibodies directed to a multitude of surface
antigens on the intact organisms; whereas a sonicated preparation consists of secretory and internal proteins (in addition to surface antigens) due to the mechanical disruption of the bacterial cell wall. This means that the antibodies measured to sonicated preparations will have a different specificity from those to intact bacteria.

In those studies in which correlation between disease activity and antibody levels has been made, disease activity has been assessed either solely on clinical grounds, by laboratory criteria (CRP or ESR) or by a combination of the two. Some groups have even included a raised serum IgA as part of the criteria for assessing disease activity (Khalafpour et al., 1988; Shodjai-Moradi et al., 1992).

The control populations with which AS patients have been compared have varied between surveys.

It is difficult, therefore, to make valid comparisons between studies which have fundamental differences in selection of controls, disease activity assessment and methodology for antibody measurement.

There have been few longitudinal studies of immunoglobulin levels in ankylosing spondylitis. In this present survey, most patients (80/112) were seen on 2 visits. Serum immunoglobulins were measured and correlations made between clinical disease activity and ESR and CRP as inflammatory markers. As there have been conflicting reports in the literature concerning the relationship between a raised serum IgA and the presence of active disease in AS, correlations of these 2 parameters were made.

Invariably, when specific antibody assays have been performed in the past, sera have been tested against laboratory strains of bacteria which have been cultured for the purpose of the assay. These bacteria might not indeed have formed part of the faecal flora of any of the subjects under investigation. Another consideration is that when bacteria are cultured in vitro surface antigens can alter and may not be the same as those antigens encountered by the putative host; therefore, serum antibodies measured to bacteria grown on artificial media may not be very meaningful.

Measurement of serum antibody levels to autologous gut bacteria from patients with AS has not previously been determined. It was of interest to examine the antibody response of AS patients to their own gut bacteria to determine the
presence of hypo- or hyper-responsiveness; and, to compare this with the antibody response of healthy individuals to their own gut flora. It was decided to harvest as many bacteria as possible from faecal samples, to kill them with formalin and to test the autologous serum samples for antibodies using an ELISA. This type of harvest would be non-selective for a particular organism and would consist predominantly of anaerobes which considerably outnumber aerobes in the intestinal flora. The bacteria would have intact cell walls and sera would be measuring antibodies predominantly to the surface antigens. An alternative option would have been to culture each stool specimen, to chose certain colonies, identify, subculture and formalinise them and use them in an ELISA. The disadvantage of this is that it would have been impossible to decide which colonies to select as the most representative of that individual’s flora. The temptation would be to select the most prolific organism on culture, with the proviso that this may not be the most numerous in vivo, as differential growth occurs in artificial media. Also, it may not be the most prolific organism which is relevant (if any are) to pathogenesis. For these reasons, a non-selective bacterial harvest was deemed the most appropriate for the purposes of the study.

In a separate analysis, serum was collected from a small group of patients on 3 occasions over a 14-month period and tested for antibodies to bacteria harvested from a single faecal sample.

The objectives of this part of the project were:

1. to measure total serum IgG, IgA and IgM in AS patients and to correlate these levels with ESR, CRP and disease activity.

2. to investigate the serum humoral immune response to autologous gut bacteria in AS patients (n = 12) and controls (n = 13).
5.2 MATERIALS AND METHODS

5.2.1 Measurement of total IgG, IgA and IgM by ELISA

The method described by Zorgani et al. (1992) was used in these studies.

5.2.1.1 Materials

Buffers used are the same as those used in the ELISA for Lewis antigens in section 2.2.5.3.

The main immunoglobulin isotypes were detected with the following:

goat anti-human IgM conjugated with horseradish peroxidase (HRP-anti-IgM) (Sigma, Poole, Dorset);
rabbit anti-human IgA conjugated with horseradish peroxidase (HRP-anti-IgA) (Dako, Glostrup, Denmark);
goat anti-human IgG conjugated with horseradish peroxidase (HRP-anti-IgG) (Sigma, Poole, Dorset).

5.2.1.2 Collection of serum

Approximately 8ml of venous blood was allowed to clot and centrifuged at 1000g for 15min. The serum was aliquoted and stored at -20°C until required.

5.2.1.3 ELISA for measurement of total IgG

Duplicate wells of a microtitre plate were coated (100μl/well) with doubling dilutions (1/1000 - 1/512000) of standard serum (Behring Diagnostics, Hounslow, Middlesex) and a dilution of each test serum. Control wells comprising a) serum but no HRP-anti-IgG and b) HRP-anti-IgG but no serum were incorporated. All dilutions were made in coating buffer and the plate stored overnight at 4°C. All subsequent stages were carried out at room temperature. The plate was washed 5 times with washing buffer using a Dynatech plate washer and 100μl blocking buffer was added to each well to block empty binding sites with BSA. After 30min incubation, the plate was washed 5 times and 50μl HRP-anti-human IgG diluted in blocking buffer added per well. After 2hr incubation, the plate was washed 8 times and 50μl of activated substrate was added to each well. The colour was allowed to develop for 10-15min and was stopped by adding 50μl sulphuric acid (12.5%).
Optical density (OD) at 490nm was determined on a Dynatech MR700 ELISA plate reader and corrected by subtracting the OD of the corresponding blank. Samples were tested in duplicate and the readings averaged. The mean of the OD ELISA readings for each dilution of standard serum (containing 1140mg/dl IgG) was plotted against the IgG concentration at that dilution and a graph drawn. Mean ELISA readings for each test serum were compared to this graph and the concentration of IgG determined for the dilution used. From this the concentration of IgG in each test serum was calculated.

5.2.1.4 ELISA for measurement of total IgA and IgM

A capture method was used to determine total total IgA and IgM. Microtitre wells were coated with 100μl of either monoclonal anti-human IgA (1/500 in coating buffer) (Sigma, Poole, Dorset) or monoclonal anti-lgM (1/500 in coating buffer) (Sigma, Poole, Dorset) and stored overnight at 4°C. All further stages were carried out at room temperature. The next day they were washed 5 times and 100μl blocking buffer added per well for 30min. The plates were washed 5 times and 50μl of doubling dilutions of standard serum (1/100 - 1/51200 in blocking buffer) were added per well to both the IgA and the IgM plates. Test sera (50μl) diluted in blocking buffer were added to the IgA plate and to the IgM plate. After 2hr incubation, the plates were washed 5 times and either HRP-anti-IgA (50μl) diluted in blocking buffer or HRP-anti-IgM (50μl) diluted in blocking buffer was added per well. After 2hr incubation, the plates were washed 8 times and the substrate was added. The plates were then treated as those for the detection of total IgG.

The graph plotting the OD of each standard serum dilution with the concentration of IgA and IgM was determined from the known concentrations of IgA (250mg/dl) and IgM (114mg/dl) in standard serum.
5.2.2 Measurement of specific serum antibodies to autologous gut bacteria in AS patients and controls

5.2.2.1 Subjects

Patients

AS patients (12) included 11 (10 male, 1 female; mean age 36 years) with primary AS and 1 female with AS associated with inflammatory bowel disease (IBD); 9 patients were secretors and 3 were non-secretors. Details of disease activity, ESR and CRP are shown in Table 5.1.

<table>
<thead>
<tr>
<th>Disease activity</th>
<th>No.</th>
<th>Mean ESR</th>
<th>Mean CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive</td>
<td>5</td>
<td>15.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Probably active</td>
<td>6</td>
<td>15.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Active</td>
<td>1</td>
<td>11.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Controls

Controls (13) (8 male, 5 female; mean age 36 years) were healthy laboratory staff with no rheumatic complaints or history of rheumatic disease; 7 were secretors and 6 were non-secretors. ESR and CRP were not determined for controls.

5.2.2.2 Harvesting of bacteria from faeces

A faecal sample was collected from each subject (section 3.2.2). Faeces (5-10g) was added to 20ml of sterile PBS and mixed thoroughly with a rotary mixer. The suspension was filtered three times through sterile glass wool to remove particulate debris. After each passage the glass wool was renewed. The filtrate was centrifuged at 320g for 7min, the pellet debris discarded and the supernatant re-centrifuged at 320g for 7min. This was repeated twice more and the final supernatant filtered through Whatman No.1 filter paper. The resultant filtrate was examined microscopically (x 40) to check that it was free of significant debris. (If debris was present, the filtrate was further centrifuged at 320g for 7min and re-filtered through Whatman No.1 paper). The filtrate was centrifuged at 1900g for
15min to pellet the bacteria. The pellet was washed three times (1900g for 15min) in 20ml sterile PBS. The bacteria were killed by resuspending them in 20ml 0.5% formalin overnight at 4°C. The bacteria were washed three times (1900g for 15min) in sterile PBS. The pellet was suspended in 2ml coating buffer and stored at 4°C until required.

5.2.2.3 Bacterial count

A 1/10 dilution of bacteria extracted from each faecal sample and suspended in coating buffer was made and the optical density (OD) at 540nm obtained. A sample from the same bacterial suspension was diluted and stained with 0.1% methyl violet and counted using a Thoma haemocytometer. From this, the concentration of bacteria in the neat suspension was calculated. Dilutions of the suspension were counted and a graph was drawn; the OD and the bacterial concentration were linear over the range 0.15-0.6. An OD reading of 0.35 was found to represent a bacterial concentration of 5 x 10^8/ml. The bacterial concentration of an unknown sample could therefore be calculated from its optical density.

5.2.2.4 ELISA for measurement of specific antibodies to autologous gut bacteria

An ELISA was used to measure specific antibodies to gut bacteria. In order to optimise conditions, titration of the bacterial concentration, serum dilution and concentration of HRP-anti-IgG, HRP-anti-IgA and HRP-anti-IgM was necessary. Unless otherwise stated, all procedures were carried out at room temperature.

Bacteria suspended in coating buffer (100μl of 2-3 x 10^8/ml) were added in triplicate to wells on three different plates which were stored overnight at 4°C. The plates were washed 5 times, blocking buffer (100μl) was added to each well for 30min and plates were washed again 5 times. For the IgG plate, 50μl test serum diluted in blocking buffer was added to each well. For both the IgA and IgM plates, 50μl of undiluted serum was added to each well. After 2hr incubation, plates were washed 5 times and 50μl of the following (diluted in blocking buffer) were added to the appropriate wells: HRP-anti-IgG, HRP-anti-IgA or HRP-anti-IgM. After 2hr, plates were washed 8 times and substrate added.

For each experiment the concentration of specific antibody of each class was calculated with reference to appropriate graphs of total IgA, IgM and IgG in a standard serum run in parallel and determined by ELISA.
5.2.3 Statistical methods

The Kendall correlation coefficient was used to correlate total immunoglobulins in patients with ESR, CRP, disease activity and secretor status. Mann-Whitney analysis was used to compare the total and specific immunoglobulins in patients and controls. The Wilcoxon rank sum test was used to correlate total and specific immunoglobulins in patients with disease activity and to compare the percentage of specific/total antibody of each class produced to autologous enteric bacteria in patients and controls.
5.3 **RESULTS**

5.3.1 **Optimisation of ELISA for measurement of total immunoglobulins**

Optimisation of reagents was necessary to ensure that reagent concentration was not limiting the detection system.

**Titration of HRP-anti-IgG for total IgG assay**

A range of dilutions of HRP-anti-IgG was tested (Figure 5.1) using serum in excess. The 1/100 dilution of HRP-anti-IgG produced the highest readings. In the interests of economy a 1/200 dilution of HRP-anti-IgG was chosen to measure total IgG to conserve reagents while still producing readings well above background.

![Figure 5.1 Titration of HRP-anti-IgG for total IgG measurement](image-url)
Titration of serum dilution for total IgG assay

Using 1/200 dilution of HRP-anti-IgG, doubling dilutions of test serum (1/100-1/51200) were tested in an ELISA and compared to dilutions of the standard serum (1/1000-1/512000) (Figure 5.2). The reading for 1/51200 dilution of test serum (dilution no.9) corresponded to the middle part of the linear graph for the standard serum; therefore 1/50000 dilution was chosen to measure total IgG. At this dilution the majority of samples fall on the linear part of the standard graph.

Figure 5.2 Titration of serum dilution for total IgG measurement. The dilutions represent doubling dilutions from 1/1000-1/512000 for standard serum and 1/100-1/51200 for test serum samples.

Titration of HRP-anti-IgM for total IgM assay

To determine the optimum concentration of HRP-anti-IgM, the following dilutions of HRP-anti-IgM were tested in an ELISA assay; 1/1000 and doubling dilutions 1/5000 to 1/40000 (Figure 5.3). Serum was used in excess (1/10) with the capture monoclonal anti-IgM antibody (1/500). The highest reading was with the 1/1000 dilution of HRP-anti-IgM and this was chosen as the working dilution to measure total IgM.
Titration of serum dilution for total IgM assay

Doubling dilutions (1/20-1/1280) in blocking buffer were made of two control sera and compared with the same dilutions of standard serum in an ELISA using HRP-anti-IgM (1/1000) and the capture monoclonal anti-IgM (1/500) (Figure 5.4). The linear part of the graph of OD plotted against concentration lay between 1/320 and 1/1280 dilutions of the standard serum. For control serum 2, readings for 3 dilutions 1/320, 1/640 and 1/1280 lay on the linear part of the graph, while for control serum 1, only the value for the 1/1280 dilution fell on the linear part. A 1/1000 dilution of serum was chosen to determine the total IgM.
Titration of HRP-anti-IgA for total IgA assay

Doubling dilutions (1/25-1/3200) of HRP-anti-IgA in blocking buffer were tested in an ELISA using two control sera in excess (1/50 dilution). The optimal dilution of HRP-anti-IgA was 1/100 (Figure 5.5).

Figure 5.5 Titration of HRP-anti-IgA for total IgA measurement

Titration of serum dilution for total IgA assay

Doubling dilutions (1/40-1/5120) of one control serum were compared with doubling dilutions (1/500-1/64000) of a standard serum in an ELISA using HRP-anti-IgA (1/100) and monoclonal anti-IgA (1/500). The results are shown in Figure 5.6. The readings for 1/2560 (dilution no.7) and 1/5120 (dilution no.8) of the control serum corresponded to points on the linear part of the graph of the standard serum. Therefore, a 1/5000 dilution of serum was used to measure total IgA.
5.3.2 Total immunoglobulin G, A and M in sera of patients at the 2 visits

Patients were assessed on 2 visits 7 months apart; 112 patients attended on the first visit and 80 also attended on the second visit. Clinical disease activity was assessed, and blood samples taken for ESR, CRP and total serum immunoglobulins. Correlations were made between total serum immunoglobulins, ESR, CRP, disease activity and secretor status.

The results were analysed in two ways; the results of the total patient group (patients with primary AS and those with secondary AS ie., AS associated with other conditions) were compared with the results of the primary AS group to see if all the spondyloarthopathies, as a group, behaved in the same way as the primary AS group. (The small numbers of patients in each subgroup of secondary AS precluded any direct comparison of them with the AS group).

As samples were available for 80 patients seen on 2 occasions, correlations between changes in variables were made.
5.3.2.1 Total immunoglobulins in each disease category

The mean immunoglobulin values at both visits for all disease subgroups are shown in Table 5.2. There was no significant difference in the mean IgG, IgA or IgM between any of the disease categories at either the first or the second visit.

Table 5.2
Mean serum immunoglobulins (mg/ml) at first and second visits for patient population

<table>
<thead>
<tr>
<th>Disease category</th>
<th>AS</th>
<th>IBD</th>
<th>ReA</th>
<th>PSA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visit 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>92</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>112</td>
</tr>
<tr>
<td>IgG</td>
<td>15.96</td>
<td>14.2</td>
<td>15.5</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>6.5</td>
<td>5.62</td>
<td>5.63</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>2.01</td>
<td>2.85</td>
<td>2.27</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td><strong>Visit 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>63</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>80</td>
</tr>
<tr>
<td>IgG</td>
<td>15.32</td>
<td>14.44</td>
<td>13.6</td>
<td>13.84</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>4.83</td>
<td>4.31</td>
<td>6.0</td>
<td>9.21</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>2.35</td>
<td>2.71</td>
<td>2.91</td>
<td>3.09</td>
<td></td>
</tr>
</tbody>
</table>

5.3.2.2 Correlations between total immunoglobulins, ESR, CRP, disease activity and secretor status

a) Total patient group:

There was no correlation between any of the immunoglobulin classes and either secretor status or disease activity. There was a weak correlation between IgA and ESR (p < 0.05, r = 0.16) and between IgG and CRP (p < 0.05, r = 0.16). There was no correlation between IgM and either ESR or CRP.

Comparing the change in IgG, IgA and IgM with changes in disease activity, ESR and CRP between the 2 visits, the only significant finding was a positive correlation between the change in IgM levels and the change in CRP (p < 0.05, r = 0.17). The change in IgM and IgA correlated (p < 0.01, r = 0.23) between the 2 visits, but neither correlated with changes in IgG.
b) AS patients:

There was no correlation between total IgG, IgA or IgM and either secretor status, disease activity or CRP in AS patients. ESR, however, correlated with IgA ($p < 0.05$, $r = 0.18$) and with IgM ($p < 0.05$, $r = 0.15$).

The only correlation between the change in serum immunoglobulins between the 2 visits and changes in ESR, CRP and disease activity, was a negative correlation between the change in IgG and the change in disease activity ($p < 0.05$, $r = -0.2$). The changes in IgM and IgG correlated with each other ($p < 0.05$, $r = 0.19$), but neither correlated with changes in IgA.

5.3.3 Measurement of total immunoglobulin levels and the humoral response to autologous gut bacteria in AS patients and controls

5.3.3.1 Total serum immunoglobulin G, A and M in AS patients and controls

The ELISA for total serum immunoglobulins was used to measure the total serum IgG, IgA and IgM in a cohort of patients ($n = 12$) and controls ($n = 13$). The values for the total immunoglobulin levels in patients and controls are shown in Table 5.3.

Table 5.3
Total serum immunoglobulins in patients and controls

<table>
<thead>
<tr>
<th>Mean total Ig (mg/ml)</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>IgG</td>
<td>17.02 (12.9-21.1)*</td>
<td>14.4 (11.7-17.1)</td>
</tr>
<tr>
<td>IgA</td>
<td>4.32 (3.3-5.3)</td>
<td>2.94 (2.0-3.9)</td>
</tr>
<tr>
<td>IgM</td>
<td>2.06 (1.5-2.6)</td>
<td>1.93 (1.3-2.5)</td>
</tr>
</tbody>
</table>

* Figures in parentheses represent the 95% confidence interval

There was no significant difference in total immunoglobulin G, A or M levels between patients and controls and no correlation between immunoglobulins and disease activity in patients.
5.3.3.2 Optimisation of ELISA for measurement of specific immunoglobulin

Optimisation of reagents was required to ensure that reagent concentration was not limiting the detection system.

Titration of bacterial concentration

A range of concentrations of bacteria (10^6-10^8 bacteria/ml) were added to wells and tested in an ELISA (Figure 5.7). The highest reading was for the concentration of 6.6 x 10^8 bacteria/ml. A concentration of approximately 2 x 10^8 bacteria/ml was used (in the interests of economy) in future specific IgM assays.

![Figure 5.7 Titration of bacterial concentration](image)

Titration of HRP-anti-IgM for specific IgM assay

A microtitre plate was coated overnight with a concentration of 10^8 bacteria/ml (100μl/well). Neat serum was added. The following dilutions of HRP-anti-IgM were tested; 1/1000, 1/2000 and doubling dilutions (1/100-1/6400) (Figure 5.8). 1/1000 dilution was chosen as the concentration of HRP-anti-IgM to use to measure specific IgM.
Figure 5.8 Titration of HRP-anti-IgM for specific antibody measurement

Titration of serum dilution for specific IgM assay

A microtitre plate was coated with $1.2 \times 10^8$ bacteria/ml (100μl/well) and tested with doubling dilutions of serum (Neat-1/32) and HRP-anti-IgM (1/1000) (Figure 5.9). When the readings were compared with a graph of standard serum, the highest ELISA reading was for neat serum and this corresponded to the lower end of the linear part of the graph for the standard serum. Neat serum was therefore used to measure specific IgM antibodies to gut bacteria.

Figure 5.9 Titration of serum dilution for specific IgM measurement
Titration of HRP-anti-IgA and serum dilution for specific IgA assay

Doubling dilutions of serum (Neat-1/32) and HRP-anti-IgA (1/25-1/200) were tested in checkerboard fashion using bacteria at a concentration of $1.2 \times 10^8$/ml. Comparing the readings obtained with those for standard serum, the optimum serum dilution was for neat serum (Figure 5.10). There was little difference in values obtained using 1/100 or 1/200 HRP-anti-IgA. 1/100 was chosen for convenience as HRP-anti-IgA (1/100) was used to measure total IgA.

![Figure 5.10 Titration of serum dilution and HRP-anti-IgA for specific antibody measurement](image)

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Titration of HRP-anti-IgG for specific IgG assay

Using bacteria at a concentration of $1.1 \times 10^8$/ml, and serum in excess (1/2), a range of dilutions of HRP-anti-IgG were tested (Figure 5.11). The optimum dilution was 1/100, but in order to save reagents without compromising results, 1/200 dilution of HRP-anti-IgG was used to assay specific IgG antibodies.
Titration of serum dilution for specific IgG assay

Doubling dilutions of serum (neat-1/512) were tested in an ELISA using bacteria at a concentration of $2.1 \times 10^8$/ml and HRP-anti-IgG (1/200). The optical density readings plateaued for the neat serum sample down to 1/32 dilution (Figure 5.12). 1/200 serum dilution was chosen to measure specific IgG antibodies as the reading lay on the straight part of the graph.

![Figure 5.11 Titration of HRP-anti-IgG for specific antibody measurement](image1)

![Figure 5.12 Titration of serum for specific IgG measurement](image2)
5.3.3.3 Specific IgG, IgA and IgM antibodies to autologous gut bacteria in AS patients and controls

The mean values for the specific serum antibodies in patients and controls are shown in Table 5.4.

Table 5.4
Specific IgG, IgA and IgM antibodies to autologous gut bacteria in AS patients and controls

<table>
<thead>
<tr>
<th>Mean specific antibody (μg/ml)</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>IgG</td>
<td>9.72 (6.4-13.1)*</td>
<td>12.57 (7.1-18.1)</td>
</tr>
<tr>
<td>IgA</td>
<td>1.85 (0.8-2.9)</td>
<td>2.22 (1.3-3.1)</td>
</tr>
<tr>
<td>IgM</td>
<td>1.17 (0.5-1.8)</td>
<td>1.71** (1.3-2.1)</td>
</tr>
</tbody>
</table>

* Figures in parentheses represent the 95% confidence interval
** excluding GS, specific Ig M = 53.4 mg/ml

There was no significant difference in the mean specific IgG or IgA antibodies to autologous gut bacteria between patients and controls but the mean specific IgM antibody level was significantly lower in patients than controls (p < 0.05).

Although the absolute levels of specific IgM to autologous gut bacteria were lower for patients than controls, the percentage of the total antibody that was directed against enteric bacteria, the specific/total antibody ratio (S/T ratio), was determined for each subject to see if patients produced a proportionately lower fraction of their total IgM as specific IgM than did controls. The same ratios were determined for specific/total IgG and IgA and are shown in Tables 5.5 and 5.6.
Table 5.5
Percentage of total serum antibody of each class produced to autologous gut bacteria in patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF</td>
<td>0.059</td>
<td>0.272</td>
<td>0.057</td>
</tr>
<tr>
<td>JD</td>
<td>0.03</td>
<td>0.02</td>
<td>0.103</td>
</tr>
<tr>
<td>AD</td>
<td>0.035</td>
<td>0.017</td>
<td>0.039</td>
</tr>
<tr>
<td>DB</td>
<td>0.034</td>
<td>0.021</td>
<td>0.048</td>
</tr>
<tr>
<td>JR</td>
<td>0.022</td>
<td>0.028</td>
<td>0.046</td>
</tr>
<tr>
<td>FB</td>
<td>0.12</td>
<td>0.018</td>
<td>0.081</td>
</tr>
<tr>
<td>RM</td>
<td>0.033</td>
<td>0.007</td>
<td>0</td>
</tr>
<tr>
<td>PF</td>
<td>0.056</td>
<td>0.022</td>
<td>0.032</td>
</tr>
<tr>
<td>JOL</td>
<td>0.074</td>
<td>0.079</td>
<td>0.056</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.033</td>
<td>0.024</td>
<td>0.028</td>
</tr>
<tr>
<td>JT</td>
<td>0.13</td>
<td>0.033</td>
<td>0.023</td>
</tr>
<tr>
<td>DD</td>
<td>0.157</td>
<td>0.106</td>
<td>0.104</td>
</tr>
</tbody>
</table>
Table 5.6  
Percentage of total serum antibody of each class produced to auologous gut bacteria in controls

<table>
<thead>
<tr>
<th>Control</th>
<th>Specific/Total antibody %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>DB</td>
<td>0.079</td>
</tr>
<tr>
<td>DM</td>
<td>0.035</td>
</tr>
<tr>
<td>VJ</td>
<td>0.008</td>
</tr>
<tr>
<td>AZ</td>
<td>0.2</td>
</tr>
<tr>
<td>GS</td>
<td>0.281</td>
</tr>
<tr>
<td>MK</td>
<td>0.098</td>
</tr>
<tr>
<td>WM</td>
<td>0.042</td>
</tr>
<tr>
<td>LC</td>
<td>0.035</td>
</tr>
<tr>
<td>JV</td>
<td>0.091</td>
</tr>
<tr>
<td>MA</td>
<td>0.089</td>
</tr>
<tr>
<td>MB</td>
<td>0.032</td>
</tr>
<tr>
<td>JS</td>
<td>0.046</td>
</tr>
<tr>
<td>JC</td>
<td>0.149</td>
</tr>
</tbody>
</table>

The S/T ratios for IgM were significantly lower for patients than controls (p < 0.01 with GS included, p < 0.05 without GS included). A high S/T ratio for IgM (2.19%) was obtained for one control (GS, the author) who had prepared all the faecal samples (Table 5.6). The specific IgM for GS was very high (53.4μg/ml) compared with other controls, but the total IgM (2.43mg/ml) was similar to that of the other controls.

The S/T ratios for IgA were also significantly lower for patients than for controls (p < 0.05). One of the controls (DB) appeared to be IgA deficient. The total IgA of another control (MK) was similar to that of the others in the group, but the specific IgA was very high (38μg/ml) resulting in a high S/T ratio. The reason for this is not clear. MK had not had a clinically evident enteric infection in the recent past.

In contrast to the lower S/T ratios for IgM and IgA in patients compared with controls, there was no significant difference in the S/T ratios for IgG between the two groups.
There was no correlation between the total or specific antibody levels of any immunoglobulin class and the disease activity of the patients.

5.3.3.4  *Serial humoral studies in patients with AS*

This experiment sought to determine the total IgG, IgA, and IgM in three serum samples taken at intervals of several months over a 14 month period in a group of 12 AS patients (the same patients as in section 5.2.2.1). Bacteria were harvested from a faecal specimen produced at the same time as the third serum sample. Specific IgG, IgA and IgM antibodies to those bacteria were measured in all three serum samples and the results compared.

**Levels of total IgG, IgA, IgM over a 14 month period**

a)  **Total IgG**

A complete series of three serum samples was collected for 11/12 AS patients. For one patients (DD) serum was only available at times 1 and 3. For most patients there is little variation in the level of IgG with time (Table 5.7). One patient (JD) did show variation, with a low level of IgG initially which rose considerably in the second and third samples. There was considerable variation in IgG levels between patients, from a mean value of all three samples of 6.3mg/ml to 26.1mg/ml. The mean total IgG of all the samples did not differ significantly at time 1, 2 or 3; 16.7, 17.6 and 18.7mg/ml respectively.
Table 5.7
Total IgG in 3 serum samples over 14 months in AS patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>TOTAL IgG (mg/ml)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum sample</td>
<td>First</td>
<td>Second</td>
<td>Third</td>
<td>Mean value</td>
</tr>
<tr>
<td>GF</td>
<td>8.5</td>
<td>10.15</td>
<td>10.15</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>JD</td>
<td>14.2</td>
<td>34.0</td>
<td>30.0</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>14.0</td>
<td>17.65</td>
<td>15.15</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>DB</td>
<td>18.75</td>
<td>19.25</td>
<td>22.15</td>
<td>20.05</td>
<td></td>
</tr>
<tr>
<td>JR</td>
<td>22.15</td>
<td>22.15</td>
<td>23.4</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>4.41</td>
<td>4.41</td>
<td>10.15</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>15.1</td>
<td>14.6</td>
<td>15.1</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>10.03</td>
<td>10.03</td>
<td>10.85</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>JOL</td>
<td>23.4</td>
<td>24.6</td>
<td>29.15</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>17.65</td>
<td>13.3</td>
<td>17.65</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>JT</td>
<td>29.15</td>
<td>23.4</td>
<td>23.4</td>
<td>25.3</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>23.4</td>
<td>—</td>
<td>17.65</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>Mean value</td>
<td>16.7</td>
<td>17.6</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
b) Total IgA

Results were complete in 11/12 patients. In one patient (DD) only two serum samples were available. There was little variation in total serum IgA with time for all patients (Table 5.8). There was little variation in IgA levels between individuals. The range in mean values of all three samples for each patient was 2.5mg/ml to 6.45mg/ml. The mean values of total IgA of the serum samples for each time interval 1, 2 and 3 were similar, 4.7, 4.6 and 4.1 mg/ml respectively.

Table 5.8
Total IgA in 3 serum samples over 14 months in AS patients

| Patient | TOTAL IgA (mg/ml) | Serum sample | | | |
| --- | --- | --- | --- | --- | |
| | FIRST | SECOND | THIRD | MEAN VALUE | |
| GF | 5.45 | 4.35 | 5.15 | 5.0 | |
| JD | 7.25 | 6.65 | 5.45 | 6.45 | |
| AD | 4.48 | 4.48 | 4.35 | 4.4 | |
| DB | 2.27 | 2.7 | 2.7 | 2.5 | |
| JR | 5.15 | 4.35 | 3.9 | 4.5 | |
| FB | 3.9 | 3.9 | 4.35 | 4.05 | |
| RM | 2.7 | 3.9 | 2.7 | 3.1 | |
| PF | 5.15 | 4.8 | 4.35 | 4.8 | |
| JOL | 3.9 | 4.35 | 3.9 | 4.05 | |
| DMCA | 5.15 | 5.45 | 4.55 | 5.05 | |
| JT | 6.65 | 5.75 | 4.55 | 5.65 | |
| DD | 3.9 | --- | 3.1 | 3.5 | |

Mean value 4.7 4.6 4.1

152
c) **Total IgM**

Results were complete in 11/12 patients. For one patient (DD) serum was only available at times 1 and 3. As for IgG and IgA, there was little variation in total serum IgM with time for any individual patient (Table 5.9). Neither was there any overall trend in the results. The range in mean values of the three samples for each patient was 0.71mg/ml to 2.5mg/ml. The mean values of total IgM for each time interval 1, 2 and 3 are very similar, 1.29, 1.34 and 1.4mg/ml respectively.

**Table 5.9**

<table>
<thead>
<tr>
<th>Patient</th>
<th>TOTAL IgM (mg/ml)</th>
<th>Serum Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>GF</td>
<td>0.87</td>
<td>0.88</td>
</tr>
<tr>
<td>JD</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>AD</td>
<td>1.97</td>
<td>1.88</td>
</tr>
<tr>
<td>DB</td>
<td>1.21</td>
<td>1.46</td>
</tr>
<tr>
<td>JR</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>FB</td>
<td>1.02</td>
<td>0.93</td>
</tr>
<tr>
<td>RM</td>
<td>0.65</td>
<td>0.82</td>
</tr>
<tr>
<td>PF</td>
<td>1.33</td>
<td>1.52</td>
</tr>
<tr>
<td>JOL</td>
<td>2.15</td>
<td>2.33</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.87</td>
<td>1.12</td>
</tr>
<tr>
<td>JT</td>
<td>1.33</td>
<td>1.28</td>
</tr>
<tr>
<td>DD</td>
<td>1.2</td>
<td>---</td>
</tr>
<tr>
<td>Mean value</td>
<td>1.29</td>
<td>1.34</td>
</tr>
</tbody>
</table>
Specific IgG, IgA and IgM antibodies to autologous gut bacteria harvested from a single faecal specimen

a) Specific IgG

In 7/12 patients results were complete for all three serum samples (Table 5.10). In 1 patient (DD) only two serum samples were obtained and in 1 patient (DMCA) the bacterial suspension had lysed, thus rendering the results invalid. In 2 patients (AD, RM) values were too low to be detected despite repeat testing of the samples at 1/50 dilution. There is little difference in the level of specific IgG antibodies between all three serum samples for any one individual. The intra-patient variation in mean values of all three samples ranged from 6.4µg/ml to 19.0µg/ml. The mean values of the serum samples for each time interval 1, 2 and 3 were similar, 8.8, 8.59 and 9.03µg/ml respectively.

Table 5.10
Specific IgG antibodies in 3 serial serum samples to autologous gut bacteria from one faecal sample

<table>
<thead>
<tr>
<th>Patient</th>
<th>SPECIFIC IgG (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum sample</td>
</tr>
<tr>
<td></td>
<td>First</td>
</tr>
<tr>
<td>GF</td>
<td>8.56</td>
</tr>
<tr>
<td>JD</td>
<td>6.4</td>
</tr>
<tr>
<td>DB</td>
<td>8.76</td>
</tr>
<tr>
<td>JR</td>
<td>11.9</td>
</tr>
<tr>
<td>FB</td>
<td>7.8</td>
</tr>
<tr>
<td>PF</td>
<td>4.3</td>
</tr>
<tr>
<td>JOL</td>
<td>6.2</td>
</tr>
<tr>
<td>JT</td>
<td>15.8</td>
</tr>
<tr>
<td>DD</td>
<td>10.2</td>
</tr>
<tr>
<td>Mean value</td>
<td>8.88</td>
</tr>
</tbody>
</table>

b) Specific IgA

For only 3 patients (JD, AD, RM) were results complete (Table 5.11). In 4 patients (DB, FB, PF, JT) results were obtained for only two serum samples. In 6 patients readings were too high to be recorded despite considerable dilution of serum
samples (1/100). Due to the sparsity of complete results little comment can be made. There is no obvious trend in the values and no wide variation between patients.

Table 5.11
Specific IgA antibodies in serum samples to autologous gut bacteria from one faecal sample

<table>
<thead>
<tr>
<th>Patient</th>
<th>SPECIFIC IgA (µg/ml)</th>
<th>Serum sample</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD</td>
<td>2.23</td>
<td></td>
<td>2.63</td>
<td>2.5</td>
<td></td>
<td>2.45</td>
</tr>
<tr>
<td>AD</td>
<td>0.96</td>
<td></td>
<td>1.15</td>
<td>1.15</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>DB</td>
<td>3.12</td>
<td></td>
<td>2.5</td>
<td>too high</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>2.1</td>
<td></td>
<td>2.87</td>
<td>too high</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>1.7</td>
<td></td>
<td>1.83</td>
<td>0.91</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>1.56</td>
<td></td>
<td>1.39</td>
<td>too high</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>JT</td>
<td>1.56</td>
<td></td>
<td>1.39</td>
<td>too high</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

c) Specific IgM

Results were complete in only 6 patients (Table 5.12). In 1 patient (AD) only the first and second samples were available. In 4 patients (JR, JOL, DD, DMCA) readings were too high (despite considerable dilution of serum samples) and in 1 (RM) too low to be detected. Again there was little intra-patient variation in specific IgM levels with time in all but one case (GF).

Table 5.12
Specific IgM antibodies in 3 serial serum samples to autologous gut bacteria from one faecal sample

<table>
<thead>
<tr>
<th>Patient</th>
<th>SPECIFIC IgM (µg/ml)</th>
<th>Serum sample</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF</td>
<td>2.68</td>
<td></td>
<td>1.78</td>
<td>1.08</td>
<td></td>
<td>1.85</td>
</tr>
<tr>
<td>JD</td>
<td>1.52</td>
<td></td>
<td>1.38</td>
<td>1.78</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>AD</td>
<td>0.72</td>
<td></td>
<td>0.65</td>
<td>too low</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>DB</td>
<td>1.97</td>
<td></td>
<td>1.52</td>
<td>1.43</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>0.87</td>
<td></td>
<td>0.87</td>
<td>0.65</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>1.7</td>
<td></td>
<td>1.74</td>
<td>1.43</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>JT</td>
<td>1.33</td>
<td></td>
<td>1.18</td>
<td>1.02</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

Mean value | 1.54 | 1.3 | 1.4 |
5.4 DISCUSSION

5.4.1 Total immunoglobulins in patients, disease activity, ESR, CRP and secretor status

In this part of the study, total serum immunoglobulins were not measured in controls, therefore no comparisons can be drawn between the levels of serum immunoglobulins in AS patients and those in healthy individuals. A number of previous surveys have demonstrated raised serum IgA in AS patients compared with controls (section 1.7.9.1). In the data presented here, there was no correlation between serum IgA, G or M and disease activity as assessed clinically, for either patients with primary AS or with AS in association with other diseases. The lack of correlation with serum IgA and disease activity in the present study agrees with others in which disease activity was also assessed on clinical grounds (Laurent and Panayi, 1983; Pease et al., 1987). The present findings partly agree with Franssen et al. (1985) who reported no correlation between both total serum IgG and IgM and a disease activity index. This index was based on the duration of morning stiffness, a pain score, chest expansion, lumbar flexion measurement and radiological changes. The index did correlate with serum IgA.

In the present longitudinal study, there was no correlation between the changes in total serum immunoglobulins and changes in disease activity between 2 visits, strengthening the observation that total serum immunoglobulin levels in AS patients bear no relationship to disease activity. This contrasts with the longitudinal study by Franssen et al. (1985) in which changes in total serum IgA with time correlated with changes in their index of disease activity. In this latter study, 38 AS patients were seen 5 times over a 48 week period as part of a clinical trial comparing two different types of NSAID.

For all patients with spondyloarthropathy and AS patients alone, there was a significant correlation between serum total IgA and ESR. This agrees with other workers (Cowling et al., 1980b; Trull et al., 1984; Mackiewicz et al., 1989). As was previously demonstrated (section 2.3.6), there is a strong correlation between disease activity and ESR, but not between disease activity and total serum immunoglobulins of any class. This suggests that the correlation between IgA and ESR, although it is statistically significant for both the total patient group (p < 0.5, r = 0.16) and for AS patients (p < 0.5, r = 0.18), it is weak (low correlation
coefficients); so the association between these 2 variables might not have much relevance. This argument can also apply to the weak correlation between total IgM and ESR in AS patients.

There was no correlation between serum total IgG, IgA and IgM and CRP in AS patients. This is in agreement with the data of others for all immunoglobulins and CRP (Laurent and Panayi, 1983) and with Sanders et al. (1987) for IgA and CRP. In contrast, Collado et al. (1987) reported a correlation between IgA (but not IgG or IgM) and CRP in AS; and Trull et al. (1984) also reported a correlation between IgA and CRP (and IgA and ESR) in AS. In the present study, a weak correlation was present between total IgG and CRP in the total patient group. As disease activity strongly correlated with CRP (section 2.3.6) but not with serum total immunoglobulins, the significance of this weak association between IgG and CRP is debatable.

Total serum immunoglobulins did not differ significantly between non-secretors and secretors. This is in agreement with findings (for total IgA and IgG) in females with recurrent urinary tract infections who had been recently referred to clinic (Blackwell et al., 1987b), although is in contrast with the lower serum IgA found in non-secretors who were carriers of N. meningitidis (Blackwell et al., 1989a).

5.4.2 Total immunoglobulins and antibody response to autologous gut bacteria

In the experiment to compare total serum immunoglobulins in a small cohort of patients with AS and age-matched controls, there was no significant difference in any of the immunoglobulin classes between the 2 groups. The common finding among several authors of a raised total serum IgA in AS compared with healthy controls was not confirmed. A criticism of the present experiment would be that the population under test was small and the standard deviations wide; therefore, one cannot be certain that in a larger group a difference would not be found.

A novel way of assessing the humoral response to autologous gut bacteria was devised by harvesting bacteria from faeces and testing the patient’s serum for antibodies to his/her organisms in an ELISA. This approach was not selective for the antibody response to a particular bacterial species, but investigated the antibody response to all types of enteric bacteria harvested from faeces, both anaerobic and aerobic organisms.
There was no significant difference in the specific IgG or IgA antibody levels between patients and controls. The only statistically significant result was a lower specific IgM response in patients to autologous gut bacteria compared with the IgM response of controls to their own gut bacteria. When the proportion of specific/total antibody of each class was calculated, it was found that patients produced proportionately less IgM and IgA to their own gut flora relative to the total IgM and IgA, than did controls to theirs. There was no difference in the proportion of the specific IgG/total IgG antibody levels between patients and controls. It is difficult to explain this result in biological terms. If patients were mounting a reduced IgM response to their own faecal flora, one would expect a consequently lower IgG (and IgA) response as B cells sequentially switch production of immunoglobulin classes as they differentiate. Conversely, if the patients had been responding over a long period to these organisms, most B cells would have undergone class-switching away from IgM; IgM levels would, therefore, be low but IgG and IgA levels should be higher.

There was no correlation between total or specific antibody levels of any class and disease activity.

Significantly reduced levels of IgM to laboratory strains of *K. pneumoniae* have been reported in sera of patients with AS (Cooper et al., 1988, Trull et al., 1983), including to the K43 strain of Geczy (Cooper et al., 1988). This is the first report of a significantly lower serum IgM response to autologous bacteria in patients with this disease compared with controls. The number of individuals in the study was low and standard deviations high, so the significance of the results is not entirely clear. If this is indeed a real phenomenon, it may suggest a defect in antigen presentation in the initiation of an immune response.

The very high specific IgM seen in one of the controls (GS) might be because GS prepared all the faecal samples. It is likely that GS became sensitised to gut bacteria from a variety of samples (despite adherence to aseptic technique). The high specific IgM in this case probably reflects IgM antibodies to shared antigenic determinants between different enteric bacteria. This is supported by the finding of a high S/T ratio for IgG in GS, as the plasma cells producing elevated IgM antibodies to determinants shared by gut bacteria would switch to producing IgG class.
5.4.3 Serial humoral studies

The total IgG levels of 3 serum samples from each of 12 patients taken at intervals of 6-7 months showed little intra-patient variation although there was considerable inter-patient variation. For total IgA and IgM, there was little intra- or inter-patient variation with time and values fell within the established normal ranges.

For each individual, IgG antibody levels in all 3 serum samples to bacteria harvested from faeces obtained at the time of the third serum sample, showed little intra-patient variation. This suggests either that bacterial flora do not alter a great deal with time, or that there are cross-reacting antibodies present in the first and second serum samples.

The results for specific IgM antibodies were similar to those for IgG, showing little intra-patient variation in the levels of antibodies in the 3 samples. Unfortunately, results were complete for only 6 patients due to lysis of the bacteria stored in coating buffer. For the IgA assay, results were available for only 3/12 patients for the same reason. The specific IgA assay was performed last in the series of experiments. It must be assumed that the longer period of time in coating buffer caused lysis of bacteria in more of the samples and consequently the results are less complete. The high ELISA readings obtained would be due to non-specific binding of reagents to bacterial intracellular proteins released on cell lysis.

The main conclusions are:

1. there is no correlation between total serum IgA, IgG or IgM and clinical disease activity or secretor status in patients with spondyloarthritis;
2. there is no correlation between any of the immunoglobulin classes and CRP in patients with AS;
3. there is a positive correlation between total serum IgA and ESR and IgM and ESR in AS patients;
4. there is no significant difference in total serum IgA, IgG or IgM between a small group of AS patients and healthy individuals;
5. AS patients had a significantly lower IgM response to autologous gut bacteria compared with controls; and, produced proportionately less IgM and IgA to their own gut flora relative to the total serum IgM and IgA, than did controls to theirs.
CHAPTER 6

GENERAL DISCUSSION

Detailed discussion of the results has been given at the end of each chapter. The objective of this chapter is to assess the general conclusions of this study with reference to the major hypotheses proposed for explaining the pathogenesis of AS and proposals for further research.

6.1 ORIGINAL OBJECTIVES

This work set out to investigate the previously reported association of non-secretion of ABO blood group antigens with AS and reactive arthritis (Shinebaum et al., 1987b); to compare the acute phase response and clinical disease assessments in secretors and non-secretors; to correlate clinical disease activity with ESR and CRP in the total patient population; to compare the faecal flora in patients with spondyloarthropathy with that of healthy individuals; to determine the prevalence of bacteria expressing molecules with structural similarities with the HLA-B27 antigen; and, to compare the humoral responses of patients and controls to their own faecal flora.

6.2 SECRETOR STATUS

The previous observation that non-secretion of ABO blood group substances was associated with AS was of considerable interest as non-secretion is a susceptibility factor for infection of mucosal surfaces; and, for many years infection has been postulated to be involved in the pathogenesis of AS. Mechanisms suggested to explain this association included the possibility that the Lewis\(^a\) antigen of non-secretors acted as a receptor for arthritogenic bacteria promoting colonisation of mucosal surfaces. It has been demonstrated here that there is no increased prevalence of non-secretors in a large population of AS patients compared with healthy individuals. Investigation of the patients who had been typed 5 years ago and also in the present study has revealed that some patients were originally mistyped as non-secretors when they were in fact secretors. The most likely explanation is that enzymatic degradation of blood
group antigens in saliva samples occurred in the initial study, probably because the samples were not promptly processed. This highlights the need for speedy processing of saliva samples prior to secretor status determination. Since the proportion of non-secretors in the AS population was the same as that in the healthy control population, further experiments to investigate the putative role of Lewisα as a receptor for arthritogenic bacteria (such as binding studies of bacteria to buccal epithelial cells of secretors and non-secretors) were not performed.

More recent studies have suggested that secretors have variable amounts of Lewisα on their epithelial cells (Blackwell et al., 1992; Saadi et al., in press). There is evidence that Leα is a receptor for some strains of S. aureus-producing pyrogenic toxin (Saadi et al., in press). The hypothesis that Leα might be a receptor for some arthritogenic bacteria cannot be eliminated.

There is no evidence from this study that non-secretors with AS experience more active disease than secretors as assessed clinically, or that they display a more intense acute phase response as determined by ESR and CRP. This contrasts with the report of elevated ESR and (especially) CRP levels in non-secretors compared with secretors with urinary tract infections (Lomberg et al., 1989); but, it is not clear whether the non-secretors in that study were more heavily infected than the secretors. In the present work, ESR and CRP did correlate with clinically-assessed disease activity in patients irrespective of secretor status. This confirms the findings of some surveys (Cowling et al., 1980a) which have shown a positive correlation between ESR and CRP and disease activity, but disagrees with others (Laurent and Panayi, 1983; Sheehan et al., 1986) all of which have assessed disease activity in a similar way to that employed here.

6.3 ROLE OF FAECAL FLORA

AS and reactive arthritis have several features in common (HLA-B27, enthesitis, sacroiliitis, peripheral arthritis, uveitis) which have been a major influence in development of the link between Klebsiella and AS. Reactive arthritis follows clinically-proven infections by gut bacteria which has not been demonstrated for AS.
The present studies of faecal flora showed that there was no major difference in the range of bacteria identified from the stools of healthy individuals and AS patients. This agrees with the findings of Ferraz et al. (1990) in Brazil who also performed a semi-quantitative analysis of stool specimens from AS patients and healthy controls and found both a similar variety of bacteria and bacterial counts/gram of faeces for both populations. Apart from the Brazilian centre, no other group has examined the faecal flora in such a way; by making dilutions of the stool sample a minimum growth of 500 organisms/gram of faeces would be detected.

In some surveys only the prevalence of *Klebsiella* was sought (Eastmond et al., 1980; Warren and Brewerton, 1980; van Kregten et al., 1991); in others stools were examined for *Enterobacter* and *Yersinia* (Ebringer et al., 1977; Ebringer et al., 1978; Hunter et al., 1981); and only one other survey has examined for a wider range of organisms and found scanty numbers of *Citrobacter*, *Proteus*, *Alkaligenes* and no isolation of *Salmonella*, *Shigella* or *Yersinia* (Kuberski et al., 1983).

*Klebsiella* is the single species which has excited the most interest as a potential trigger of flare-ups of the disease. This followed the original observation of increased isolation of *Klebsiella* in active AS (Ebringer et al., 1977; Ebringer et al., 1978). These workers concluded that a positive *Klebsiella* culture in a patient with inactive disease was a harbinger of active disease developing 2-3 months later (Ebringer et al., 1978). The validity of this conclusion remains controversial. Criticisms of this report were that controls attended on only 1 occasion whereas patients attended on 2-3 occasions; patients with active disease attended more often than those with inactive disease and had more samples taken; therefore, they were more likely to have at least 1 sample positive (Brewerton and Warren, 1978). Kuberski et al. (1983) also found an increased isolation rate of *Klebsiella* in active AS.

In the present survey, there was no increase in the isolation of *Klebsiella* from the faecal samples of patients compared with controls. This agrees with many studies (Warren and Brewerton, 1979; Eastmond et al., 1980; Warren and Brewerton, 1980; Hunter et al., 1981; Brewerton, 1983; van Kregten et al., 1991) but disagrees with the results of Ebringer et al. (1977) and (1978) and Kuberski et al. (1983). In the data presented here, in those patients from whom *Klebsiella*
was isolated, there was no relationship between its presence and disease activity. Although studies vary with regard to *Klebsiella* isolation in active disease, there is agreement in the literature that AS patients with inactive disease do not have increased carriage of *Klebsiella* and indeed many normal individuals carry faecal *Klebsiella*.

The lower *Klebsiella* isolation rate in patients (8.5%) compared with controls (19.4%) in the present work might be partly due to delays in sample arrival, as patients' samples were delivered by post whereas controls' samples were delivered in person. The isolation rate of faecal *Klebsiella* from controls in the present survey was 19.4% and has varied worldwide from 13% in Arizona (Kuberski, 1983) to 60% in the Netherlands (van Kregten et al., 1991). Differences between studies are probably due to a combination of factors: different types of controls; different faecal sampling methods; different culture methods; and geographical variations in the prevalence of *Klebsiella*.

### 6.4 CROSS-TOLERANCE HYPOTHESIS

Ebringer's cross-tolerance hypothesis (Ebringer, 1983) was based on studies of faecal flora in AS patients and on a number of pieces of evidence of antigenic similarity between HLA-B27-positive lymphocytes and *K. pneumoniae* and other Gram-negative enterobacteria. Antibodies to HLA-B27 cross-reacted with *K. pneumoniae* (Avakian et al., 1980; Ebringer et al., 1976). Rabbit antisera to *Klebsiella* showed cytotoxicity to B27-positive lymphocytes (Welsh et al., 1980) although this was not confirmed by Beaulieu et al. (1983). Cross-reactivity has also been demonstrated by Chen et al. (1987) who found that a membrane protein of *Y. pseudotuberculosis* reacted with an anti-HLA-B27 monoclonal antibody (Ye-2). Zhang et al. (1988) reported that the 35kDa bacterial outer membrane protein reactive with several anti-HLA-B27 monoclonal antibodies was the outer membrane protein A (OmpA) common to many Gram-negative enterobacteria. Although such reports of serological cross-reactivity exist, antibodies to HLA-B27 are not found in the sera of patients with a recent Gram-negative enteric infection (Cavender and Ziff, 1986; Kapasi et al., 1988).

The cross-tolerance hypothesis suggests that a humoral response is directed to antigenic determinants on enteric bacteria and some of these antibodies cross-react with HLA-B27 molecules on the surface of cells in the vicinity of sacroiliac
joints. Complement-mediated inflammatory reaction occurs leading to tissue damage. This does not explain the selectivity of tissue damage in spondylarthropathies as most of the cells in the body bear MHC class I antigens (Daar et al., 1984).

If inflammation in AS is initiated by cross-reactions between bacterial antigens and HLA-B27, then one might expect the tissue distribution and relative density of these cross-reacting epitopes to play a role in tissue localisation and the extent of inflammation. Although MHC class I antigens can be detected on most nucleated cells (Daar et al., 1984), the relative tissue density of the antigens is little known. The major sites affected in AS are ligamentous, articular and synovial structures within the axial skeleton, sacroiliac joints and tendon insertions. Husby et al. (1989) reasoned that if the series of events postulated by the molecular mimicry theory contributed to the pathogenesis of AS, these cross-reacting epitopes should be concentrated at these sites. Synovial tissues from HLA-B27-positive AS patients were examined by an immunoperoxidase technique using antisera to synthetic peptides representing antigens shared between HLA-B27.1 and K. pneumoniae nitrogen reductase. Positive staining was localised with either antiserum to the synovial lining cells, endothelium of small and medium vessels and to a lesser extent within some inflammatory cells. The control synovial tissues from HLA-B27-negative patients with rheumatoid arthritis, juvenile chronic arthritis or osteoarthritis did not show any significant staining. Examination of skin biopsies from 2 HLA-B27-positive normal individuals showed staining in epidermal cell membranes but this was much less pronounced than in synovial tissues from AS patients. It is not clear, however, whether the epitope was the native HLA-B27 molecule or a bound peptide. It is not clear whether the prominent expression of HLA-B27 antigen in synovial tissue is primarily related to pathogenesis of HLA-B27-related arthropathy or whether it represents a secondary phenomenon related to the inflammatory process.

The reason for uveal involvement in some patients with spondyloarthropathy is not clear. MHC class I antigens are not normally expressed on uveal cells in vivo although they are present throughout the blood vessel endothelium of the uveal tract (Abi-Hanna and Wakefield, 1988). These antigens are expressed on tissue-cultured uveal cells and interferon-α and interferon-γ can enhance the in vitro expression of MHC class I antigens. Several tissues which normally lack
HLA antigens are found to express these in disease states. Skeletal muscle is normally devoid of HLA antigens but expresses class I antigens in Duchenne muscular dystrophy. It might be that induction of HLA antigens on uveal cells is a prerequisite for the initiation of an autoimmune response in the eye.

In the present work, a search was made for faecal bacteria bearing epitopes which show antigenic similarity to HLA-B27. A monoclonal anti-HLA-B27 antibody was used as a probe to identify HLA-B27-like antigens on enteric bacteria. Although initial immunoblotting experiments detected positively-staining colonies, it became apparent that the detection system was identifying organisms bearing a peroxidase enzyme; the monoclonal antibody was superfluous to the reaction. Flow cytometry using fluorescein-labelled anti-mouse immunoglobulin is a sensitive method of detecting mouse antibodies bound to bacteria. Flow cytometry experiments on those strains that were positive in the initial screening step did not reveal any evidence of HLA-B27-like antigens on any of the faecal flora (either anaerobic or aerobic organisms). No evidence was found therefore to support the cross-tolerance theory.

Much attention has been focussed on the hexameric amino acid sequence which is shared by HLA-B27.1 (B*2705 subtype) and the nitrogen reductase enzyme of *K. pneumoniae*. It has been calculated that the probability of two proteins sharing a 6 amino acid sequence is 1 in $64 \times 10^6$ (Schwimmbeck et al., 1987). The protagonists of the molecular mimicry theory have proposed that this remote probability must have some biological significance. It was suggested that this hexameric sequence provides an epitope that would induce an immune response against the same 6 amino acids in B*2705 in vivo. Sera from 7/24 (29%) AS patients and 18/34 (53%) Reiter's syndrome patients had antibodies to a synthetic peptide containing the amino acid sequence in amounts above those seen in normal individuals. A significant overlap, however, was found between patients and controls for borderline values. Ewing et al. (1990) produced similar results but the overlap between patients and controls was large. In contrast, some groups have failed to detect such antibodies (de Vries et al., 1990; Tsuchiya et al., 1989) and there is no evidence for cytotoxicity directed against HLA-B27-positive lymphocytes (Kono et al., 1984; Cavender and Ziff, 1988). Although antibodies in the sera of some patients react with synthetic peptides, there is no evidence that they react with the intact HLA-B27 molecule. In the intact HLA-B27 molecule the homologous sequence is coiled inside an
alpha helix structure in contrast to the linear conformation of the hexamer in a synthetic peptide.

The cross-reactivity described by Ebringer appears to involve a protein in the outer membrane of Klebsiella, not the sequence in the nitrogen reductase enzyme which is homologous with HLA-B27; therefore, Ebringer's work is not supported by the serological findings mentioned above.

If cross-reactivity with intact HLA-B27 proteins cannot be firmly established, it would suggest that the molecular mimicry between HLA-B27 and Klebsiella has no pathogenetic relevance whatsoever. Molecular mimicry alone cannot explain the disease. Much effort has been concentrated on the homology of K. pneumoniae nitrogen reductase and HLA-B27.1 while it would appear that little attention has focussed on the fact that the enzyme is found in only a small proportion of faecal strains, is expressed only in anaerobic conditions and in protein-free mediums and, therefore, not in the human host. The Klebsiella strains that carry the particular enzyme are predominantly soil bacteria (Brill, 1980).

It is likely that the important regions of HLA-B27 are those that make it different from the other HLA-B antigens and are conserved between subtypes (Benjamin and Parham, 1990). This is suggested by the observation that there is no selective association of AS with a particular subtype (Breur-Vriesendorp et al., 1987) or different subtype distribution in patients compared with normals (Grumet et al., 1981). The hexameric sequence is part of the antigen binding groove and is situated in the hypervariable region of the alpha 1 domain of B*2705. Amino acid substitution here markedly alters T cell recognition suggesting that some peptide binding capabilities are also modified in the different HLA-B27 subtypes (Lopez de Castro, 1990; Calvo et al., 1990). It is likely that it is those amino acid sequences in the peptide groove that are both unique to HLA-B27 and common to all its subtypes that are important, not directly, but because they help specify the nature of the peptides that can bind in the antigen binding groove of all HLA-B27 variants.
Antibodies to *Klebsiella* and other enterobacteria have been found to be elevated in AS by some researchers but other groups have not confirmed these observations (reviewed in section 1.7.8).

In the present study, AS patients were shown to mount a lower IgM response to their own faecal flora than healthy individuals did to theirs. Also, proportionately less of the total IgM and IgA was directed to autologous gut bacteria in patients than in controls. As there was no significant difference in the total immunoglobulins between patients and controls, it suggests, that if this is indeed a real phenomenon there may be a defect in antigen presentation in AS patients. One might expect that a low IgM response to an antigen would result in a low IgG titre to that antigen as B cells sequentially switch in immunoglobulin classes as they differentiate, but this was not observed. Low IgM responses to *Klebsiella* K43 have been previously noted but not explained (Cooper et al., 1988). Few hard conclusions can be drawn from the present findings in view of the small numbers of subjects studied. If a larger group was examined the results might be different. There is no convincing evidence from any source that AS is a humorally-mediated disease.

There was no significant difference in total serum IgA between patients and controls. This confirms other reports, but disagrees with some groups who have found elevated serum IgA which in some cases has been related to disease activity. There is evidence to suggest that raised IgA in AS might be an epiphenomenon. NSAIDs increase intestinal permeability (Bjarnason et al., 1986) causing blood and protein loss (Bjarnason et al., 1987). Increased intestinal permeability in AS patients has been demonstrated using $^{51}$Chromium-labelled EDTA (Wendling et al., 1990). A comprehensive ileocolonoscopic study revealed inflammatory lesions of the gut in high frequency in both ReA (65%) and in AS (57%) patients especially those with peripheral arthritis and also those not on NSAIDs (Mielants et al., 1988). Disturbed gut permeability (from whatever cause) could result in increased absorption of enteric bacterial antigens and a polyclonal increase in IgA. This could also explain the raised IgA antibodies to *Klebsiella* (Trull et al., 1983) and *Yersinia* (Wagener et al., 1985).
which have been found in AS. The raised IgA could be part of the immune response to a subclinical infection in the ileum in the presence of chronic ileitis.

6.6 Future Work

While examples of molecular mimicry are common in nature (Oldstone, 1989), there is little evidence that such antigenic similarity contributes to disease. One notable exception is rheumatic fever in which anti-streptococcal antibodies cross-react with an identical sequence in cardiac structures as is present in the M protein of group A streptococci (Bronze et al., 1988). Cross-reactivity between HLA-B27-positive lymphocytes and *K. pneumoniae* and other Gram-negative enterobacteria has been demonstrated by different laboratories, but many other groups have failed to confirm these findings. In the current work, there was no evidence for the presence of a HLA-B27-like epitope on enteric bacteria from the faeces of either AS patients or healthy controls.

It is intuitively unlikely that an individual would mount an immune response to their own MHC class I antigens. If cross-tolerance is involved, then cross-reactivity is more likely to decrease the response to *Klebsiella*, perhaps thereby allowing undue persistence of the organism and disease than inducing anti-self MHC class I reactivity. In addition, cross-reactivity does not explain the distribution of disease. Future research into the pathogenesis of AS should be directed away from further investigating cross-reactivity between HLA-B27 and enterobacteria.

The majority of evidence (including the present work) suggests that *Klebsiella* carriage is not increased in active AS. In the instances that such an observation has been made, it is more likely to be an epiphenomenon than having any biological significance. Further studies of faecal flora are unlikely to advance our understanding of this disease.

The most exciting recent developments in AS have been the discovery by X-ray crystallography that peptides bound to HLA-B27 appear to be nonamers and are in a largely extended conformation (Madden et al., 1991). A series of these peptides have been identified as self peptides derived from proteins which are in abundance in the cell such as histones, ribosomal proteins and members of the 90K heat shock proteins (Jardetzky et al., 1991). It would be of interest to determine if there is any difference in the range of peptides bound to the HLA-
B27 molecule in healthy and diseased HLA-B27-positive individuals. One could speculate that peptides which form part of a self protein derived from articular tissue/enthesis might be identified in the antigen binding groove of the HLA-B27 molecule.

As there is no convincing evidence that AS is a humorally-mediated disease, further studies into T cell function and antigen presentation may improve our understanding of the disease process.

There is considerable evidence that non-secretion of ABO substances is a susceptibility factor for certain mucosal infections and autoimmune diseases with a proposed infective aetiology (Blackwell, 1989). The only other rheumatic disease examined so far which is associated with non-secretion is psoriatic peripheral arthropathy (but not psoriatic spondyloarthritis or uncomplicated psoriasis (Shinebaum et al., 1987a)). Non-secretion is not associated with rheumatoid arthritis. It might be that the Lewis[^3^] antigen on the cells of non-secretors acts as a receptor for micro-organisms triggering psoriatic arthropathy and further research could be directed towards this.

In summary, further research into the nature of the peptides bound by the HLA-B27 molecule, and into antigen presentation and T cell immunity in AS may elucidate the pathogenesis of this fascinating disease.
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