THE ROLE OF ENTEROBACTERIACEAE IN ANKYLOSING SPONDYLITIS

by

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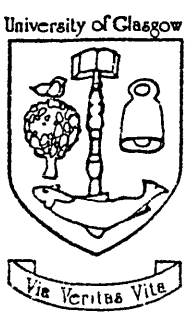
A thesis presented to the University of Glasgow for the degree of Doctor of Philosophy on a study carried out in the Department of Medicine, Glasgow Royal Infirmary

October 1990



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Acknowledgements

I would like to thank the following people who have helped me in the last three years :

My supervisors Dr Roger Sturrock and Dr Curtis Gemmell for their guidance

My colleague Lynn Annandale for her useful advice and practical help and - most of all - for keeping me sane!

The Ankylosing Spondylitis patients attending the Rheumatology clinic for donating samples, and the laboratory and medical staff who made up the "normal" population

The medical staff, past and present, of the Centre for Rheumatic Diseases for their co-operation - particularly Stuart Ralston, Janusz Jankowski and Liz Murphy

Rajan Madhock for his advice, for the use of his computer and for taking numerous blood samples

John Smith for his useful advice in many areas

All my friends and colleagues in the Department of Medicine for their support and their company - particularly Lynda Mowat, Susan Lennie, Margaret Frame and Seamus Caine

Tyrone Pitt of the Central Public Health Laboratory for providing anti-Klebsiella antisera and P.Klouda of the UK Transplant Service for tissue-typing sera

The Biochemistry Department, Glasgow Royal Infirmary for measuring CRP and immunoglobulin levels

Finally, a special thanks to my husband Gavin, not only for proof-reading this thesis and preparing several of my graphs but also for doing all the household chores for the last four months!

SUMMARY

In this study, we investigated the immune response of patients with Ankylosing Spondylitis (AS) and looked for evidence to support the proposal that AS is a form of reactive arthritis in which Klebsiella or other Enterobacteriaceae play a role.

Immunoglobulin levels in patients' sera were measured. Using the laboratory parameters erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels as indicators of disease activity, it was found that patients with active disease had considerably higher levels of IgA and IgG than patients with inactive disease or controls. This suggests that these immunoglobulin levels could be another useful indicator of disease activity and they were therefore taken into account in all subsequent patient studies.

It has been suggested that these high serum immunoglobulin levels indicate an ongoing immune response to antigen in patients with active disease, but such **marked** elevations are more likely to be accounted for by non-specific mechanisms. We had hoped to investigate any cellular basis for this by co-culturing AS and normal lymphocytes. However, in vitro immunoglobulin production by peripheral blood lymphocytes (PBLs) did not reflect the serum studies : we were unable to demonstrate any abnormality in the production of immunoglobulin by patients' lymphocytes.

The possibility of involvement of Enterobacteriaceae in AS was studied by measuring the levels of specific antibody in patients' sera to sonicated preparations of several bacteria. An enzyme-linked immunosorbent assay (ELISA) was used. When total antibodies were measured (i.e. all immunoglobulin classes), no differences were found between patients and controls. However, when antibodies of the

IgA class only were studied, patients were found to have elevated antibody levels to Klebsiella and the three arthritogenic bacteria Salmonella, Shigella and Yersinia. This was not simply a result of the general elevation in serum IgA found in patients since normal levels of IgA antibodies to E.coli and Proteus were found. Using SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting, an attempt was then made to identify any abnormality in the level of serum antibodies to individual Klebsiella proteins. No obvious difference was found in the blotting pattern of sera from controls, patients with inactive disease and patients with active disease.

Since Klebsiella and the other bacteria implicated in AS are found in the gut, a study of the gut immune response should provide a better indication of whether antigenic stimulation by such bacteria has occurred. We studied patients' saliva, the only gastrointestinal secretion readily available. In an ELISA, neither patients as a whole nor the active disease group had high levels of total salivary IgA or IgA antibodies to formalin-killed or sonicated Klebsiella or Yersinia.

If, as it has been suggested, the immune response to Enterobacteriaceae plays a pathogenic role in the disease, we would expect to find evidence for this immune response at the site of inflammation - the joints. We measured antibodies to sonicated and formalin-killed preparations of Klebsiella, Salmonella, Shigella and Yersinia in synovial fluids. These antibody levels were no higher in AS patients than in patients with Rheumatoid Arthritis or other inflammatory joint diseases. Moreover, the antibody response to these four bacteria was similar to that found to E.coli, a bacterium which has not been implicated in the seronegative spondyloarthropathies. Further studies using immunoblotting revealed no difference between AS and other patients in the level of synovial fluid antibodies to individual Klebsiella proteins .

In the known reactive arthritides, lymphocytes from the joint demonstrate an elevated **cellular** immune response to the causative organism. We therefore measured the *in vitro* proliferative response to *Klebsiella* of AS synovial T-cells. This was not significantly different from the response of synovial lymphocytes from patients with other diseases.

The humoral immune response in AS was further studied by measuring the levels of circulating immune complexes (CIC) in patients' sera. Using a Raji assay, patients were found to have elevated levels of IgG-CIC which were associated with active disease while an assay based on polyethylene glycol (PEG) precipitation and radial immunodiffusion revealed a difference between patients and controls which was independent of disease avtivity. All patients had normal serum levels of IgM-CIC. Significant levels of both IgG- and IgM- immune complexes were found in AS synovial fluids. These immune complexes may have no pathogenic relevance but they could be useful in the identification of any environmental factors associated with the disease. We concentrated immune complexes from serum and synovial fluids by PEG precipitation; using SDS-PAGE and immunoblotting, we studied the reactivity of anti-Klebsiella antisera to proteins within these complexes. No evidence for the presence of *Klebsiella* antigens was found.

As well as a study of the immune response to Enterobacteriaceae in AS, the presence of the bacteria themselves was investigated using rectal swabs. Klebsiella was isolated from only 17% of AS patients and apart from *E.coli*, no other bacterium was found in more than 10% of cases.

The main theory proposed to explain the involvement of Enterobacteriaceae in AS is that of molecular mimicry which is dependent on an immunological cross-reaction between these bacteria and the B27 antigen. We investigated this by measuring the ability of anti-Klebsiella antisera to

bind to B27-positive lymphocytes in an immunofluorescence assay and the ability of anti-B27 tissue-typing sera to bind to various Enterobacteriaceae in an ELISA. No evidence for cross-reactivity was found. In addition, we looked at a six amino acid sequence which is found to be shared by the B27 antigen and a Klebsiella protein. We looked for antibodies in patients' sera reactive to a synthetic peptide representing this sequence but no significant levels of such antibodies were found.

In conclusion, our studies lend little support to the proposal that AS is a form of reactive arthritis in which Klebsiella or other Enterobacteriaceae play a pathogenic role.

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Abbreviations

A	Patients with active disease
AHG	Aggregated human Immunoglobulin G
AS	Ankylosing Spondylitis
BSA	Bovine serum albumin
C	Control subjects
CFU	Colony forming units
CIC	Circulating immune complexes
C.L.E.D.	Cysteine-Lactose-Electrolyte-Deficient
cpm	counts per minute
CRP	C-reactive protein
E.coli	Escherichia coli
ESR	Erythrocyte sedimentation rate
HLA	Human Leucocyte Antigen
IA	Patients with inactive disease
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
NHS	Normal human serum
NSAIDs	Non-steroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PBLs	Peripheral blood lymphocytes
PEG	Polyethylene glycol
PEG-SRID	Polyethylene glycol and single radial immunodiffusion
PHA	Phytohaemagglutinin
PsA	Psoriatic Arthritis
PWM	Pokeweed mitogen

RA	Rheumatoid Arthritis
ReA	Reactive Arthritis
RPMI	Rosewell Park Memorial Institute (culture medium)
RS	Reiter's syndrome
SDS	Sodium duodecyl sulphate
SLE	Systemic Lupus Erythematosus

CHAPTER 1

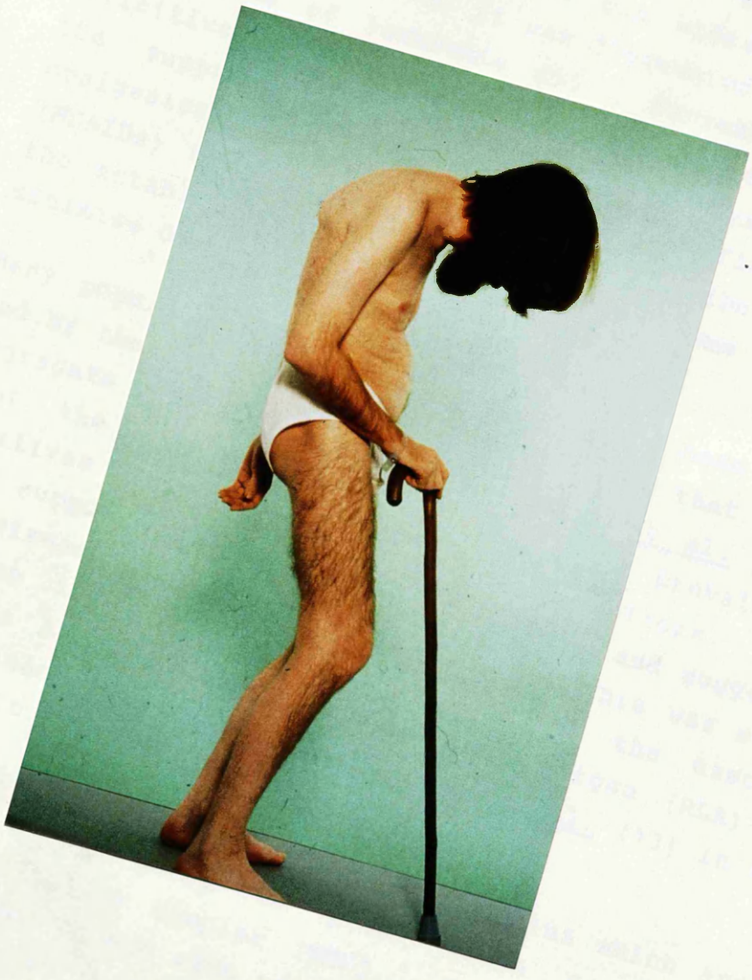
INTRODUCTION

Ankylosing Spondylitis (AS) is a chronic inflammatory joint disease of unknown aetiology which primarily affects the spine and sacroiliac joint. The name is derived from the Greek words "ankylos" which means bent and "spondylos" which means vertebra. Within the joint, repeated bouts of inflammation of the entheses (bone-ligament junctions) occur and the lesions are repaired by deposition of fibrous tissue and bone, leading to immobilisation of the joint. The disease, which is predominantly found in males, usually has an insidious onset in early adulthood. The most common clinical features are lower back pain and morning stiffness. As the disease progresses, the entire spine can become stiff and the patient loses normal posture, often developing a forward stoop of the neck (kyphosis). Figure I shows a patient with severe AS with classical kyphosis. Other joints, such as hips, shoulders and knees can be involved in up to 35% of patients (1) and in some cases, inflammation of extra-articular tissue such as the eyes, heart and lungs can occur (2).

AS has probably existed since ancient times since it has been documented in the remains of Egyptian mummies (3), but the first accurate account of the disease is thought to be that of Bernard Connor in 1695 (cited by Blumberg (4)), and it was not until the 1930s that the full clinical picture of AS emerged.

Since the start of this century, a number of theories on the aetiology of AS have been proposed and a variety of treatments have been used. At the turn of the century, an abnormal reaction to trauma was proposed as the cause of the disease (5). So too was focal sepsis (6,7) which led to the removal of teeth, tonsils, appendices and even

Figure I. A patient with severe Ankylosing Spondylitis



colons from many unfortunate patients. Some patients underwent parathyroidectomy in the 1930s because of the proposal that high calcium levels were responsible for the disease (8). In the 1950s, treatment with deep X-ray therapy had a considerable degree of success although how it worked is not clear and the widespread use of such treatment ceased after it was discovered that it conferred a high risk of leukaemia (9). Currently, there is no definitive treatment for AS. Management is directed at the suppression of pain and stiffness with simple analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and phenylbutazone, and at the establishment of an exercise programme to prevent or minimise deformities of the spine.

Many population and family studies have been carried out and by the 1950s, it was well established that AS tends to aggregate within families. Stretcher et al. (10) found that the disease was 30 times more prevalent among relatives of AS patients than among controls. This has been supported by recent studies (11,12) and suggests that the disease is under genetic control. This was supported by the landmark discovery in 1973 of the association between AS and the human leucocyte antigen (HLA) B27 - simultaneously reported by Brewerton et al. (13) in the UK and Schlosstein et al. (14) in the USA.

HLA antigens are cell-surface glycoproteins which are the products of a group of genes known as the major histocompatability complex (MHC). The class I antigens HLA-A, -B and -C are 44kD transmembrane proteins which are found on the surface of most nucleated cells, non-covalently linked to the 12kD glycoprotein beta-2-microglobulin. Class II or HLA-D/DR antigens consist of two polypeptide chains of 28kD and 32kD and are found mainly on B-lymphocytes and antigen-presenting cells. HLA antigens have several important roles in the immune response. D/DR antigens are thought to be the analogues of Ia antigens in mice which are involved in antigen

presentation : they interact with foreign antigens to form an epitope recognised by helper T-lymphocytes (15). Similarly, class I antigens are involved in the recognition of antigen by T_s or cytotoxic T-cells.

A large number of diseases, involving every medical speciality, are associated with particular HLA alleles (16). The list of HLA-disease associations is rapidly expanding, partly due to the increasing availability and specificity of tissue-typing techniques. The largest group is associated with D/DR antigens and includes the putatively autoimmune diseases Rheumatoid Arthritis (RA), Systemic Lupus Erythematosis (SLE), Myasthenia Gravis and Graves' Disease (16). The remaining group is associated with class I, mainly HLA-B antigens, and includes AS. In most cases, the role played by the HLA antigen is not known.

The association between B27 and AS is the strongest HLA-disease association known : as many as 95% of Caucasoids with AS are B27-positive while this antigen is found in less than 8% of healthy Caucasoids (13,14). The association holds true for all racial, geographical and ethnic groups studied although in some groups, the percentage of patients with B27 is considerably lower than 95% (17).

Two main theories have been put forward to explain the relationship between AS and B27. The two gene theory, first proposed by McDevitt and Bodmer (18), postulates that the B27 gene is not itself involved in AS but is in linkage disequilibrium with another gene which **is responsible** for the disease. Linkage disequilibrium between genes is not uncommon : the HLA association of congenital adrenal hyperplasia can be explained by linkage of the MHC with the gene for 21-hydroxylase enzyme (19). Similarly, idiopathic haemachromatosis is related to a defect in a gene invoved in iron metabolism and this gene is closely linked to the MHC (20).

It has been proposed (18) that the B27 gene is linked to a D/DR gene which acts as an immune response gene. In this way, an abnormal immune response to an environmental agent such as an infectious organism could account for development of AS in B27+ individuals. However, studies of HLA-D/DR alleles show no correlation with AS (21, 22) and no mechanism has been proposed to explain how any other linked gene might mediate the disease. Moreover, despite an extensive search, there is no evidence of recombinant families where dissociation of AS from the B27 gene has occurred (23). In view of these findings, the two gene theory is unlikely to explain the association of AS with B27 and the one gene theory has now gained quite widespread acceptance.

The one gene theory postulates that the B27 gene is directly involved in precipitating the disease and two main mechanisms for this have been proposed. In the **receptor theory**, first proposed by Seager et al. (24), the B27 antigen acts as a receptor for some disease-inducing factor. In the **molecular mimicry theory** (25), it is suggested that the B27 antigen cross-reacts with an environmental factor such as a micro-organism and this leads to an aberrant immune response. This response could be specific tolerance to the factor since the immune system is often unresponsive to antigens which resemble "self". In this way, a micro-organism could escape normal immune mechanisms and be allowed to persist in a B27+ host and cause tissue damage. Alternatively, this environmental factor could induce a positive immune response which also recognises the cross-reactive B27 antigen. Normal tolerance to B27 may then be overridden, resulting in autoimmunity. Such a mechanism operates in Rheumatic Fever where cross-reaction between Streptococcal cell walls and human cardiac tissue leads to an autoimmune response (26).

Evidence that environmental factors as well as genetic factors are involved in AS comes from the discordance

observed between monozygotic twins (27, 28) and the fact that less than 2% of B27+ individuals develop the disease (29, 11). As far as it is known, there is no difference in the B27 antigens of AS patients and normal B27+ individuals (30).

It has long been suspected that the environmental factors associated with AS may be bacteria. AS is one of a group of arthritic diseases known as the seronegative spondyloarthropathies (which are "negative" for rheumatoid factor) and these include the reactive arthritides. Reactive arthritis (ReA) is normally an acute arthritis which has a well established association with an infection with an enteric or sexually-transmitted micro-organism such as Salmonella (31), Yersinia (32), Shigella (33) or Chlamydia (34). The role played by these micro-organisms is not clear and all attempts to culture them from the joints have failed (35, 36) suggesting that local infection is not the cause of the joint inflammation. These reactive arthritides share certain features with AS: 60-80% of patients with ReA possess the HLA-B27 antigen (37); the peripheral arthritis of AS and ReA are histologically similar; Reiter's Syndrome (RS) is a chronic form of ReA which is associated with urethritis and conjunctivitis, and around 30% of patients with RS develop spondylitis (38). Many investigators therefore believe that AS may also be a reactive arthritis, triggered by an infectious agent (39).

To investigate the possibility that an infectious organism could induce AS through molecular mimicry with the B27 antigen, Ebringer et al. (25) raised antisera to B27+ lymphocytes and tested their ability to interact with a range of bacteria. By immunodiffusion, the antisera were found to react with isolates of Klebsiella, Enterobacter and Yersinia. This reactivity was later confirmed using other techniques such as haemagglutination and radiobinding assays (40). The same group then reported that AS patients have a higher than normal rate of faecal

carriage of Klebsiella pneumoniae, particularly during phases of active disease (41). These studies led to the proposal that Enterobacteriaceae such as Klebsiella play a pathogenic role in AS through molecular mimicry with the B27 antigen.

In the last decade, a large number of studies have been carried out in an attempt to confirm these findings and to look for further evidence for the role of Enterobacteriaceae in AS.

The possibility of cross-reaction between Klebsiella and B27 and has been extensively studied. Ebringer's group have found evidence for this by using a number of techniques to demonstrate the ability of rabbit and human anti-B27 antisera to interact with Klebsiella (25, 42) and the ability of anti-Klebsiella antisera to interact with B27+ cells (25, 40). Some further evidence has come from studies with monoclonal antibodies (43, 44) and one group has recently found a possible molecular basis for such cross-reaction by identifying a six amino acid sequence shared by the B27 antigen and a Klebsiella protein (45).

An Australian group led by Geczy (46) have repeatedly demonstrated that anti-Klebsiella antisera react with B27+ cells from AS patients but not from healthy donors and they propose that AS patients have a Klebsiella-derived product on the surface of their cells in association with the B27 antigen. However, several investigators have been unable to confirm the findings of either Ebringer's or Geczy's group or to find any evidence for cross-reaction between Klebsiella and the B27+ cells of AS patients (47, 48, 49, 50).

In their more recent studies, Ebringer et al. (51, 52) have been able to confirm their early report of high faecal carriage of Klebsiella in AS patients and Kuberski et al. (53) also demonstrated that Klebsiella could be isolated more frequently from patients with active disease than from controls. Several groups, however, have failed

to find an association between Klebsiella carriage and either the presence or activity of AS (54, 55, 56).

AS patients have been found to have elevated levels of immunoglobulins, particularly IgA (57, 58, 59, 60). Since the majority of IgA is derived from mucosal surfaces such as the gut (61), it is suggested that this indicates antigenic stimulation at such sites and thus provides further support for the involvement of gut bacteria in the disease (57, 62).

The possibility of an immune response to bacteria in AS has been studied more directly. It has been shown that patients with ReA have high levels of serum antibodies which react with the organism that precipitated the disease (63, 64, 65) and several investigators have measured antibodies to Klebsiella and other Enterobacteriaceae in AS patients. Higher than normal levels of IgA antibodies to Klebsiella (66, 67, 39) and Yersinia (68, 69) have been detected in the sera of patients with active disease and one study demonstrated elevated levels of anti-Klebsiella antibodies in patients' saliva (65).

Further evidence for an ongoing humoral immune response in AS comes from several reports that patients have high levels of circulating immune complexes (CIC) (70, 71, 72, 73, 74, 75). These complexes may or may not have any pathogenic relevance but they could be of great value in the identification of any aetiological agent. One study (76) demonstrated that CIC from AS patients cross-react with each other but not with CIC from other disease groups which suggests that the complexes contain disease-specific antigens. So far, however, little attempt has been made to identify the antigens within these complexes.

The possibilty of a role of the cellular immune response in AS has also been studied. The in vitro proliferative response of patients' peripheral blood lymphocytes (PBLs) have been measured but no consistent abnormality has been

demonstrated in either the non-specific response to mitogen (77, 78, 79) or the specific response to bacterial antigens (24, 80, 79).

As in many current studies in AS, the main aim of our project was to look for evidence which might support the proposal that Klebsiella or other Enterobacteriaceae play a role in the disease. This involved studies of patients' humoral and cellular immune response to bacteria (particularly in the joint), an attempt to demonstrate bacterial antigens within immune complexes and a study of the immunological cross-reactivity between B27 and Klebsiella.

Note on the Assessment of Disease Activity

There are no internationally recognised criteria for the assessment of disease activity in patients with AS. A variety of clinical measurements are used including pain, morning stiffness, physical measurements of mobility, the level of non-steroidal anti-inflammatory drugs (NSAIDs) required and the presence of peripheral arthritis. The main laboratory parameters used are the erythrocyte sedimentation rate (ESR) and the serum levels of C-reactive protein (CRP) and immunoglobulins. The use of different criteria by different investigators may explain some of the conflicting findings in AS.

In many studies, disease activity is assessed using only the laboratory measurements ESR or CRP or a combination of these (57, 67, 73). ESR and CRP serve as non-specific indicators of inflammation and are useful in assessing disease activity in RA (81). However, although AS patients with clinically active disease do tend to have higher ESR and CRP levels than those with inactive disease (57, 82), the serial studies of Scott et al. (83) suggest that these measurements are not very reliable as indicators of disease activity in AS. This is supported by the findings of Ogryzlo (1). Moreover, Laurent et al. (84) reported that ESR correlates better with the peripheral arthritis of AS than the spondylitis.

Clinical measurements can be subjective and it is not always clear whether pain/immmobility is due to acute inflammation or to joint damage caused by previous inflammatory attacks.

Rather than relying on any one criterion to assess disease activity in our studies, medical staff in our group designed a point system which took several clinical and laboratory factors into account. (See " Point System for Assessment of Disease Activity ", Appendix A.) These

included morning stiffness, NSAID requirements, the presence of peripheral arthritis, and ESR and CRP. IgA and IgG levels were also included because of the finding by us and other groups that high levels of these immunoglobulins are associated with high ESR and CRP or clinically active disease (57,59,60, and see Chapter 2 of this thesis). These immunoglobulin levels were considered to be elevated when they were above the normal range (> 15mg/ml IgG ; > 4.5mg/ml IgA).

This point system for assessing disease activity was used in all our studies unless otherwise stated. All patients were diagnosed as having AS using the New York criteria (85).

CHAPTER 2

IMMUNOGLOBULIN STUDIES

2.1 Introduction

An early study of serum immunoglobulins using immunoelectrophoresis found broader and more intense IgM precipitation lines in sera of AS patients than in normal control sera (86). More recently, Veys et al. (87) used a linear plate immunodiffusion technique to demonstrate elevated levels of all three major classes of immunoglobulin in patients' sera. In this study, no correlation was found between immunoglobulin levels and any of the four criteria for disease activity used (ESR, CRP, subjective complaints and scanning of joints).

Several groups, however, have demonstrated differences in serum IgA levels between patients with active disease and those with inactive disease using a number of different criteria for disease activity. Cowling et al. (57) found elevated IgA in patients with an ESR above 15mm/hr or a CRP above 15 µg/ml and similar findings were reported by Calguneri et al. (58). Hicking et al. (59) defined patients with active disease as those with an ESR above 30mm/hr, early morning stiffness and a necessity for increased doses of non-steroidal anti-inflammatory drugs (NSAIDs). Such patients were found to have higher serum IgA levels than patients with inactive disease. In a longitudinal study (60), it was found that serum IgA correlated with a composite index of disease activity which took into account chest expansion, lumbar flexion index, morning stiffness and spinal pain. The author concluded that regular measurement of IgA would be useful in the assessment of disease activity. These studies all measured immunoglobulins by radial immunodiffusion. Similar high IgA levels were found in AS patients with

active disease when an enzyme linked immunosorbent assay (ELISA) was used (52). Recently, Collado et al. (88) and Mackiewicz et al. (62) have both demonstrated a statistically significant correlation between IgA and CRP levels in AS.

As well as IgA, serum IgG and IgM levels have been studied. Several groups have shown increased concentrations of IgG in AS patients (78, 87, 89, 90) and some have found this to be associated with active disease. (58,59,60). No direct correlation between this class of immunoglobulin and any of the laboratory parameters of disease activity has been reported. IgM appears to be normal in AS. Only one study (87) found high levels of IgM in patient sera and this was not associated with active disease.

One group sought to determine whether the serum studies were reflected by immunoglobulin production by patients' peripheral blood lymphocytes (PBLs) in vitro (91). They found no difference in IgA or IgG production between patient and control cells in unstimulated or pokeweed mitogen (PWM)-stimulated cultures. PWM is one of a group of lectins which non-specifically activate lymphocytes. One significant finding was, however, a low IgM response to PWM. In a study designed to measure specific antibodies to Enterobacteriaceae and B27 in mitogen-stimulated cultures, Cavender et al. (92) found no abnormality in the secretion of total IgA, IgG or IgM by patient's lymphocytes (although only a small number of patients were used in this study).

Most studies of immunoglobulins in AS have looked for abnormalities in serum levels. However, because of the suspected association of the disease with gut bacteria and the fact that the serum immunoglobulin most consistently raised in AS is IgA (a class of immunoglobulin normally associated with antigenic stimulation at mucosal sites), it may be more useful to look at gastrointestinal

secretions which contain antibodies of the gut mucosal immune system. Clearly, it would be very difficult to obtain intestinal fluid from volunteers. A gastrointestinal secretion which can, however, be readily obtained is saliva. Evidence that salivary antibodies reflect intestinal responses comes from a study by Mestecky et al. (93) which showed a clear antibody response in saliva after oral vaccination with Streptococcus. The major immunoglobulin found in saliva is secretory IgA (sIgA). Unlike serum IgA, this is a dimer bound to a secretory component. One of its main functions is to inhibit the adherence of microorganisms to mucosal surfaces and thus prevent their entry into host tissue.

Two groups have looked at total sIgA levels in saliva of AS patients. Calguneri et al. (58) found significantly more IgA in patients with active disease than those with inactive disease. Pease et al. (69) demonstrated no difference between patients and controls but no comparison was made between patients of different disease activities.

2.2 Aims of Study

1) To see if the reports of an association of high serum levels of immunoglobulins with elevated ESR and CRP in AS can be supported.

2) To measure spontaneous and mitogen-stimulated immunoglobulin production in vitro.

3) To measure IgA levels in patients' saliva.

2.3 Subjects and Methods

Note : Unless otherwise stated, the chemicals described in this and all other method sections were obtained from BDH, UK.

2.3.1 Measurement of serum immunoglobulins

Subjects

Group A consisted of AS patients with an ESR below 30mm/hr and a CRP below 25µg/ml, Group B of patients with an ESR above 30mm/hr and a CRP above 25µg/ml and the control group of normal healthy laboratory and medical staff.

In this and other studies, we have attempted to make the male:female ratio in the control group comparable to that of the patient groups since it is well recognised that sex can influence the immune response (as reviewed by Cohn (94). However, because of the large predominance of males with AS, the male:female ratio in the patient group is often higher. The details of the subject groups used in this study are given below.

	Group A	Group B	Controls
Number	65	60	60
Mean age in years (Range)	31 (21-53)	37 (25-67)	36 (24-56)
Male:Female ratio	7.5:1	5.8:1	4.5:1

Assay

Serum IgA, IgG and IgM levels were measured in the Biochemistry Department, Glasgow Royal Infirmary by an immunoturbidimetric assay using an ENCORE centrifugal analyser (Baker Instruments, UK).

2.3.2 Measurement of in vitro immunoglobulin production by PBLs

Subjects

Patients with active disease (A) and patients with inactive disease (IA) as well as normal healthy laboratory and medical staff (C) were studied. Disease activity was assesed using the " Point System For Assessment Of Disease Activity " (see Appendix A). Patients with a score of ≥ 5 were considered to have active disease and those with a score of < 5 were considered to have inactive disease. This system is used in all further studies in this thesis. The details of the groups are given below.

	IA	A	C
Number	20	20	20
Mean age in years (Range)	36 (25-36)	34 (25-54)	32 (22-41)
Male:Female ratio	6:1	5.2:1	3.9:1

Preparation of lymphocytes

The cells were isolated under sterile conditions in a laminar flow hood (Microflow Pathfinder, Inter Med, USA) using sterile reagents. Heparinised whole blood was carefully layered over equal volumes of Lymphoprep (Nycomed, Norway) and centrifuged at 450g for 45 mins at room temperature. The buffy layer (mononuclear cells) was washed three times in Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco, Scotland), spinning the cells each time for 10 mins at 250g. The cells were counted in an Improved Neubauer Chamber (Hawksley, UK) using 0.05% trypan blue (Gibco, Scotland) in PBS and resuspended in RPMI containing 10% Foetal Bovine Serum (Northumbria Biologicals, UK) and 50IU/ml penicillin / 5µg/ml streptomycin (Gibco, Scotland) then adjusted to the required concentration.

Cell culture

500µl of the cell suspension were added in duplicate to the wells of sterile flat-bottom 24-well plates (Nunc, Denmark). 500µl of medium, with or without PWM, were added to each well and the plates incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for the required number of days. The contents of each well were then transferred to tubes and centrifuged at 800g for 10 mins. The supernatants were taken off and stored at -20°C until assayed.

ELISA.

A sandwich ELISA was used for the quantitation of immunoglobulin in the culture supernatants. 100µl of a 1:100 dilution of goat anti-human IgA, IgG or IgM (Sigma, UK) diluted in 50mM carbonate buffer (pH9.6) were added to the wells of flat-bottom 96 well-plates (Sterilin, UK) and left to coat at 37°C for 1 hour. The wells were emptied

by inverting the plate over a sink and tapping briskly on absorbent paper, then washed three times by filling with phosphate buffered saline containing 0.05% Tween 20 (PBS-Tween 20) and emptying in the same way. Non-specific binding sites were blocked by adding 200µl of PBS containing 1% Bovine Serum Albumin (BSA) and incubating for 30 mins at 37°C. 100µl of the culture supernatants, either undiluted or diluted in PBS-Tween 20, or IgA, IgG or IgM standards (Sigma, UK) were added in triplicate to the wells and incubated for 60 mins at 37°C. After three washes, 100µl of a 1:100 dilution of goat anti-human IgA-, IgG- or IgM-alkaline phosphatase conjugate (Sigma, UK) in PBS-Tween 20 were added and the plate incubated at 37°C for a further 60 mins. After another three washes, 100µl of a 1mg/ml solution of p-nitrophenyl phosphate disodium (Sigma, UK) in carbonate buffer containing 1mM magnesium chloride were added to each well and the plates incubated at 37°C until the colour developed. The reaction was stopped by the addition of 50µl of 3M sodium hydroxide and the absorbance at 405nm measured using a Titertek Multiskan spectrophotometer (Titertek, Finland).

2.3.3 Measurement of IgA in saliva

Subjects

Patients with active disease (A), patients with inactive disease (IA) and controls (C) were studied. The details are given over the page.

	C	IA	A
Number	47	28	30
Mean age in years (Range)	30 (21-42)	38 (25-67)	37 (25-57)
Male:Female ratio	3.6: 1	5: 1	7.2 :1

Saliva collection

Saliva was collected in the mornings only to obviate any effect of diurnal variation in salivary function and was taken at least one hour after eating. Only subjects who had their own teeth were studied since it is known that people with dentures lack many of the minor salivary glands. Each subject was given 2ml of a 10% solution of citric acid to stimulate secretion. The saliva was not spat out - subjects were asked to stop swallowing and gently "dribble" their saliva into a universal container until 5ml had been collected. Each sample was clarified by centrifuging at 2000g for 15 mins and then stored at -20°C until assayed.

ELISA

The amount of IgA in the saliva was measured by a sandwich ELISA (as in section B, this chapter) using known concentrations of IgA as standards. Three dilutions of each sample - 1:400, 1:800 and 1:1600 in PBS-Tween 20 - were tested and the mean result taken.

2.3.4 Statistical Analysis

The Mann-Whitney and Spearman-Rank tests were used (see Appendix B).

2.4 Results

2.4.1 Measurement of serum immunoglobulins

Patients were divided into two groups : those with an ESR < 30mm/hr and a CRP < 25µg/ml (Group A) and those with an ESR > 30mm/hr and a CRP > 25µg/ml (Group B). Patients who fell between the two groups - e.g. an ESR < 30mm/hr but a CRP > 25µg/ml - were excluded.

As shown in Table 1, serum IgA and IgG levels were found to be considerably higher in Group B patients than Group A patients and controls ($p < 0.001$). Group A did not differ from the control group. There was no significant difference in IgM levels between any of the groups.

To determine whether there was a direct correlation between IgA or IgG and either ESR or CRP levels in these patients , a Spearman-Rank correlation test was performed. With ESR, $r = 0.47$ ($p = < 0.001$) for IgA and $r = 0.46$ ($p < 0.001$) for IgG. With CRP, $r = 0.46$ ($p < 0.001$) for IgA and $r = 0.42$ ($p < 0.001$) for IgG. This indicates a moderate correlation of both values with IgA and IgG.

Table 1 Serum immunoglobulin levels (mg/ml) in patients and controls.

	Controls	Patients		
		Group A	Group B	Statistical Significance
Number	60	65	60	
IgA	2.2 (1.7-2.5)	2.4 (2.0-3.1)	4.3 (3.4-4.9)	Controls v GpA : NS Controls v GpB : p = <0.001 GpA v GpB : p : <0.001
IgG	12.45 (10.7-14.3)	12.4 (11.4-14.0)	20.1 (15.3-26.3)	Controls v GpA : NS Controls v GpB : p = <0.001 GpA v GpB : p : <0.001
IgM	1.55 (1.1-2.35)	1.4 (1.0-2.0)	1.6 (1.25-2.0)	Controls v GpA : NS Controls v GpB : NS GpA v GpB : NS

Group A = AS patients with a CRP<25 ug/ml and ESR<30 mm/h
Group B = AS patients with a CRP>25 ug/ml and ESR>30 mm/h
Medians and interquartile ranges shown
NS = not significant.

2.4.2 Measurement of in vitro immunoglobulin production by PBLs

ELISA standard curves

Figure II shows an example of a typical standard curve for each of the immunoglobulin classes studied. The most linear part of the curve lies within 10-800ng/ml and only this area was used in the assay. Any sample lying to the right of this area was diluted and measured again and any sample lying to the left was recorded as 10ng/ml.

Determining optimal conditions for cell culture

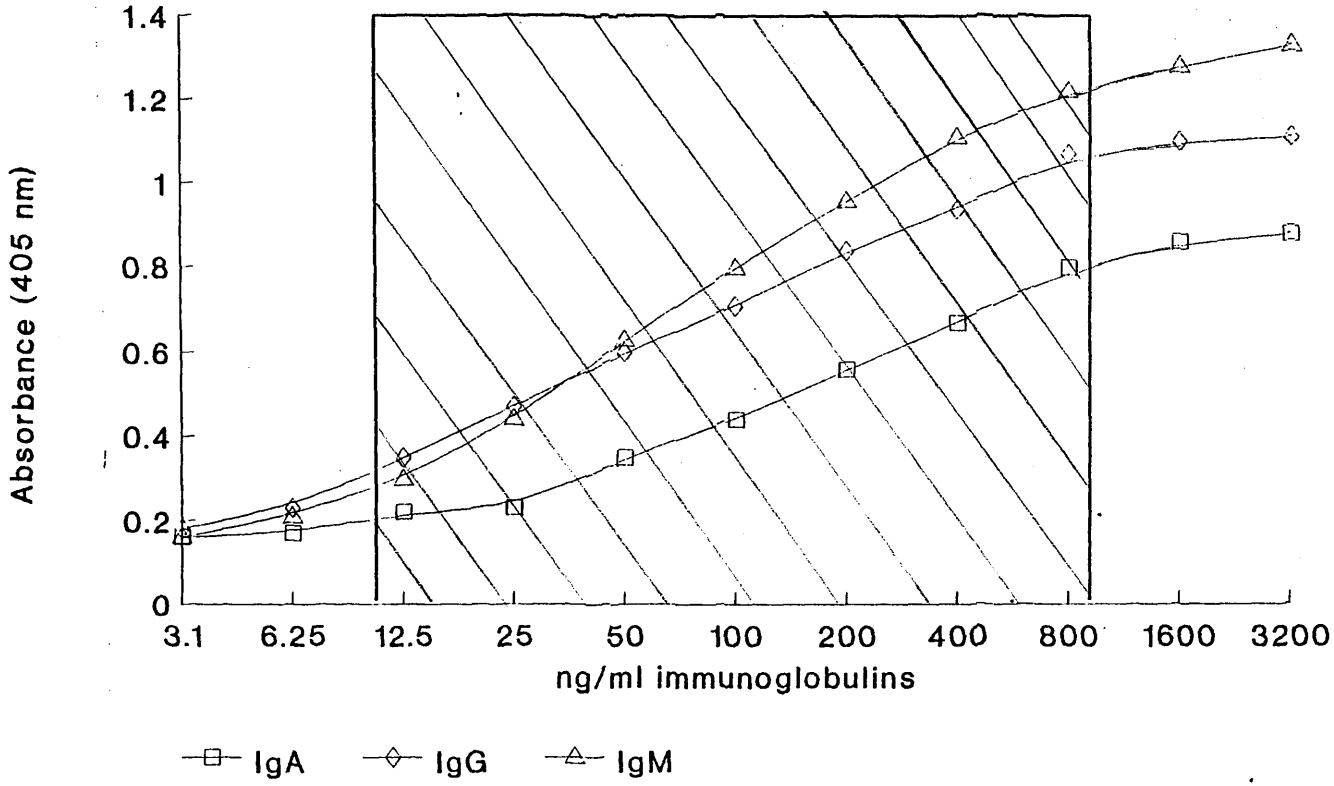
a) Cell number

$1x10^5$, $5x10^5$ and $1x10^6$ mononuclear cells from five control subjects were stimulated with a range of PWM dilutions and the immunoglobulin production measured after 10 days. The results are shown in Figure III. Clearly, a minimal response is shown by $1x10^5$ cells and an optimal response by $1x10^6$ for each immunoglobulin class. However, in order to limit the amount of blood required for the assay, $5x10^5$ cells were used since this was sufficient for significant immunoglobulin production.

b) PWM Dilution

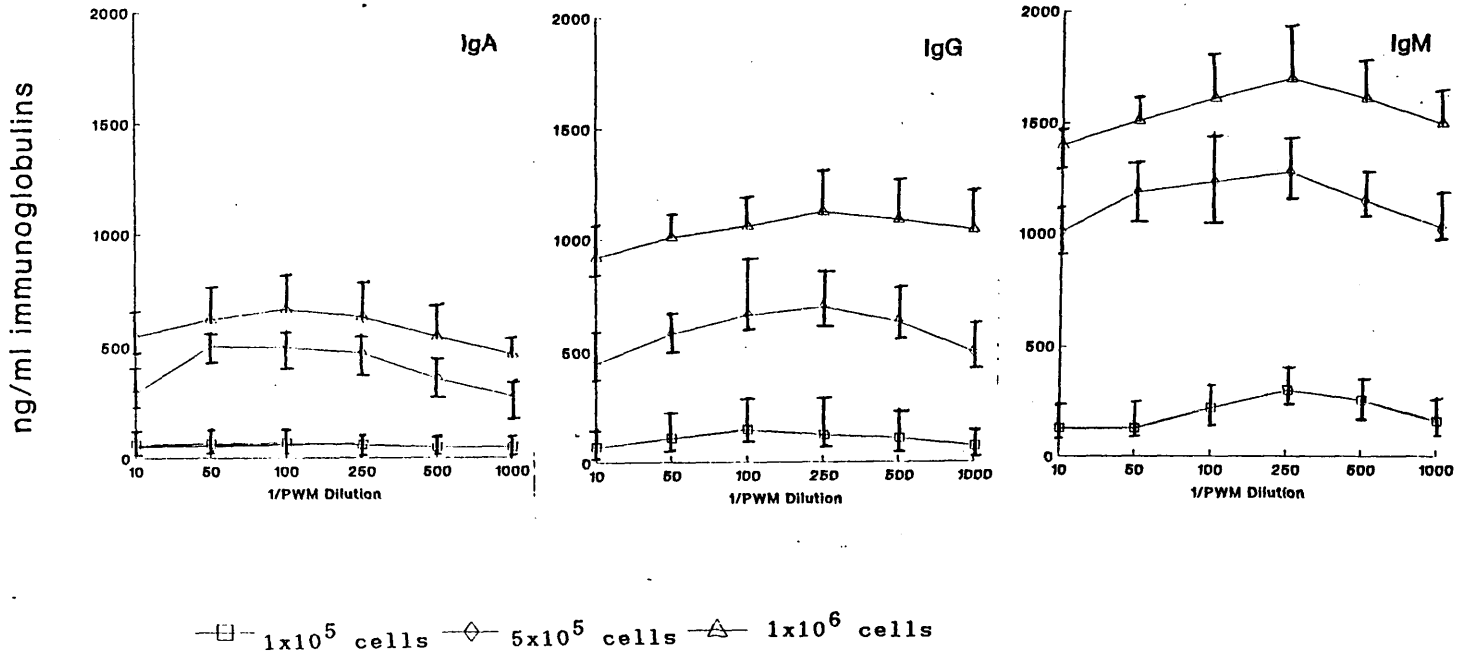
$5x10^5$ mononuclear cells from five control subjects were stimulated with a range of PWM dilutions. The immunoglobulin production after 10 days is shown in Figure IV. There is considerable variation in the optimal PWM dilution for different individuals : 1:50, 1:100 or 1:250 was shown to be optimal depending on the cells and the immunoglobulin class. It was therefore decided to use

Figure II. Standard ELISA curve of immunoglobulins



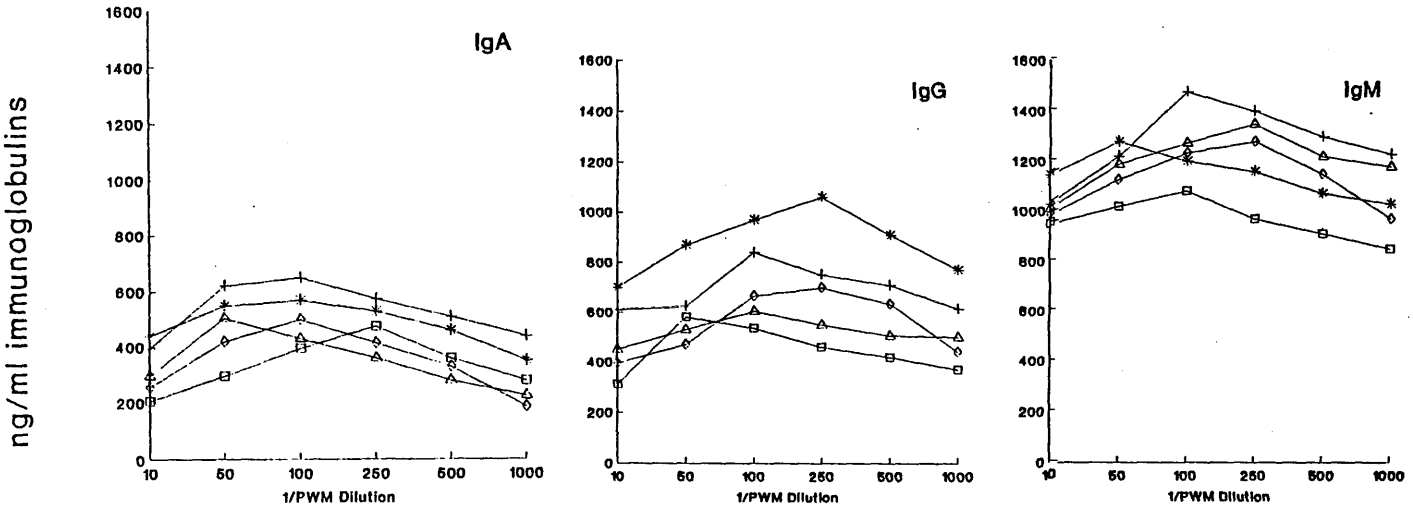
Shaded area shows part of curve used

Figure III. Effect of cell number on PWM-stimulated immunoglobulin production by peripheral blood lymphocytes



Medians and interquartile ranges shown

Figure IV. Effect of PWM concentration on immunoglobulin production by peripheral blood lymphocytes



each of these dilutions in the assay.

c) Incubation time

$5x10^5$ cells from five control subjects were stimulated with a 1:100 dilution of PWM and incubated for 5, 7, 10, 12 or 15 days. The results are shown in Figure V. Day 12 appeared to be the appropriate day for harvesting since any further immunoglobulin production by day 15 was minimal. The slightly higher median IgM production at day 10 (1220ng/ml compared to 1150ng/ml at day 12) was not significant.

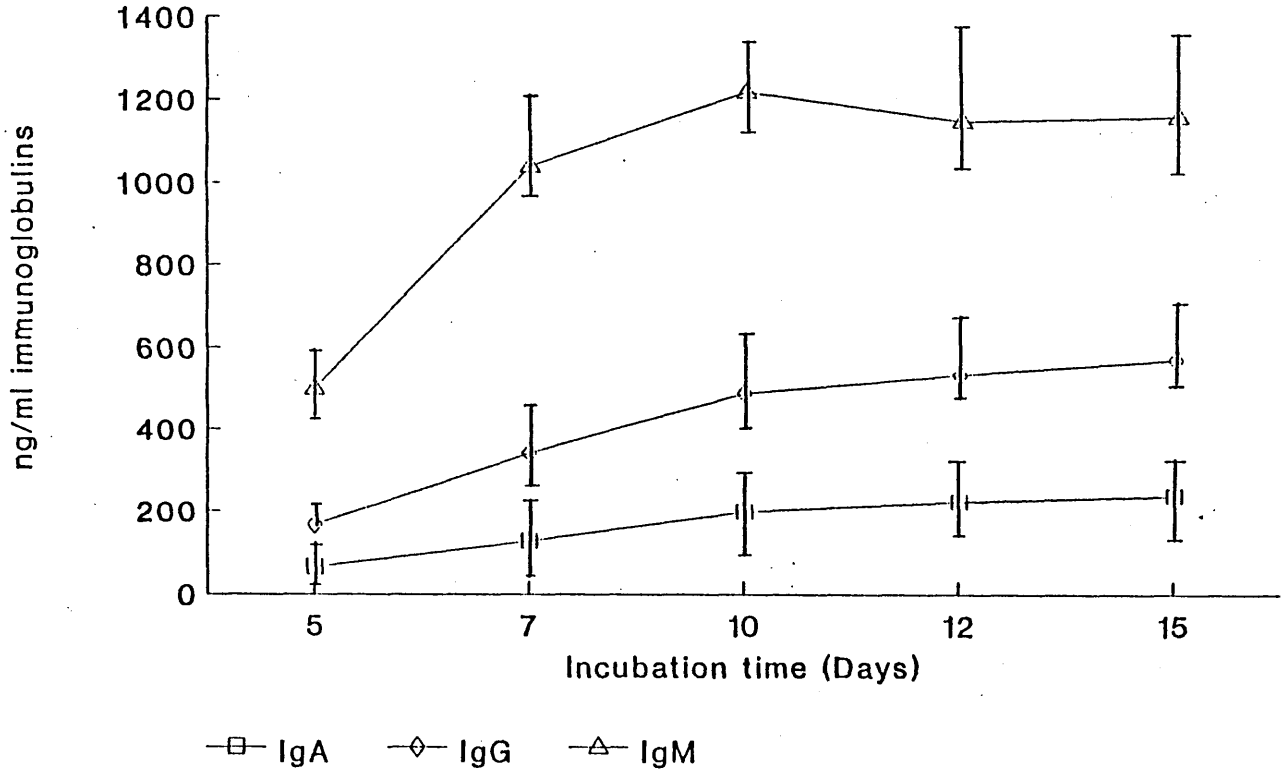
Comparing patient and control lymphocytes

$5x10^5$ cells from 20 patients with active disease, 20 patients with inactive disease and 20 controls were cultured alone or in the presence of a 1:50, 1:100 or 1:250 dilution of PWM for 12 days. Table 2 shows the amount of immunoglobulin produced spontaneously, the amount produced in response to a particular PWM concentration (1:100) and the maximum produced at any concentration of mitogen. In none of the groups was a difference found between controls, patients with active disease and patients with inactive disease for any of the three immunoglobulin classes. When the serum immunoglobulin levels of the subjects used in this study were measured, it was found that patients with active disease had significantly higher IgA and IgG levels than patients with inactive disease and controls ($p < 0.001$ for both immunoglobulin classes), reflecting the trend seen in Table 1.

2.4.3 Measurement of IgA in saliva

As shown in Figure VI, the salivary IgA levels in patients with active disease are comparable to those of patients with inactive disease and controls. When patients as a

Figure V. Effect of incubation time on PWM-stimulated immunoglobulin production by peripheral blood lymphocytes



Medians and interquartile ranges shown

Table 2 Spontaneous and PWM-stimulated immunoglobulin production (ng/ml) by patient and control peripheral blood lymphocytes

	Spontaneous			PWM-Stimulated					
				1:100 PWM			Maximum Immunoglobulin*		
	C	IA	A	C	IA	A	C	IA	A
No.	20	20	20	20	20	20	20	20	20
IgA	24 (15-44)	27 (11-57)	31 (21-86)	245 (178-390)	265 (164-340)	186 (141-470)	410 (237-510)	475 (280-520)	370 (185-620)
IgG	72 (28-200)	84 (36-165)	105 (53-173)	460 (180-670)	370 (105-605)	330 (150-720)	660 (324-870)	660 (390-950)	560 (290-740)
IgM	69 (22-180)	67 (20-155)	80 (27-165)	1100 (700-1250)	770 (420-930)	1200 (650-1650)	1300 (675-1450)	1175 (800-1550)	1450 (890-1700)

C = controls; IA = patients with inactive disease; A = patients with active disease

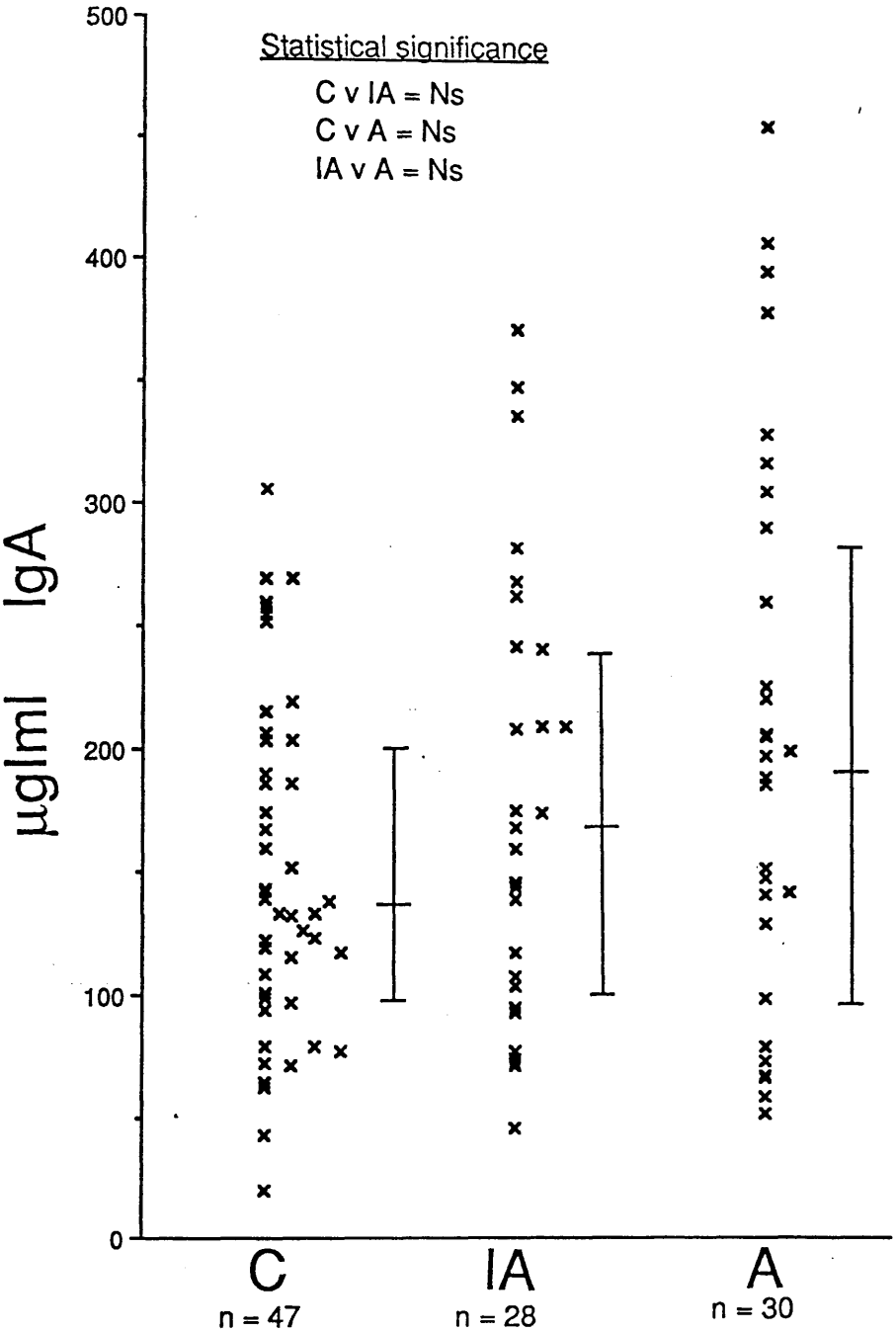
* Maximum immuglobulin produced at any of the PWM dilutions used.

Medians and interquartile ranges shown.

For each immunoglobulin class, C, IA and A subjects were compared within the spontaneous and the two PWM stimulated groups.

No significant differences were found.

Figure VI: IgA levels in saliva of patients and controls as measured by ELISA



C = controls; IA = patients with inactive disease;
A = patients with active disease
Ns = not significant
Medians and interquartile ranges shown.

whole were compared to controls, there was also no significant difference. It should be pointed out that the ELISA assay used detects IgA heavy chains. It is assumed that most of this is the secretory form of the immunoglobulin although no attempt was made to check this by measuring the secretory component.

2.5 Discussion

This study confirms previous reports of high IgA and IgG levels in the sera of patients who are thought to have active disease on the basis of elevated levels of ESR and CRP (57, 59). In addition, a moderate correlation was found between IgA and both ESR and CRP which is similar to that reported by two other groups (62, 68) but is contrary to the findings of Sanders et al. (95) who reported a very poor correlation with CRP ($r = 0.24$). The discordance between our results and those of Sanders could be related in part to the different number of patients included in each study (120 in our study compared to only 22 in theirs). Interestingly, IgG was found to correlate as well as IgA with ESR and CRP. As far as we know, we are the only group who has previously reported such a finding (96).

These high levels of immunoglobulins could reflect an ongoing immune response in patients with active disease. IgA levels are especially elevated : the median level in patients with active disease is almost double that of controls. It has been suggested (57, 62) that such findings indicate antigenic stimulation at a mucosal surface such as the gut since the majority of serum IgA is derived from plasma cells at such sites (61). This has lent support to the theory that an immune response to gut bacteria is involved in the pathogenesis of AS. However, one might therefore expect to find high levels of IgA in sera from patients with gastroenteritis. Neumann et al. (97) found this not to be the case. The same study showed

elevated IgA levels in Rheumatoid Arthritis (RA) and Psoriatic Arthritis (PsA) and the author concluded that these high concentrations of IgA do not indicate a gut infection but are a result of the inflammatory process associated with rheumatic diseases. Several groups (60, 87) have found that the raised serum IgA levels in AS do not differ significantly from those in RA, and Collado et al. (88) suggests that IgA should be regarded as an acute phase reactant like CRP. Ebringer's is the only group to report that elevated serum IgA is restricted to AS and not found in other inflammatory diseases (52).

It does seem very likely that non-specific mechanisms are operating. The amount of specific antibody produced in response to antigenic stimulation, by a gut bacteria for example, is not likely to account for such a major elevation in immunoglobulin levels. On the other hand, a large number of B-cells can be non-specifically induced to secrete immunoglobulins by lymphokines, such as B-cell differentiation factor, which are produced during inflammation. The fact that IgA and IgG are raised while IgM is normal may reflect the maturity of the cells which are stimulated : it is known that cells which produce IgA and IgG are more mature than IgM-secreting cells.

Whatever the cause, it is clear that high immunoglobulin levels are associated with active disease as defined by common laboratory parameters. This supports the use of immunoglobulin measurements in aiding the assessment of disease activity, as was suggested by Franssen et al. (60). All further studies in this thesis have used the " Point System For Assessment Of Disease Activity " (see Appeddix A) which takes immunoglobulin levels into account.

Another of our studies looked to see if the production of immunoglobulins by patients' PBLs in vitro supported the in vivo (serum) findings. If this was the case, it might be possible, using co-culture experiments, to investigate

the role of different lymphocyte populations in producing these high levels of immunoglobulins. Patients with Systemic Lupus Erythematosus (SLE) have high serum levels of immunoglobulins and they have been shown to have elevated numbers of cells which spontaneously secrete immunoglobulins *in vitro* but have a reduced response to PWM (98). By co-culturing SLE PBLs and mitomycin-treated normal T-cells, one group reported that the reduced response to PWM was due to decreased T-cell help (98). Nies *et al.* (99), on the other hand, found that this low response was not improved by co-culturing with normal T-cell populations and concluded that the abnormality lay with the B-cells themselves.

In our study, both spontaneous and PWM-induced immunoglobulin production by PBLs were measured as indicators of both the activation state of the cells *in vivo* and their capacity for further stimulation. No abnormality in production of any of the three major classes of immunoglobulin could be demonstrated by the PBLs of AS patients. This was despite the fact that patients in the active disease group had elevated levels of ***serum*** IgA and IgG. This supports the findings of Vuento *et al.* (91) for IgA and IgG but fails to support their demonstration of a low IgM response to PWM. They found that this low response was corrected by addition of hydrocortisone, suggesting that it was due to suppressor T-cells.

Clearly, our *in vitro* study does not reflect the findings in serum. There may be several explanations for this. In the case of IgA, it is believed that the majority of plasma cells producing serum IgA are located at mucosal surfaces and studying cells from the peripheral blood may not therefore be the most useful approach. It is also possible that a high activation state of the cells is dependent on serum factors such as interleukins which are not produced *in vitro*. Alternatively, it could be that the levels of immunoglobulin produced *in vitro* are too low

to reveal a difference between groups. Although significant amounts of immunoglobulins can be measured in the supernatants, they are in ng/ml concentrations while the serum levels are measured in mg/ml. Whatever the reason, it is clearly not possible to use co-culture experiments to investigate the cellular basis for the high levels of serum immunoglobulins found in AS patients.

IgA in patients' saliva was measured to see if it reflected the high levels found in serum. However, no significant difference was observed between controls, patients with active disease and patients with inactive disease. This is contrary to the findings of Calguneri et al. (58) which demonstrate high levels of IgA in the serum of patients with active disease. Interestingly, the levels found in our study are considerably higher than those reported by Calguneri : the median level in our active disease group was 193 µg/ml compared to a mean value of 55 µg/ml in theirs. The reason for this is not clear although different methods of collecting the saliva and a different assay were used : they used Curby cups (100) for collection and single radial immunodiffusion for their assay. Moreover, when comparing the groups, we used non-parametric analysis, appropriate to the spread of the data while t-tests were employed in their study. This may help to explain the different findings of the two studies.

Our finding of normal salivary IgA in AS does not support the proposal of antigenic stimulation of the gut mucosal immune system. It may seem surprising that serum IgA is raised while the IgA in mucosal secretions is not since this immunoglobulin is derived from mucosal sites. However, there is considerable doubt as to how well saliva reflects the intestinal immune response. As with their serum studies, Neumann et al. (97, 101) demonstrated that patients with gastroenteritis had normal levels of salivary IgA. Another study of acute gastroenteritis (102) found no evidence that bacterial-specific salivary antibodies correlated with antibodies in the intestinal

fluid. Trull et al. (65), however, demonstrated elevated salivary IgA antibodies to Salmonella in patients with Salmonella reactive arthritis. Clearly, an accurate measurement of the intestinal immune response requires studies of secretions and tissue from the gut itself. Access to these is difficult, but some such studies have been carried out in AS. In a preliminary study, O'Mahoney et al. (103) measured IgA in the intestinal fluid and showed that the levels in AS were normal and Stodell et al. (104) reported that AS patients have increased numbers of IgG-containing cells in their rectal lamina propria.

In conclusion, high serum levels of IgA and IgG are found in AS and are associated with active disease but the PBL of patients do not secrete high levels of immunoglobulins and patients have normal levels of IgA in their saliva.

This study has looked at non-specific immunoglobulins in AS. Perhaps more relevant than this is the specific antibody response to the environmental factors suspected of involvement in the pathogenesis of the disease : Klebsiella and other Enterobacteriaceae.

CHAPTER 3

ANTI-BACTERIAL ANTIBODIES

3.1 Introduction

In Reactive Arthritis (ReA), it has been shown that serum antibodies to the causative organism are elevated and can persist for several months. In one study, IgA antibodies to *Yersinia* were found in all 13 patients with Yersinia-arthritis and only 1 out of 12 patients who had an uncomplicated Yersinia infection. (64). These high levels could still be detected in the arthritic patients up to three years later. Trull *et al.* (65) found high serum levels of Salmonella-specific antibodies (of all classes) in suspected cases of Salmonella-arthritis. Similarly, high titres of IgG and IgM antibodies to *Chlamydia* have been found in sexually-acquired ReA (63).

If, as many authors propose, AS is a form of reactive arthritis which is associated with *Klebsiella* or other Enterobacteriaceae (39), one would expect to find high levels of antibody to these organisms in patients' sera. Several groups have investigated this. In a series of studies which have been recently reviewed (52), Ebringer's group have used several techniques to demonstrate the presence of high levels of antibody to *Klebsiella pneumoniae* in the sera of patients with active AS. In the first ELISA study (105), AS patients with an ESR $\geq$ 15mm/hr were found to have higher levels of IgA anti-Klebsiella antibodies than AS patients with an ESR $<$ 15mm/hr, patients with RA or Psoriatic Arthritis (PsA) and healthy controls. This antibody could be specifically absorbed out of the sera with *Klebsiella* and normal levels of antibody to the control organisms *E.coli* and Candida were found in these patients. In a subsequent ELISA study in which disease activity was based upon CRP levels (65), patients with active disease were found to have more IgA antibodies to

Klebsiella than patients with inactive disease. Antibodies to the other Enterobacteriaceae Salmonella, Yersinia and Pseudomonas were similar in patients and controls, regardless of disease activity.

Absorption studies (39) demonstrated that the level of total IgA in patients' sera was significantly reduced after overnight incubation with Klebsiella but not Proteus or Streptococcus. A fourth study by the same group (106) which used ^{125}I-labelled Klebsiella antigen in an inhibition assay, was said to show higher levels of specific antibody in the sera of patients with active disease (although the details of this study have never been published). Trull and Panayi (66) reported not only raised IgA antibodies to Klebsiella in the active disease group but a correlation between such antibodies and CRP levels.

In Scandinavian countries, the most frequent causative agent in ReA is Yersinia and here it is this organism rather than Klebsiella which is most strongly implicated in AS (68). High levels of Yersinia-specific antibodies have been detected in Scandinavian AS patients (68). In addition, in a small study of British patients, Pease *et al.* (69) found that serum antibodies to Yersinia but not Klebsiella oxytoca were slightly raised in AS although this was not related to disease activity.

In addition to the large number of studies on bacterial-specific antibodies in the serum, two groups have looked at **salivary** antibodies in AS. Trull et al. (65) found that patients had raised salivary antibodies to Klebsiella and a normal response to E.coli and Pseudomonas. (No comparison was made between patients with active and inactive disease.) In contrast, Pease et al. (69) found no elevation in the antibody response to either Klebsiella or Yersinia.

These studies of anti-bacterial antibodies arose out of reports by Ebringer's group of an increased faecal carriage of Klebsiella pneumoniae in AS patients with active disease

(41, 51, 107). One study (107) showed that these bacteria were isolated in 79% of cases of active disease compared to 40% of cases of inactive disease and only 16% of controls. Another study showed that patients with a positive culture for Klebsiella had higher mean CRP and ESR values than those with negative cultures (51). A similar association with active disease in a native American population was reported by Kuberski et al. (53). However, several groups have failed to find an association between Klebsiella and the presence or activity of AS (54, 55, 56).

3.2 Aims of Study

1) To study anti-bacterial antibodies in serum by ELISA and immunoblotting.

2) To measure anti-bacterial antibodies in saliva and lymphocyte culture supernatants.

3) To investigate the rate of carriage of Klebsiella and other Enterobacteriaceae.

3.3 Subjects and Methods

3.3.1 Bacterial preparations

The bacteria used were : Klebsiella pneumoniae K43, Yersinia enterocolitica O:3, Shigella flexneri 2a, Proteus mirabilis (all obtained from the National Collection of Type Cultures, U.K.), Escherichia coli 2387 (originally supplied by Dr. H.Mayer, Max-Plank Institute for Immunobiology, West Germany) and Salmonella typhimurium SH892 (originally supplied by Dr. P.H.Makela, Central Public Health Laboratory, Helsinki, Finland).

The bacteria were maintained in the laboratory on Columbia Blood Agar or Cysteine-Lactose-Electrolyte Deficient (C.L.E.D.) plates (supplied by Belvidere Hospital,

Glasgow). Several loopfulls were innoculated into 100ml of Mueller-Hinton Broth (Oxoid, UK) and incubated overnight at 37°C. The bacteria were centrifuged at 1500g for 10mins, washed twice in PBS and counted. The concentration of each bacterium was calculated from its absorbance at 405nm. The relationship between concentration and absorbance for S.flexneri 2a is shown in Appendix C. For the other bacteria, the concentration was calculated from standard curves previously drawn up in our laboratory (50). Two bacterial preparations were made : formalin-killed and sonicated. For formalin treatment, the bacteria were made up at 10^8 colony-forming units (CFU)/ml in PBS containing 1% formalin and left overnight at room temperature. After three washes in PBS (centrifuging at 1500g for 10 minutes), the cells were resuspended in PBS, recounted and stored at -20°C. For sonicated preparations, the cells were made up to 10^9 CFU/ml in PBS and sonicated on ice in 5ml volumes for 10 minutes at 10 microns. The preparation was centrifuged at 1500g for 10 minutes to remove intact cells. The protein concentration of the supernatant was measured using the Lowry method (205) and adjusted to 1mg/ml. This was stored at -20°C until used.

3.3.2 Measurement of anti-bacterial antibodies in sera, saliva and culture supernatants.

Subjects

a) Serum studies

30 controls (C), 29 patients with inactive disease (IA) and 30 patients with active disease (A) were studied. The details of the subjects are given over the page.

	C	IA	A
Number	30	29	30
Mean age in years (range)	34 (26-50)	39 (24-56)	46 (29-67)
Male:Female ratio	4:1	7.6:1	8.1:1

b) Saliva studies

The subjects were those used in section 2.3.3

c) Culture supernatants

The subjects were those used in section 2.3.2

Serum collection

10ml of venous blood were collected in glass tubes and allowed to clot at room temperature for two hours then centrifuged at 2000g for 10 mins. The serum was removed and stored at -70°C until tested.

ELISA

An indirect ELISA was used. 100µl of the bacterial preparation, diluted in 50mM bicarbonate buffer (pH9.6), were added to the wells of 96-well flat-bottom plates (Sterilin, UK) and left to coat at 37°C for 1 hour. The plates were washed and the non-specific binding sites blocked as previously described (Section 2.3.2). 100µl of the test sample, diluted in PBS-Tween 20, were added in triplicate to the wells and the plates were incubated at 37°C for 60 minutes. The plates were washed three times and 100µl of a 1:5000 dilution of biotinylated sheep anti-

human immunoglobulin (Amersham, UK) or a 1:2500 dilution of biotinylated goat anti-human IgA (Amersham, UK) in PBS-Tween 20 were added and the plates left for a further 60 minutes at 37°C. After a further three washes, 100µl of a 1:100 dilution of a streptavidin and biotinylated alkaline phosphatase complex (Strep ABC Complex-Alkaline Phosphatase, Dakopatts, Denmark) in 50mM tris pH7.6 were added and the plates incubated for 30 mins at 37°C. The substrate was added and the absorbance read as described in Section 2.3.2. In each test, the sample was added to wells which had been coated with bacteria and to uncoated (blank) wells. The absorbance of the blank wells was subtracted from that of the test sample.

3.3.3 Immunoblotting of Klebsiella proteins with patients' sera

Subjects

From the subject groups described in Section 3.3.2, 20 control samples, 20 samples from patients with inactive disease and 20 samples from patients with active disease were studied.

Immunoblotting

Sodium duodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Laemmli system (108) with a glass plate cassette (Gibco, Scotland) of dimensions 15x17x0.15cm. A 10% polyacrylamide running gel was prepared using the following formula : 50% acrylamide mix (48.75% acrylamide,1.25% N,N,-methylene bisacrylamide), 4ml; 1M tris HCL pH8.8, 15ml; 10% SDS, 0.4ml; 1.5% ammonium persulphate (Sigma, UK), 0.9ml; N,N,N,N-Tetramethylethylenediamine (TEMED) (Sigma, UK), 0.025ml; deionised water (dH_2O), 15.7ml. 32ml of this was poured into the cassette, overlayed with isobutanol and allowed to set. The isobutanol was poured off and the surface of the

gel was rinsed with dH_2O before 5ml of stacking gel was poured on top. The stacking gel consisted of : 50% acrylamide mix, 1.0ml; 1M tris HCL (pH6.8), 1.25ml; 10% SDS, 0.1ml; TEMED, 0.02ml; dH_2O, 7.15ml. This was overlayed with isobutanol and allowed to set. After rinsing the stacking gel surface, the cassette was attached to the gel tank and both chambers were filled with electrode buffer (0.193M glycine, 0.025M tris base, 0.1% SDS). 1.5ml of a 500µg/ml preparation of sonicated Klebsiella which had been diluted in sample buffer (tris-HCL (pH6.8) containing 6% SDS, 6% 2-mercaptoethanol, 40% sucrose and approximately 0.01% pyronin Y (Sigma, UK)) was boiled for 5 minutes and carefully layered onto the surface of the gel. The electrodes were connected to a power pack and run at a constant current of 40mA at room temperature for 4 hours or until the dye had reached the bottom of the gel.

The proteins on the gel were then electrophoretically transferred to nitrocellulose membranes (BIO-RAD, USA) using a Multiphor II Electrophoresis Unit (Pharmacia/LKB, UK) according to the manufacturer's instructions. The transfer buffer was 39mM glycine, 48mM tris base, 0.0375% SDS, and 20% methanol, and the electrophoresis was continued for 25 minutes at 200mA.

After transfer, the membrane was incubated at 4°C overnight in 50mM tris (pH10.3) containing 150mM sodium chloride and 0.025% Tween-20. It was then cut into 1cm strips and each of these was sealed in polythene tubing (Jencons, UK) with 5ml of the test sera, diluted in 50mM tris containing 150mM sodium chloride and 0.1% Tween 20 (incubation buffer). After 90 minutes at room temperature, the nitrocellulose strips were washed for 30 minutes in four changes of incubation buffer. They were then incubated for 60 minutes in a 1:500 dilution of biotinylated sheep anti-human immunoglobulins (Amersham, UK) or a 1:250 dilution of biotinylated goat anti-human IgA (Amersham, UK) in incubation buffer, using sufficient volume to cover the

strips (usually 30ml). After three washes, they were placed in a 1:100 dilution of a streptavidin and biotinylated alkaline phosphatase complex (Strept ABC Complex-Alkaline Phosphatase, Dakopatts, Denmark) in 50mM tris pH7.6 and left for 30 minutes before washing a further three times. The substrate solution was prepared using the following formula : 0.1M ethanolamine buffer (pH9.6), 50ml; 1g/l nitrobluetetrazolium (Sigma, UK), 5ml; 0.4% 5-bromo-4-chloro-3-indolyl phosphate (Sigma, UK) in 2 parts methanol:1 part acetone, 0.75ml; 1M magnesium chloride, 0.2ml. The strips were incubated in the substrate solution and the colour allowed to develop for 20 minutes. They were then rinsed in H_2O and left to dry. The resulting banding pattern was assessed by eye.

3.3.4 Isolation of Enterobacteriaceae from rectal swabs

Subjects

Patients with inactive (IA) and active disease (A) were studied. The details are given below.

	IA	A
Number	24	27
Mean age in years	43	43
(Range)	(26-29)	(20-77)
Male:Female ratio	7:1	4.4:1

Method

Rectal swabs were taken by the patients themselves using cotton-tipped applicators ("Transwab", Medical Wire and Equipment, UK). They were innoculated into several culture media : MacConkey Agar (without salt), Desoxycholate Citrate Agar (Hynes), C.L.E.D. Medium, Simmons Citrate Agar with 1% inositol, Columbia Blood Agar Base with Campylobacter skirrow and horse blood, and Selenite Broth Base with sodium biselenite. These were all supplied by Belvidere Hospital, Glasgow, and made up according to "The Oxoid Manual" (109). The Campylobacter plates were placed in an anaerobic jar (Don Whitely Scientific, UK) containing a hydrogen and carbon-dioxide generator envelope (BBL Microbiological Systems, USA) and left at 37°C for 48 hours. The remaining cultures were incubated for 24 hours under aerobic conditions. A sample from each resulting bacterial colony was cultured onto plates containing Columbia Blood Agar Base with horse blood and left for 24 hours. If necessary, further passage onto these plates was carried out until pure colonies were obtained. Colonies which were found to be Gram-negative were identified using an Analytical Profile Index (API) 20E (API-Bio-Merrieux, UK). The Klebsiella isolates were sent to the National Collection of Type Cultures for identification of species and type.

3.3.5. Statistical Analysis

The Mann-Whitney and Chi-squared tests were used (see Appendix B).

3.4 Results

3.4.1 Measurement of anti-bacterial antibodies in sera

Effect of bacterial concentration

In order to determine the concentration of sonicated bacteria required to coat the ELISA plates, 1:100 dilutions of five control sera were tested against a range of dilutions of Klebsiella, Salmonella, Shigella, Yersinia and E.coli. The results are shown in Table 3. For each bacteria, 200µg/ml was the concentration chosen for the assay since this produced high levels of antibody binding which were not significantly increased when 500µg/ml were used.

Effect of serum concentration

Using 200µg/ml Klebsiella to coat the wells, various dilutions of five control sera was tested for both total and IgA antibodies. The results are shown in Figure VII. For total antibodies (using anti-human immunoglobulins as the 2nd antibody), the most linear part of the curve lies between a 1:100 and a 1:500 dilution of sera and for IgA antibodies (using anti-human IgA as the 2nd antibody), it lies between a 1:50 and a 1:200 dilution. Similar curves were obtained with the other bacteria. A 1:200 dilution was therefore used for the total antibody assay and a 1:100 dilution for the IgA antibody assay.

Comparing patient and control sera

a) Total antibodies

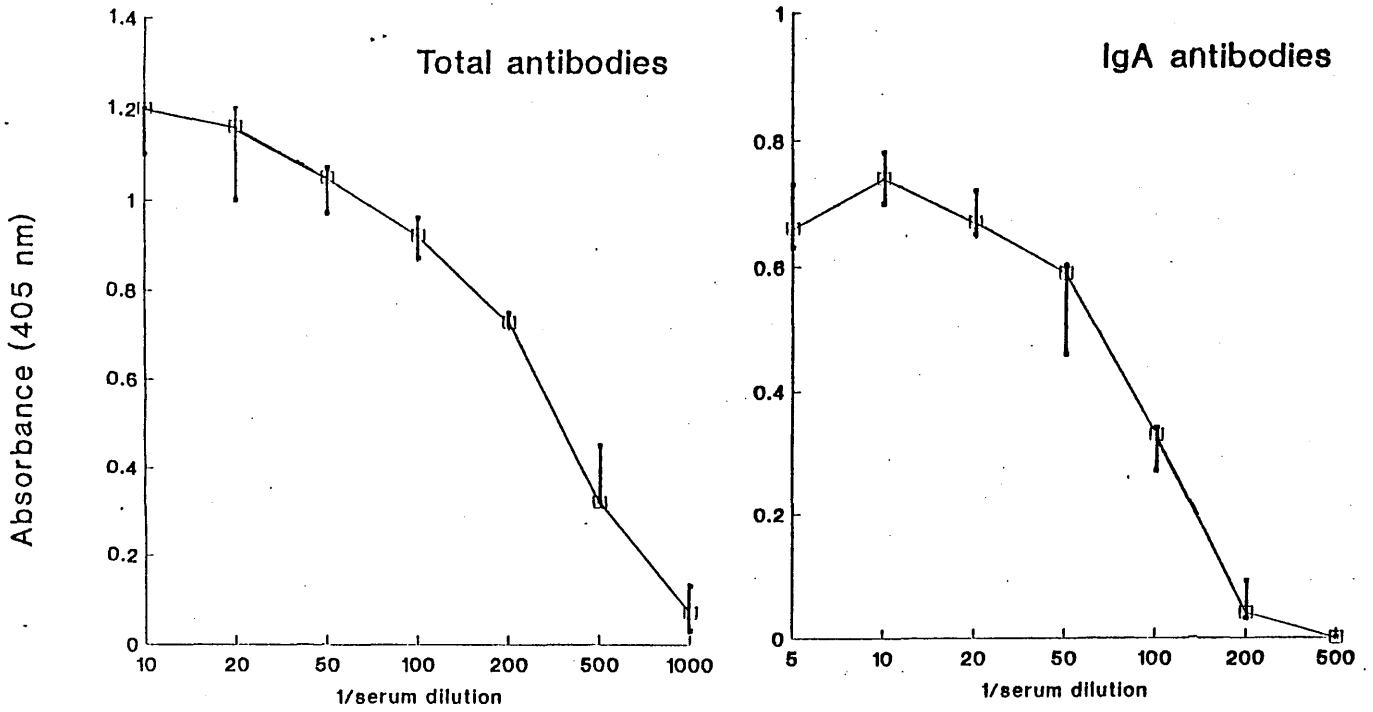
The amount of Klebsiella-specific antibodies in the sera of 59 patients and 30 controls was measured using the bacteria and serum dilutions outlined above. A patient's serum which was known to have significant levels of

Table 3. Effect of bacterial concentration on measurement of antibodies to sonicated bacteria by ELISA ($Absorbance_{405nm}$).

Conc. sonicated bacteria (ug/ml)	1	10	100	200	500
Klebsiella	0.12 (0.1-0.15)	0.39 (0.22-0.41)	0.78 (0.44-0.94)	1.03 (0.68-1.1)	1.01 (0.75-1.16)
Salmonella	0.12 (0.09-0.21)	0.66 (0.56-0.77)	0.99 (0.86-1.05)	1.00 (0.98-1.01)	1.08 (1.03-1.1)
Shigella	0.16 (0.13-0.26)	0.68 (0.56-0.76)	0.83 (0.77-1.06)	0.87 (0.86-1.06)	0.94 (0.85-1.16)
Yersinia	0.18 (0.11-0.25)	0.73 (0.58-0.86)	0.85 (0.2-1.12)	0.87 (0.78-1.10)	0.93 (0.79-1.1)
E. coli	0.13 (0.10-0.18)	0.62 (0.56-0.78)	0.72 (0.6-0.81)	0.94 (0.80-1.0)	0.95 (0.9-0.99)

Medians and interquartile ranges shown.

Figure VII. Effect of serum dilution on measurement of antibodies to sonicated Klebsiella by ELISA



The medians and interquartile ranges of five control sera are shown

Enterobacteriaceae-specific antibodies was used as a standard. The standard was included in each plate and the result for each sample was expressed as : Absorbance(405nm) of sample / Absorbance(405nm) of standard (after subtracting the absorbance of the blank wells). The colour was allowed to develop until the absorbance of the standard was approximately 1.0. This practice was used in most of our ELISA studies of bacterial-specific antibodies.

Figure VIII shows the amount of total antibodies to sonicated Klebsiella in controls, patients with inactive disease and patients with active disease. There was no difference between any of the groups.

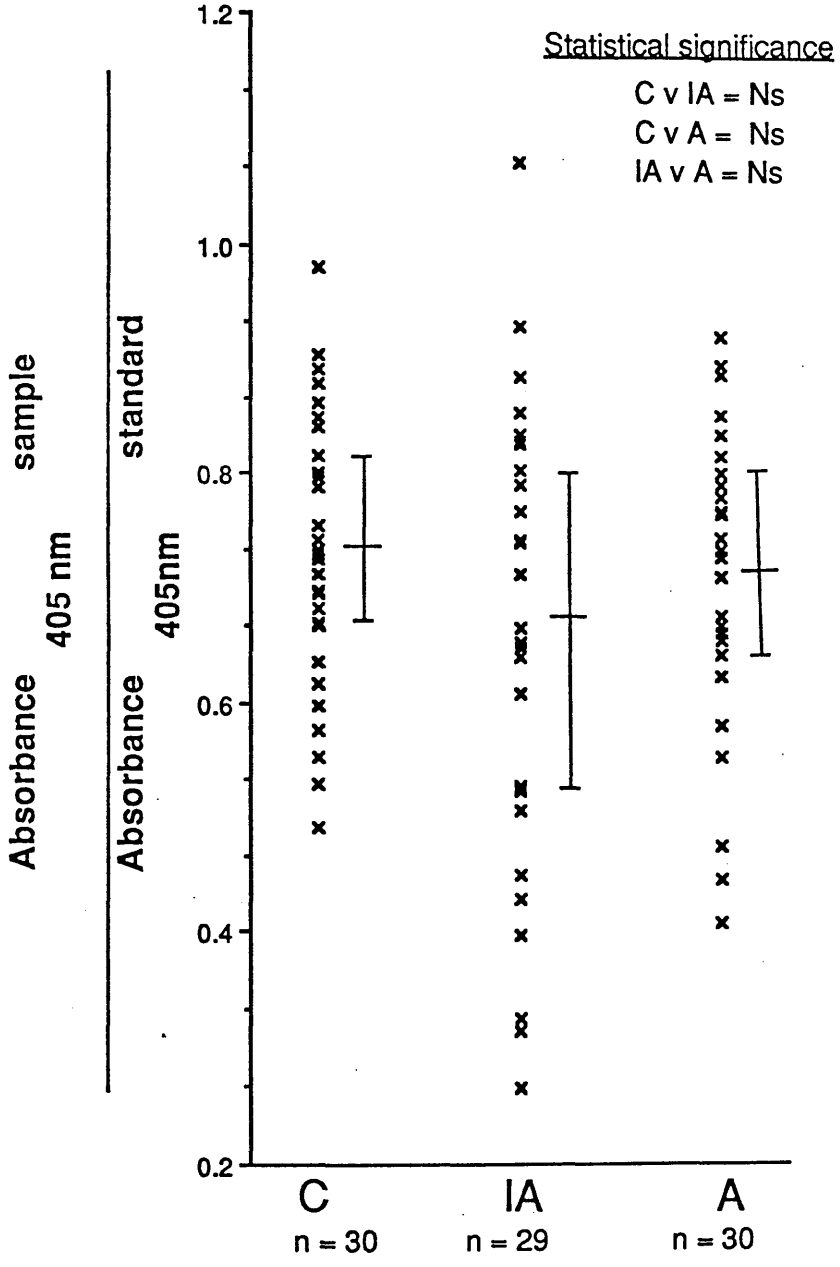
b) IgA antibodies

Klebsiella-specific antibodies which were exclusively of the IgA class were also measured (Figure IX). The patient group as a whole was found to have an elevated median leval of specific antibody ($p < 0.01$) and individually both the active and the inactive disease group had more specific antibodies than the controls. However, when patients with active disease were compared to patients with inactive disease, no significant difference was found.

Comparing patient and control serum IgA antibodies to other bacteria

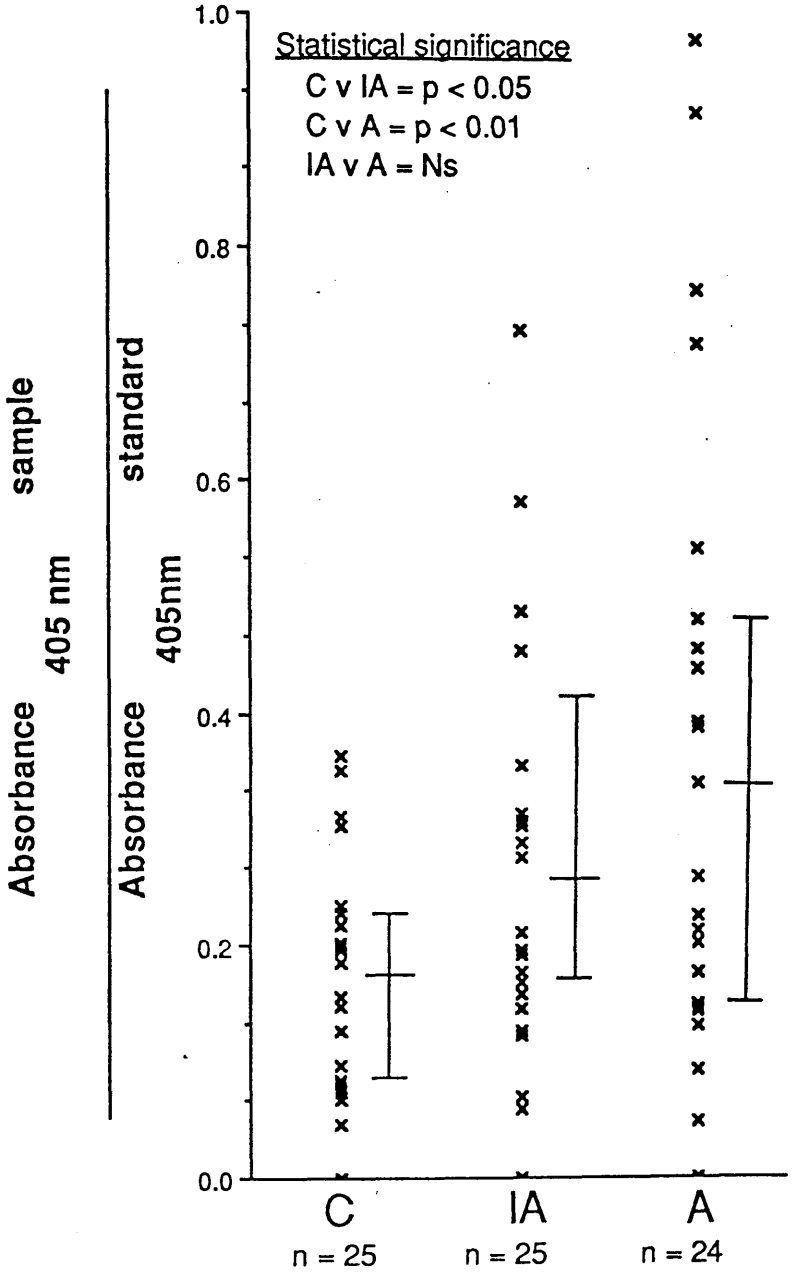
To see if an elevated IgA response was also found against other bacteria, IgA antibodies to several other members of the Enterobacteriaceae family were measured. Compared to controls, patients were found to have higher levels of IgA antibodies to Salmonella, Shigella and Yersinia but not to E.Coli or Proteus (Table 4). Like the response to Klebsiella, the elevated antibody levels to Salmonella are not related to the activity of the disease since there is no significant difference between the active and the inactive disease group. In the case of Shigella and Yersinia, however, patients with active disease have more specific antibodies than patients with inactive disease and

Figure VIII: Total antibodies to sonicated Klebsiella in the sera of patients and controls as measured by ELISA.



C = controls; IA = patients with inactive disease;
A = patients with active disease
Ns = not significant
Medians and interquartile ranges shown.

Figure IX : IgA antibodies to sonicated Klebsiella in the sera of patients and controls as measured by ELISA.



C = controls; IA = patients with inactive disease;
A = patients with active disease
Ns = not significant
Medians and interquartile ranges shown.

Table 4. IgA antibodies to sonicated bacteria in sera of patients and controls as measured by ELISA (Absorbance$_{405nm}$ sample/Absorbance$_{405nm}$ standard).

	Subjects				Statistical Significance			
	C	P	IA	A	CvP	CvIA	CvA	IAvA
n	25	49	25	24				
Salmonella	0.16 (0.1-0.33)	0.33 (0.24-0.55)	0.27 (0.18-0.45)	0.42 (0.29-0.57)	p<0.001	p<0.05	p<0.001	NS
Shigella	0.24 (0.14-0.42)	0.29 (0.17-0.52)	0.2 (0.14-0.35)	0.38 (0.2-0.67)	p<0.05	NS	p<0.05	p<0.01
Yersinia	0.20 (0.15-0.41)	0.42 (0.21-0.67)	0.24 (0.15-0.41)	0.65 (0.42-0.96)	p<0.05	NS	p<0.001	p<0.001
E. coli	0.60 (0.52-0.84)	0.73 (0.51-0.93)	0.67 (0.43-0.83)	0.78 (0.62-0.95)	NS	NS	NS	NS
Proteus	0.23 (0.15-0.36)	0.26 (0.13-0.45)	0.26 (0.08-0.48)	0.26 (0.13-0.45)	NS	NS	NS	NS

C = controls; P = all patients; IA = patients with inactive disease; A = patients with active disease; NS = not significant.

Medians and interquartile ranges shown.

these patients with inactive disease have antibody levels comparable to that of the control group.

Absorption study

A small absorption study of three patient sera was carried out to determine whether the IgA antibody response to Klebsiella was cross-reactive with the other bacteria. 1ml of a 1:50 dilution of the sera was added to wells of a 24-well plate which had been coated with 200µg/ml sonicated Klebsiella. After 2 hours at 37°C, the serum was transferred to a second Klebsiella-coated well. This was repeated four times at 2 hourly intervals and the final incubation was at 4°C overnight. By ELISA, the levels of antibody to various sonicated bacteria were measured in these serum samples (post-absorption) and compared to serum samples which had been incubated in the same way in uncoated wells (pre-absorption). For Klebsiella, it was found that the mean ELISA value (Absorbance(405nm)) of the post-absorption samples was 40% lower than that of the pre-absorption samples. For Salmonella, Shigella, Yersinia and E.coli, the levels were 23%, 20%, 19% and 19% lower, respectively.

3.4.2 Measurement of anti-bacterial antibodies in mitogen-stimulated PBL culture supernatants.

Effect of concentration of formalin-killed bacteria

Table 5 shows the absorbance (405nm) of five control sera (1:100) tested in wells coated with a range of dilutions of formalin-killed bacteria. For each bacterium, 10^9 CFU/ml were found to be sufficient to produce maximum antibody binding and this concentration was used in all subsequent ELISAs of formalin-killed bacteria.

Table 5. Effect of bacterial concentration on measurement of antibodies to formalin-killed bacteria by ELISA ($Absorbance_{405nm}$)

Conc bacteria (CFU/ml)	10^7	10^8	5×10^8	10^9	5×10^9
Klebsiella	0.23 (0.21-0.24)	0.27 (0.27-0.34)	0.47 (0.42-0.48)	0.73 (0.71-0.82)	0.74 (0.54-0.78)
Salmonella	0.53 (0.46-0.88)	0.74 (0.55-0.79)	0.94 (0.77-1.14)	1.07 (1.03-1.12)	1.07 (1.06-1.11)
Shigella	0.32 (0.28-0.34)	0.37 (0.36-0.39)	0.73 (0.68-0.78)	0.87 (0.84-0.89)	0.89 (0.87-0.90)
Yersinia	0.34 (0.32-0.57)	0.53 (0.42-0.58)	0.87 (0.74-0.93)	1.01 (0.93-1.02)	1.13 (1.03-1.18)
E. coli	0.33 (0.32-0.37)	0.62 (0.61-0.65)	0.71 (0.68-0.75)	0.70 (0.66-0.77)	0.71 (0.59-0.74)

CFU = colony forming units.

Medians and interquartile ranges of five control sera shown.

Comparing patient and control supernatants

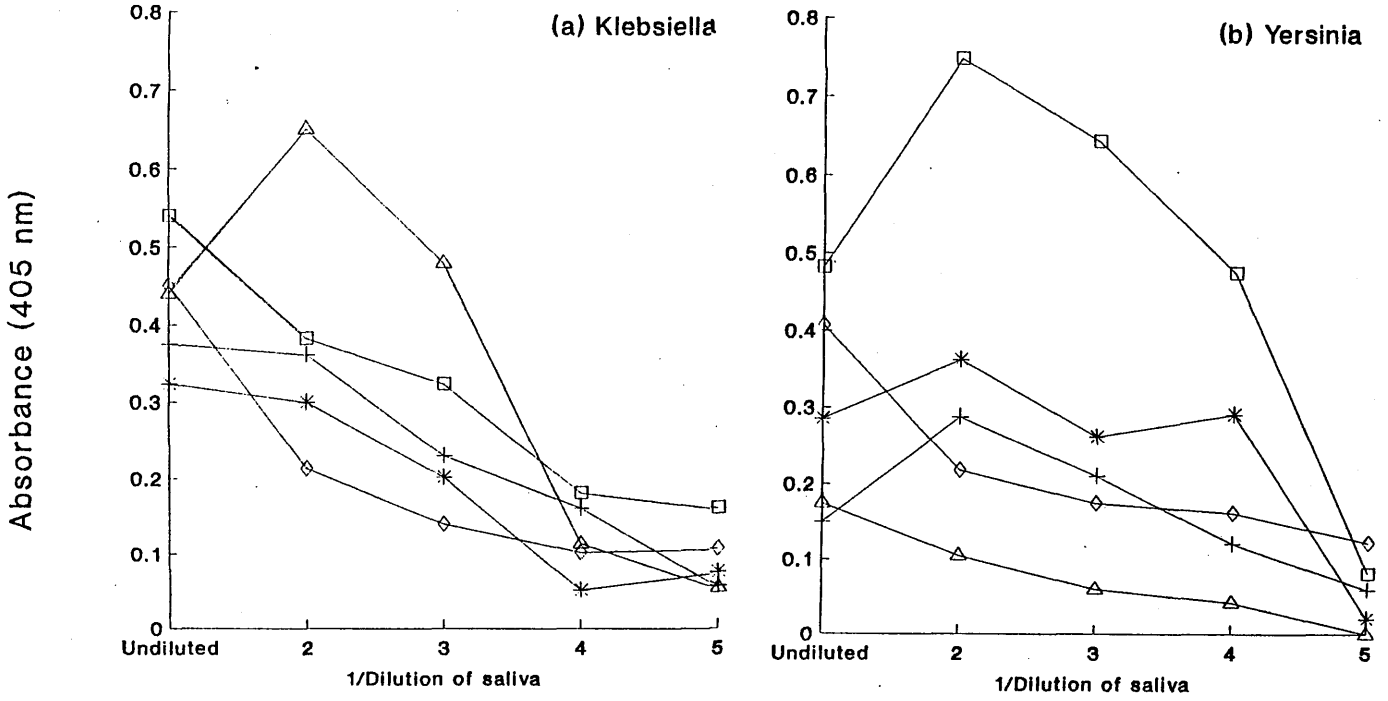
The mitogen-stimulated PBL culture supernatants obtained in Section 2.3.2 were studied for the presence of total antibodies to both sonicated and formalin-killed Klebsiella, Salmonella, Shigella and Yersinia. 1:2 dilutions of samples from 10 patients and 10 controls were studied and for each individual, the culture supernatant which had been found to contain the maximum amount of immunoglobulin was used. The absorbance (405nm) readings obtained were all below 0.1 (The equivalent reading for the serum standard was approximately 1.0.) indicating that the culture supernatants contained negligible amounts of antibodies reactive with these bacteria. We nevertheless compared patients and controls and no differences were found.

3.4.3 Measurement of anti-bacterial antibodies in saliva

Effect of saliva concentration

Figure X shows the ELISA readings obtained when various dilutions of control saliva (undiluted - 1:5) are tested for antibodies to sonicated Klebsiella and Yersinia. Since different patterns were found, individual curves for each sample, not median values, are shown. Specific antibodies to these bacteria can be detected in the saliva but only at high concentrations, falling off as the sample is diluted to 1:5. In some cases, the antibody levels detected in the undiluted sample were lower than those found at a 1:2 dilution. This is most likely due to the problem of high background (i.e. non-specific binding of antibody) in such concentrated samples. It was decided to use a dilution of 1:3 for the assay. Although the readings obtained with this dilution are relatively low, we can be quite confident that it lies within the linear part of the curve.

Figure X. Effect of saliva dilution on measurement of salivary IgA antibodies to sonicated bacteria by ELISA



The results of five control saliva are shown

Comparing patient and control saliva

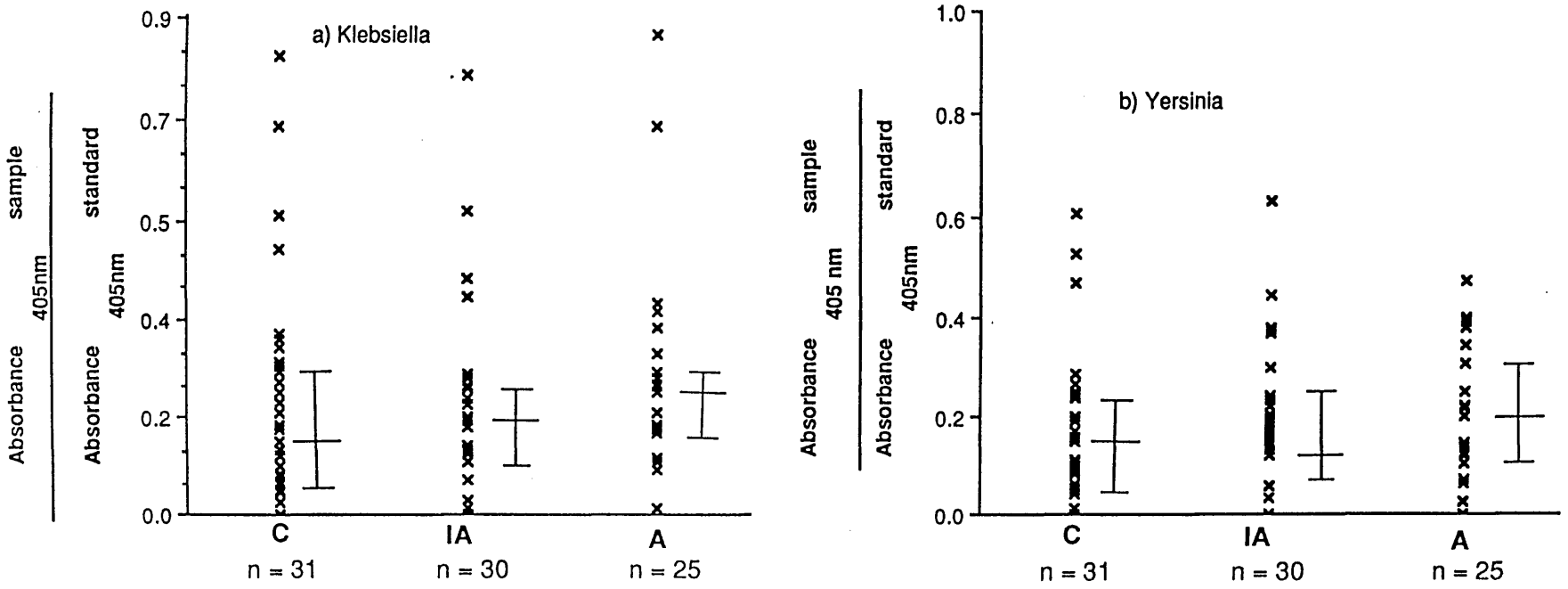
Figure XI compares the levels of IgA antibodies to sonicated Klebsiella and Yersinia in 20 controls, 20 patients with inactive disease and 20 patients with active disease. No significant differences were found between any of the groups. Figure XII shows the equivalent results for formalin-killed bacteria and once again, no difference were found. There was also no significant difference when patients as a whole group were compared to controls.

3.4.4 Immunoblotting of Klebsiella proteins with patient sera

a) Total antibodies

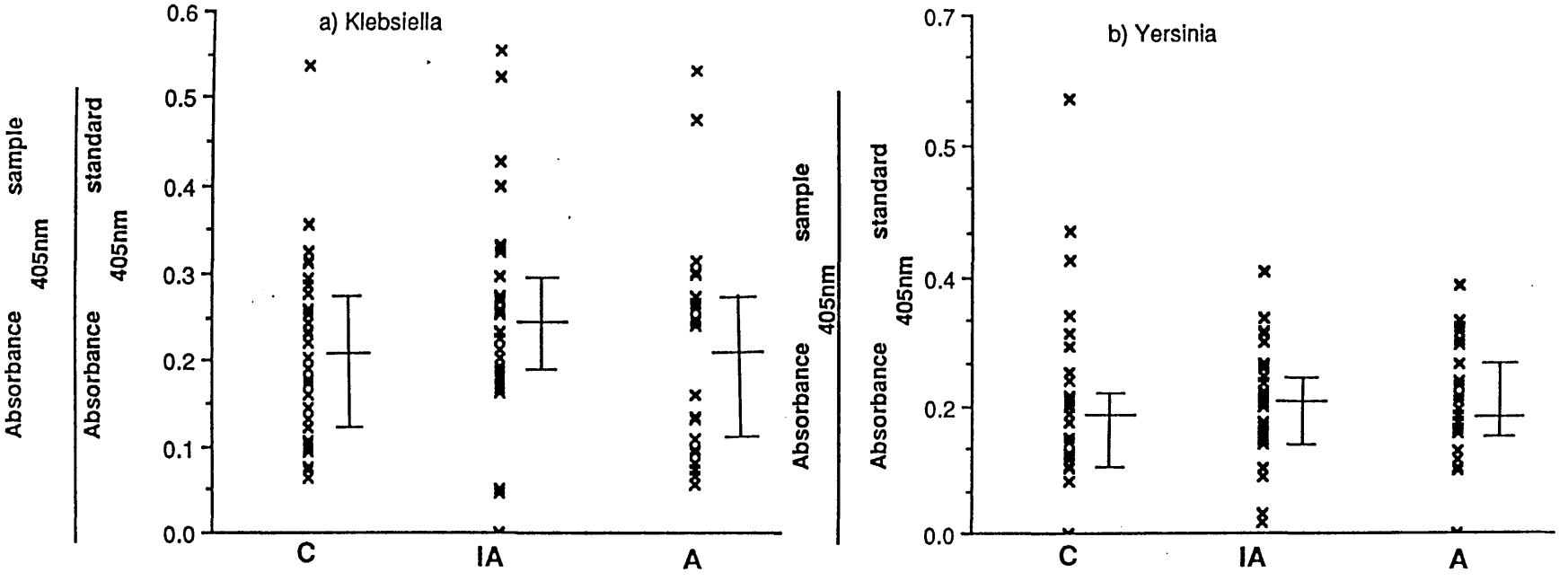
A serum dilution of 1:200 was used as in the ELISA (Section 3.3.1). Figure XIII shows an example of an immunoblot obtained with a patient's serum. We found a total of 24 distinct proteins bands (although no immunoblot contained all 24 bands) and the presence and intensity of each of these bands was noted for each serum sample. In Table 6, we have shown the frequency with which each band occurs in the control and patient groups. Clearly, some bands are present in the majority of the samples (e.g. bands 14-16 which have molecular weights of around 30kD) and some are rarely present (e.g. band 3 which has a molecular weight of approximatley 92kD). However, no band is found significantly more frequently in any of the subject groups. (This was confirmed by chi-squared tests.) This suggests that neither the presence nor absence of antibodies to any of these separated proteins is associated with the presence or activity of AS. The intensity of the staining of each band was also studied (assessed by eye and scored as +-, + or ++). The strength of an antibody response to any of the proteins did not appear to be associated with any of the subject groups.

Figure XI : IgA antibodies to sonicated bacteria in saliva of patients and controls as measured by ELISA



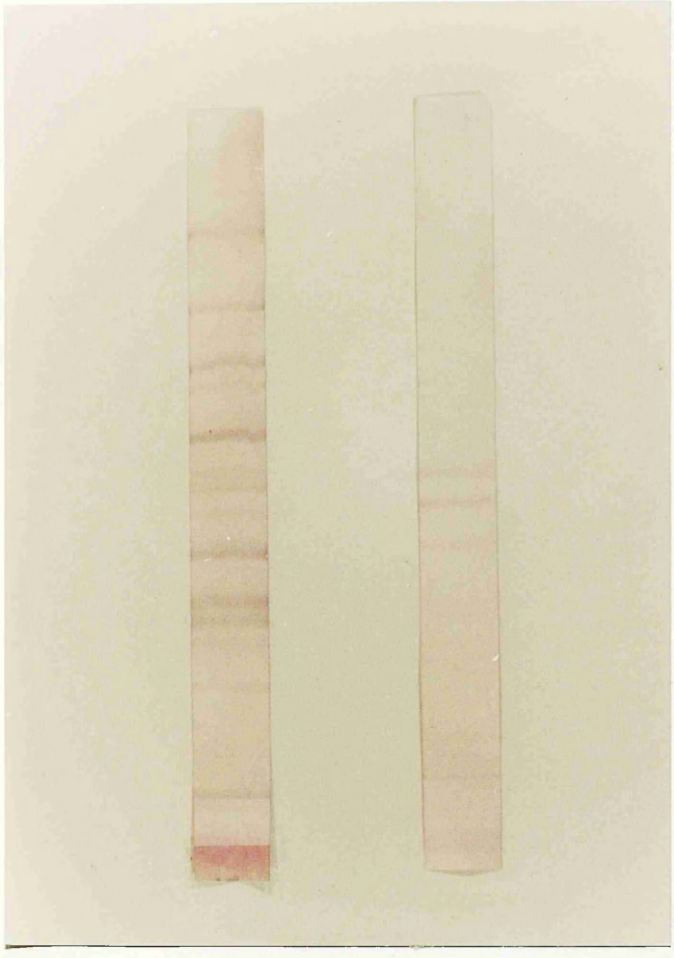
C = controls; IA = patients with inactive disease; A = patients with with active disease.
Medians and interquartile ranges shown.
No significant differance was found between the C, IA and A groups for either bacteria

Figure XII : IgA antibodies to formalin - killed bacteria in saliva of patients and controls as measured by ELISA



C = controls; IA = patients with inactive disease; A = patients with with active disease.
Medians and interquartile ranges shown.
No significant differance was found between the C, IA and A groups for either bacteria

Figure XIII. Immunoblotting of Klebsiella proteins with a patient's serum



A = Total antibodies; B = IgA antibodies

Table 6. Immunoblotting of Klebsiella proteins with patient and control sera. (Total antibodies).

Protein Band No.	Approx Mol wt.	Sera C n=20	IA n=20	A n=20
1		3	5	4
2		3	1	3
3	96kD	2	3	2
4		8	4	5
5		12	14	16
6		11	6	8
7		4	2	1
8		8	8	12
9		2	2	5
10		9	11	10
11		4	1	4
12	45kD	4	6	4
13		6	6	4
14		19	16	15
15		15	17	19
16	29kD	17	18	18
17		5	7	9
18		5	7	7
19		4	2	2
20	24kD	10	11	10
21		6	11	10
22		1	2	2
23		3	5	2
24		2	0	4

C = controls; IA = patients with inactive disease; A = patients with active disease.

The protein bands were labelled consecutively in descending order of molecular weight.

The number of samples in each group in which the band was found is shown.

b) IgA antibodies

A serum dilution of 1:100 was used as in the ELISA (Section 3.3.1). Figure XIII shows an example of an immunoblot obtained with a patient's serum. In the IgA immunoblots, only 10 of the 24 protein bands found in the total antibody blots could be seen and many of these bands were present at a much lower frequency. Table 7 shows which bands are found and the frequency with which they occur in each of the subject groups. Clearly, no band is found more frequently in any one of the groups. Similarly, the intensity of staining of any of the bands did not appear to differ between the groups.

3.4.5 Isolation of Enterobacteriaceae from rectal swabs

In our study of rectal swabs, Klebsiella was isolated from 9/51 (17.6%) AS patients - 6/24 (25%) patients with inactive disease and 3/27 (11.1%) patients with active disease. The difference between these two groups was not statistically significant when compared in a chi-squared test. All these isolates were of the K.pneumoniae group and included serotypes K10, K11, K15, K21, K47, K53, K60 and K61. No K43 was identified.

Apart from E.coli which was found - not surprisingly - in all cases, no bacterium was isolated in large numbers. Both Proteus and Enterobacter were found in 5/51 (9.8%) of patients and their presence was not associated with active disease. Other Enterobacteriaceae isolated included Citrobacter, Seratia and Hafnia but none of these was found in more than 5% of cases.

3.5 Discussion

To investigate the possible involvement of Klebsiella in AS, antibodies to this bacteria were measured in patients' sera. An ELISA test, first described by Engvall and Perlman (110), was used because of its high sensitivity in detecting antibody (111). The use of laboratory and

Table 7 Immunoblotting of Klebsiella proteins with patient and control sera (IgA antibodies)

Protein Band No.	Approx Mol wt.	Sera C	IA	A
		n=20	n=19	n=20
6	66kD	0	1	0
8		0	0	2
10		3	0	2
14		17	18	16
15		11	13	13
16	29kD	10	10	12
18		2	3	3
20	24kD	2	2	1
21		0	0	1
22		0	0	1

C = controls; IA = patients with inactive disease; A = patients with active disease.

The protein bands are those described in Table 6.

The number of samples in each group in which the band was found is shown.

medical staff as controls was appropriate because of the reports suggesting that people who frequent health care facilities have increased intestinal colonisation with Klebsiella (54, 112).

Patients were found not to differ from controls when total anti-Klebsiella antibodies were studied. However, when antibodies of the IgA class only were measured, patients were shown to have significantly elevated antibody levels. In contrast to the findings of Ebringer et al. (52), these elevated anti-Klebsiella antibodies were not associated with active disease.

It is difficult to compare our results with those of Ebringer. In the four studies by Ebringer's group discussed (39, 67, 105, 106), three different methods of assessing disease activity were used. In addition, the bacterial preparation used in their assay was different from ours. This is a common problem with studies of Klebsiella in AS: different groups use different bacterial preparations and serotypes, and the antigenic similarities and differences between these serotypes are not entirely clear. We used K43 since it is this serotype in particular which has been shown to cross-react with the B27 antigen and is, therefore, most strongly implicated in AS (43, 46). We also chose to use both a sonicated and a formalin-killed preparation in most of our studies. The formalin-killed preparation is thought to represent surface antigens on intact bacteria while the sonicated sample is a collection of all bacterial antigens including secretory and internal proteins which could be exposed within a host.

Another area of conflict with Ebringer's group is our finding that the high IgA antibody levels are not restricted to Klebsiella : higher than normal levels of IgA antibodies to the known arthritogenic bacteria, Salmonella, Shigella and Yersinia were also found. In an attempt to determine whether the antibody response was elevated to all of these four bacteria simply because the response to one

cross-reacted with the others, a limited absorption study was carried out. By incubating patient sera with sonicated Klebsiella, we reduced the antibody response to the autologous preparation by 40%. The response to each of the other bacteria was reduced by only approximately half of this. This suggests that the serum antibody response to Klebsiella consists of both antibodies specific only for Klebsiella and antibodies which cross-react with each of the other three bacteria. A considerable degree of cross-reactivity is known to exist between these bacteria (113). However, it is not possible to conclude too much from this absorption study since the Klebsiella was unable to absorb out more than 40% of the anti-Klebsiella reactivity. This may be partly due to the fact that the Klebsiella - since it was a sonicated preparation - had to be used on solid phase.

Further evidence that the IgA antibody responses to these four bacteria are different comes from our finding that the elevated antibody levels in AS to Shigella and Yersinia are associated with active disease while the high responses to Klebsiella and Salmonella are not.

Evidence that the high level of serum IgA antibodies to these bacteria are not simply a consequence of the general elevation in IgA seen in patients comes from the finding that the responses to E.coli and Proteus were normal. Neither of these two bacteria have been implicated in AS. It might be tempting to conclude that the abnormal IgA anti-bacterial response in AS is restricted to Klebsiella and arthritogenic bacteria but further studies on a range of organisms would be necessary before any such statement could be made. However, our findings do suggest that if Enterobacteriaceae are involved in some way in AS, there is probably nothing unique about Klebsiella. Although this organism has been the focus of a great number of studies over the last two decades, many now believe that a group of Enterobacteriaceae may be involved (114).

Our ELISA assay and the techniques used in the other antibody studies discussed are limited in that they provide information only about the collective antibody response to whole bacteria or a group of bacterial antigens. One group has used a gel-immuno-radioassay to investigate the serum antibody response in AS to individual bacterial proteins which have been separated on polyacrylamide gels. They have shown (206) that while their Scandinavian AS patients did not have raised antibody titres to several whole Enterobacteriaceae, they did possess more frequently than controls antibodies to a 35kD heat-modifiable major outer membrane protein (h-momp) which is highly cross-reactive amongst many Enterobacteriaceae (116). This abnormal reactivity was confirmed by excising the protein from gels and testing by ELISA (117).

Another study used immunoblotting to compare the serum antibody response to Yersinia antigens of patients with Yersinia arthritis and patients with an uncopmplicated infection. Although no obvious difference was found in the blotting pattern of sera from the two groups, the IgA response to certain Yersinia antigens tended to persist longer in the arthritis patients (118).

We decided to follow this approach and used immunoblotting to study the serum antibody response in AS to separated Klebsiella proteins. In the case of IgA where high levels of antibodies to sonicated Klebsiella had been detected by ELISA, it might be possible to identify which antigens this elevated antibody response is directed towards. It could be that only patient sera recognise a particular antigen or that they make a higher than normal response to it. In the case of antibodies of other immunoglobulin classes, the overall response to the bacteria could be normal (as we found in our total antibody ELISA) while an abnormal antibody response to individual antigens is revealed by immunoblotting.

Our results revealed no obvious difference between the immunoblots of controls, patients with inactive disease and patients with active disease, suggesting that neither the presence nor the activity of the disease is associated with a particular antibody response to any of the separated proteins. However, there were several problems with the interpretation of these blots. Firstly, the presence and the intensity of the staining of the bands could not be quantitatively determined but was assessed by eye, causing much subjectivity. Different levels of background staining made scoring difficult and in some cases, there was poor resolution between bands. It is probably safe to say, however, that any gross abnormality would have been revealed.

Clearly, the blots do not reflect the ELISA results. The serum samples from patients with active disease used in this study had demonstrated an elevated median level of IgA antibodies to Klebsiella in the ELISA (Section 3.4.1). When the IgA blots of the 10 patient sera which had given ELISA values above 0.4 (see Figure VIII) were compared to those of control sera (all control sera values below 0.4), there was no obvious difference. There could be several explanations for this. The elevated antibody response may be directed towards non-protein antigens such as capsular polysaccharides, to protein antigens not present on the blot (e.g. because they are too large to get into the gel) or to protein antigens in their native state only - before dissociation by 2-mercaptoethanol and denaturation by SDS.

This immunoblotting study was also limited because of its use of such a complex antigenic preparation. Sonicated Klebsiella is made up of a very large number of proteins and some of the bands may represent several proteins of similar molecular weight. It would perhaps be more useful to immunoblot a selection of proteins, particularly those thought to be most immunogenically relevant such as membrane proteins. We attempted to isolate outer membrane proteins from Klebsiella by treatment with sarkosyl (119)

but were unable to obtain reproducible preparations for immunoblotting.

In conclusion, this immunoblotting study has not provided us with any more information on the immune response to Klebsiella in AS patients.

Anti-bacterial antibodies were also measured in the supernatants of PWM-stimulated PBL cultures to determine whether patient lymphocyte populations contain a high number of bacterial-specific clones. Although significant levels of immunoglobulins had been detected in these supernatants (e.g. µg/ml quantities of IgM), negligible amounts of antibodies to Klebsiella, Salmonella, Shigella and Yersinia were found in both patients and controls by an ELISA. A similar finding was reported by Cavender et al. (92). However, it is perhaps unreasonable to expect to find detectable levels of such antibodies in these supernatants. Using our ELISA, antibodies to these bacteria are hardly detectable in patients' sera at dilutions higher than 1:1000 and such serum samples contain more total immunoglobulin than we detected in the supernatants (see Section 2.4.2).

A more useful study of the in vitro humoral immune response to Enterobacteriaceae in AS might involve stimulating the cells specifically so that only bacterial-specific clones would be expanded, as in the Jerne plaque assay (120).

IgA antibodies to Klebsiella and Yersinia were measured in saliva and most subjects were found to have detectable amounts of such antibodies (although these were considerably lower than serum levels). Neither patients as a whole nor the active disease group had higher than normal levels. This supports the findings on Klebsiella of Pease et al. (69) and contrasts with those of Trull et.al. (66). The use of different bacterial preparations might explain these different findings. Like Pease's group, we studied sonicated Klebsiella (as well as a formalin-killed preparation) while Trull's group used Klebsiella culture

supernatants as their antigen. It is perhaps possible that the patients' salivary antibody response is elevated only to certain secreted bacterial proteins and that this is not apparent when a complex mixture of antigens - such as a sonicated preparation - is used.

We encountered, however, a number of problems in our ELISA measurements of salivary antibodies. One problem was high non-specific binding of some samples. For example, the test sample (i.e. in wells coated with bacteria) could have an absorbance reading of 0.6 while the background sample (i.e. in uncoated wells) was as high as 0.4. It is not clear how meaningful an absorbance value for specific binding of 0.2 would be in such a case. This high non-specific binding is due in part to the high saliva concentration required for the assay. Moreover, although the saliva dilution used in the assay lay within a linear part of the ELISA curve, in some cases this did not have a steep gradient (see Figure X).

Finally, although we have looked at antibodies in the saliva in the hope that they may be more relevant than the **serum** antibody response to intestinal pathogens, there is some controversy over how well salivary antibodies reflect the humoral response in the gut (as discussed in Chapter 2).

With these problems in mind, our findings of normal salivary antibodies to Klebsiella and Yersinia in AS are in contrast to our serum studies and do not lend support to the proposal of an involvement of these gut bacteria in the disease.

As well as the many studies on antibodies to Klebsiella in AS, there has been much interest in the faecal carriage of this organism. Much controversy exists over whether patients with active disease differ from those with inactive disease or controls in their rate of faecal carriage of Klebsiella, and this study sought to determine the isolation rate in the patients who attend our out-

patient clinic. Most studies have been concerned exclusively with Klebsiella, using culture conditions selective for the growth of this organism. However, because of the possibility that other bacteria may be involved in AS, we looked to see if any other Enterobacteriaceae were found at a significant level in patients. A range of different culture media were used in the hope of isolating as many of the bacteria of interest as possible.

The results obtained provide no evidence for a high rate of faecal carriage of Klebsiella in AS and this supports previous findings in our laboratory (121). We isolated this organism from patients at a frequency (17.6%) lower than that reported by many other groups (51, 107) and found no difference between patients with active and inactive disease. It is notable that none of the isolated Klebsiella were K43, the serotype which has been most implicated in AS. In addition, no other member of the family Enterobacteriaceae was isolated at a rate high enough to suspect it of any association with the disease or to warrant further investigation (e.g. by looking at healthy controls). In no case were any of the known arthritogenic bacteria found.

It could be argued that the low recovery rate of organisms is due to the use of rectal swabs. It is certainly possible that rectal swabs do not provide as good an indicator of bowel flora as faecal samples. In the majority of studies in AS, faecal samples have been used although a few (53, 55) have used rectal swabs. Our choice of rectal swabs was a purely practical one : it was simpler for the patients and they were therefore much more likely to co-operate.

The only finding in this series of studies which suggests any association between Enterobacteriaceae and AS is that of high levels of antibody to sonicated bacteria in patients' sera. This finding does not necessarily imply

that the bacteria play any pathogenic role in the disease. One study (122) has demonstrated high levels of complement-fixing antibodies to Yersinia and Campylobacter in several seronegative spondyloarthropathies and the author suggests that this could be a consequence of the tendency of rheumatic patients to be prone to some infections. Recently, Cooper et al. (50, 123) found raised serum IgA antibodies to Klebsiella and Yersinia in RA, Ulcerative Colitis and Crohn's Disease as well as AS, and the author concluded that increased permeability of the gut to bacterial antigens may be responsible for the elevated immune response. There is some evidence that AS patients do have abnormal bowel permeability (124) and that this is related to the use of NSAIDs (125, 126). These findings conflict with those of Ebringer's group who have demonstrated a normal anti-Klebsiella response in other rheumatic diseases such as RA and PsA (52).

The findings of raised serum anti-bacterial antibodies in AS cannot be interpreted until we have established whether this is restricted to AS and exactly which group of organisms are involved.

This study has measured antibodies in the serum and saliva but AS is a disease of the joints. Our next approach was therefore to look for any evidence of a local immune response to Enterobacteriaceae in these joints.

CHAPTER 4

ANTI-BACTERIAL RESPONSES IN THE JOINT

4.1 Introduction

Chronic arthritis can be induced in rats by the injection of streptococcal cell walls but the disease does not develop in athymic rats unless they are given infusions of T-lymphocytes (127). This indicates that the cellular immune response of the host can play an important role in arthritis.

Both specific and non-specific cell-mediated immune responses of peripheral blood lymphocytes have been studied quite extensively in AS. There are conflicting reports on the *in vitro* proliferation of such cells after non-specific stimulation with mitogens. In general, when measured by uptake of tritiated (^3H) thymidine, the response to PWM and Concavalin A are normal (77, 79) but some groups have demonstrated decreased lymphocyte proliferation in response to Phytohaemagglutinin (PHA) (24, 77) while others (78, 19, 128) have reported normal responses to this mitogen.

Because of the proposal of a bacterial involvement in AS, several groups have looked for an abnormality in the specific response of patients' blood lymphocytes to bacterial antigens with equally conflicting results. The earliest report was by Nikbin *et al.* (78) who found a significantly reduced proliferative response to *Yersinia* in AS patients. This was associated with the B27 antigen rather than the disease itself. Another group (24, 43) demonstrated a reduced response to several isolates of *Klebsiella* which was independent of B27. However, there have been several reports of normal proliferation in response to *Klebsiella* (79, 80) and *Yersinia* (80, 129).

Unlike many of the investigators who failed to take into account factors which can influence cell-mediated immunity such as age, sex and drug treatment, Kinsella et al. (79) used age- and sex-matched patient-control pairs and withdrew patients from medication prior to the study. In addition, only Klebsiella which had been directly isolated from AS patients were used as antigen in this study, and in some cases the cells were stimulated with autogenous faecal isolates. No difference was found in the proliferative response of patients and controls.

When another indicator of lymphocyte function was studied, an **increased** response to Klebsiella was found in AS. Gross et al. (130, 131) showed that while T-cell proliferation in response to various preparations of Klebsiella was generally poor in both patients and controls, the bacteria induced a significantly higher production of Leucocyte Migration Inhibitory Factor (LIF) in patients' cells. LIF is a lymphokine, produced mainly by T-cells, which plays an important part in cell-mediated immunity : it inhibits the migration of macrophages, causing them to accumulate at the site of inflammation. This heightened LIF response in AS was said to correlate with disease activity.

In a study of ReA, the same group (131) showed that the LIF response of PBLs to Yersinia was higher in patients with Yersinia-arthritis than in patients with uncomplicated yersiniosis. In addition, proliferation studies by Brenner et al. (129) revealed a marked increase in the response to Yersinia in Reiter's Syndrome. This was found only with the arthritogenic strain Yersinia enterocolitica O:3 and not with Yersinia enterocolitica O:8, a serotype which has not been implicated in arthritis. However, one group found a normal response to a range of Enterobacteriaceae in RS (80) and another group has reported a significantly lower proliferative response to Yersinia in Yersinia-arthritis (132).

Clearly, much controversy exists over the cell-mediated responses to bacteria in both AS and ReA. However, these studies have been concerned with the immune response of lymphocytes from the peripheral blood. What about the site of inflammation - the joint? In ReA, there is increasing evidence that bacterial antigens are present in the joints and that synovial lymphocytes respond much more vigorously to these antigens than blood lymphocytes do.

Attempts to culture viable organisms from joints have failed (35, 36) but recent immunohistochemical studies using rabbit antisera and monoclonal antibodies have indicated the presence of chlamydial elementary bodies in the synovial tissue of patients with sexually-acquired ReA (36) and RS (133). In Yersinia-arthritis, one group has demonstrated bacterial components in synovial cells by both immunofluorescence and immunoblotting (134) and also found these antigens within immune complexes derived from synovial fluid (135). In these studies, the bacterial antigens were associated with polymorphonuclear or mononuclear phagocytes. Similar findings have been reported in chronic arthritis in rats which has been induced by injection of streptococcal cell walls (127).

Several investigators have found evidence for a local cell-mediated immune response in the joints of ReA patients to the organism which precipitated the disease. In several cases of enteric ReA, synovial lymphocytes have been shown to proliferate most vigorously in response to the causative organism, make a marked response to other, cross-reactive Enterobacteriaceae and to make little or no response to the sexually-transmitted organisms Chlamydia and Ureaplasma (136, 137). Cells from patients with sexually-acquired ReA responded to Chlamydia and Ureaplasma but not to Enterobacteriaceae. In RA (138) and arthritis associated with inflammatory bowel disease (139), synovial cells did not respond significantly to any of these organisms. The authors concluded that the

synovial lymphocyte response could be used to diagnose the cause of the precipitating infection in ReA.

Gaston et al. (140) have reported very similar results. By inhibiting with Cyclosporin A and monoclonal antibodies, they have shown that the synovial response in ReA is mediated by class II restricted T-cells. They also demonstrated marked proliferation in the presence of a 65kD heat shock protein in several cases of enteric ReA, suggesting that the synovial cell response is directed, in part, to this highly cross-reactive bacterial antigen (141). In addition, Hermann et al. (142) were recently able to generate a number of Yersinia-specific T-cell clones from the synovial fluid of patients with Yersinia-arthritis.

In these studies, marked synovial lymphocyte responses were often present in the absence of a significant response by PBLs. For example, the stimulation index (cpm of stimulated cultures / cpm of unstimulated cultures) of synovial cells could be > 200 while that of PBL was < 3 for the same organism (143). This suggests that the bacterial-specific synovial lymphocytes are not derived from the circulation but are either selectively recruited to the joint or, more likely, clonally expanded within the joint itself.

Despite these studies on cell-mediated immunity in ReA, as far as it is known, only one group has looked at the **humoral** response in the joints. Keat et al. (63) measured antibodies to Chlamydia in the synovial fluid from patients with sexually-acquired arthritis. They found titres of specific antibody similar to that found in the sera, providing no evidence for local antibody production.

So far, very little attempt has been made to study the local immune response - either cell-mediated or humoral - in AS. Ford et al. (137) showed that synovial lymphocytes from a small group of six AS patients were unable to make

a significant proliferative response to Klebsiella. As far we know, no other such studies have been carried out.

4.2 Aims of Study

1) To study the proliferative response of synovial lymphocytes to Klebsiella.

2) To study anti-bacterial antibodies in the synovial fluid by ELISA and immunoblotting.

4.3 Subjects and Methods

4.3.1 Collection of synovial fluids and isolation of synovial lymphocytes

All the synovial fluids were aspirated from knee joints. Each fluid was collected in universals containing 200µl of 20% ethylenediaminetetraacetic acid (EDTA) and centrifuged at 250g for 15 minutes. The supernatant was taken off, centrifuged again at 2000g for 10 minutes to remove any remaining cells then aliquoted and stored at -70°C until required. Under sterile conditions in a laminar flow hood ("Microflow Pathfinder", Inter Med, USA), the cell pellet from the 250g spin was resuspended in RPMI 1640 medium and layered over equal volumes of Lymphoprep. The mononuclear cells were separated, washed and counted as described in Section 2.3.2. They were then resuspended in RPMI 1640 medium containing 10% dimethylsulphoxide (Sigma, UK) and 20% pooled human serum at a concentration of $5X10^6$-10^7 cells/ml. The cell suspension was transferred to polypropylene cryogenic vials (Bibby, UK) and immediately stored at -70°C. The following day, the vials were transferred into liquid nitrogen and maintained in this (at -196°C) until required.

4.3.2 Measurement of synovial lymphocyte proliferative responses to Klebsiella

Subjects

Synovial lymphocytes were collected from the following patients.

	AS	Others
Number	6*	11
Mean age in years (range)	33 (13-63)	47 (22-57)
Male:Female ratio	All male	0.8:1

* Synovial fluid was collected twice from two of the patients (6 months and 14 months apart) giving a total of 8 AS samples.

The " Others " group was made up of 7 RA and 4 Psoriatic arthrtis patients.

Bacterial preparations

Formalin-killed Klebsiella was prepared as in section 3.3.1 except that after incubation in formalin, the bacteria were kept under sterile conditions. Sterile PBS was used for resuspension and all manipulations were carried out in a laminar flow hood. A sonicated preparation was made up as in section 3.3.1 except that the final preparation was sterile- filtered using 0.45µm disposable filters (Gelman Sciences, USA).

Cell culture

The cryopreserved mononuclear cells (Section 4.3.1) were thawed at 37°C and immediately added to 10ml RPMI 1640 and centrifuged at 250g for 10 minutes. They were washed twice in the medium, recounted and made up to the required concentration in medium containing 10% pooled human serum and 50IU penicillin / 5µg/ml streptomycin (complete culture medium). 100µl of the cell suspension were added in triplicate to the wells of 96-well round bottom plates (Corning, UK). 100µl of one of the Klebsiella preparations, made up in complete culture medium, were added to the wells. The plates were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for the required number of days. A solution of tritiated thymidine was made up as follows : (methyl ^3H) thymidine at 1mCi/ml (Amersham, UK), 0.5ml ; 5µM "cold" thymidine, 0.1ml ; complete culture medium, 9.4ml. 20µl of this were added to each well and the plates were incubated for a further 18 hours. The cells were harvested onto filter mats (Skatron, UK) using an automated cell harvester (Skatron, UK). After drying overnight, the paper discs were transferred to scintillation vials (Packard, UK) and the vials filled with 4ml toluene (May and Baker, UK) containing 4g/l of 2,5-diphenyloxazole (Packard, UK). The radioactivity in the vials was measured using a beta scintillation counter (Rackbeta II Liquid Scintillation Counter, LKB, UK). The uptake of tritiated thymidine by the cells gave a measure of their proliferation. The results were expressed as counts per minute (cpm) or as the stimulation index. The stimulation index was :

cpm of stimulated cells / cpm of unstimulated cells.

4.3.3 Measurement of anti-bacterial antibodies in synovial fluids

Subjects

Synovial fluids from the following patients were studied :

	AS	RA	Others
Number	13	15	12
Mean age in years (range)	39 (13-70)	53 (41-72)	41 (38-47)
Male:Female ratio	12:1	0.5:1	1.2:1

The patients in the " Others " group were made up of : 3 PsA, 3 Adult Stills Disease, 2 Osteoarthritis, 2 SLE, 2 Gout and one of unknown origin.

ELISA

The ELISA was carried out as for serum antibodies as described in Section 3.3.2. 10^9 CFU/ml of formalin-killed or 200µg/ml of sonicated bacteria were used to coat the ELISA plates.

4.3.4 Immunoblotting of Klebsiella proteins with synovial fluids

Subjects

12 AS, 10 RA and 10 "Other" samples were taken from the subjects described in the previous section.

Immunoblotting

Immunoblotting (total antibodies) was carried out as for serum antibodies as described in Section 3.3.3.

4.3.5 Statistical Analysis

The Mann-Whitney test was used (see Appendix B).

4.4 Results

4.4.1 Measurement of synovial lymphocyte proliferative responses to Klebsiella

Effect of cell number and bacterial concentration

10^5, $5x10^5$ and 10^6 synovial cells from three non-AS patients were stimulated with a range of concentrations of formalin-killed and sonicated Klebsiella. Table 8 shows the results from one patient. There was a wide scatter in the values obtained with the different patients but the same trend was found in all three. The maximum response was obtained when 10^6 cells were stimulated with 10^7 CFU/ml of formalin-killed or 20µg/ml of sonicated bacteria. These antigen concentrations were therefore used in our assay. Cell numbers higher than 10^6 were not tested since we had insufficient numbers of most of the frozen cells to use more than this in the assay.

Effect of incubation time

Figure XIV shows the results obtained when 10^6 synovial cells from three non-AS patients were incubated with 10^7 CFU/ml of formalin-killed or 20µg/ml of sonicated Klebsiella for a period of 3,4,5 or 6 days. In two out of three cases, a 4-day incubation was optimal for both antigen preparations. The third sample gave maximum proliferation at day 5 but this was only slightly higher than the proliferation obtained at day 4. A 4-day incubation period was therefore used in the assay.

Table 8 Effect of cell number and bacterial concentration on the proliferative response of synovial lymphocytes to Klebsiella (as measured by uptake of ^{3}H-Thymidine).

(a) Formalin-killed bacteria.

	Conc. of bacteria (CFU/ml)				
no. synovial cells	10^6	5 x 10^6	10^7	5 x 10^7	10^8
10^5	2.1* (154)	2.1 (155)	2.4 (183)	0.8 (62)	1.4 (109)
5 x 10^5	6.5 (931)	8.3 (1191)	10.6 (1517)	6.4 (926)	1.5 (209)
10^6	7.1 (1477)	8.7 (1798)	**12.7**** (2616)	8.6 (1792)	1.6 (327)

(b) Sonicated bacteria.

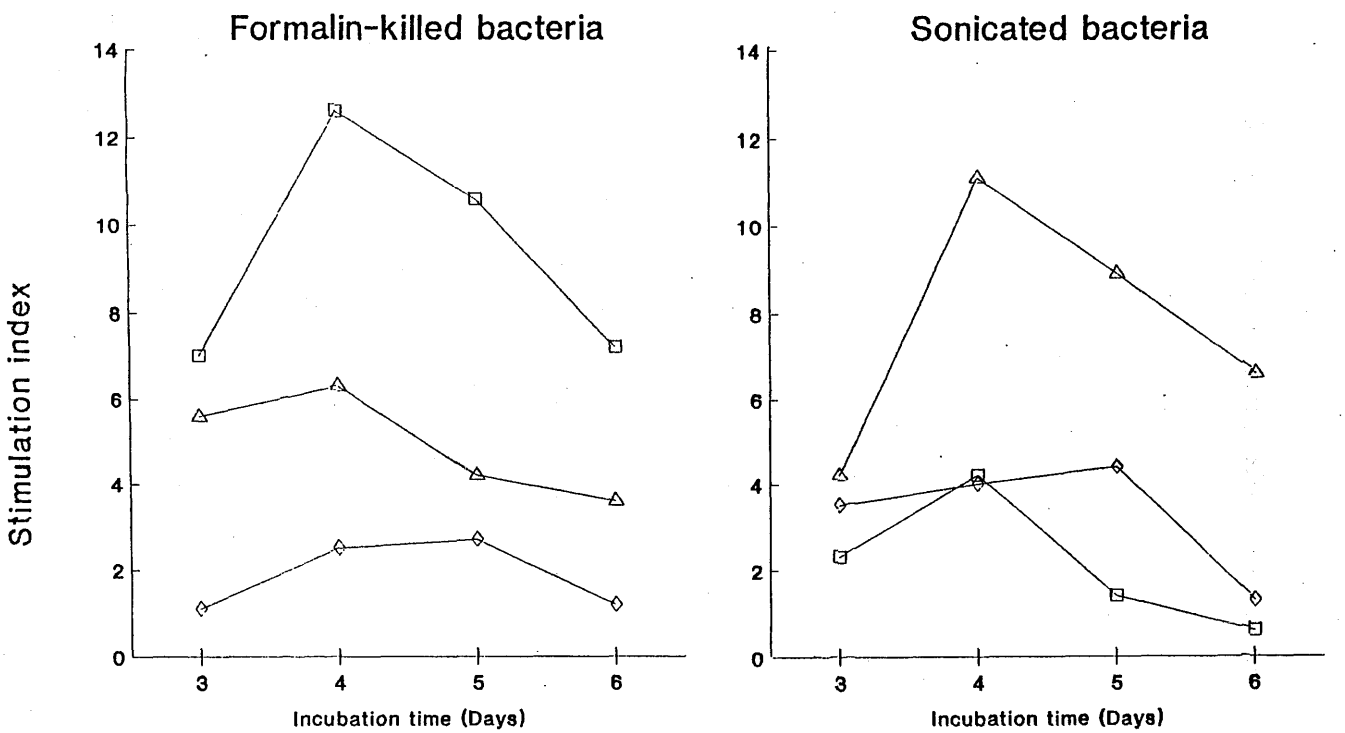
	Conc. of bacteria (µg/ml)					
no. synovial cells	1	5	10	20	50	100
10^5	1.3 (526)	1.4 (567)	1.7 (688)	1.9 (770)	1.9 (769)	1.0 (405)
5 x 10^5	1.9 (1430)	2.3 (1732)	2.2 (1657)	3.8 (2861)	1.8 (1356)	0.9 (678)
10^6	2.7 (4555)	5.4 (9109)	5.1 (8603)	**8.5**** (14339)	6.3 (10628)	2.1 (3542)

*Results expressed as: Stimulation index (cpm)

Stimulation index = $\frac{\text{cpm of stimulated cells}}{\text{cpm of unstimulated cells}}$

CFU = colony forming units
** Maximum values.

Figure XIV. Effect of incubation time on the proliferative response to Klebsiella of synovial lymphocytes



Stimulation index = cpm of stimulated cells / cpm of unstimulated cells

Effect of PHA concentration

10^6 cells from three non-AS patients were stimulated with a range of concentrations of PHA in a 4-day incubation. The results are shown in Table 9. 25µg/ml PHA induced a maximum response in all three cases and this was used for the assay.

Comparing lymphocytes from AS and other patients

Using the optimal conditions outlined in the previous sections, we measured the proliferative response to Klebsiella of synovial lymphocytes from 8 AS and 11 other patients. The stimulation indices are shown in Figure XV. The data are quite widely scattered and the responses are generally low. No significant difference was found between the two groups for either formalin-killed or sonicated bacteria.

PHA responses

To determine whether the synovial cells studied were **capable** of a high proliferative response, they were stimulated non-specifically with PHA. The median stimulation index for the AS patients was 38 and for the control patients was 28. These values are not much lower than those reported (144) for PBL from healthy donors. Synovial cells which had a low stimulation index in response to Klebsiella often had a high PHA response. This suggests that a low response to Klebsiella indicates a lack of antigen-specific clones rather than a poor ability of the cells to proliferate (for example, because of damage caused by the freezing of the cells).

Table 9. Effect of PHA concentration on non-specific stimulation of synovial lymphocytes.

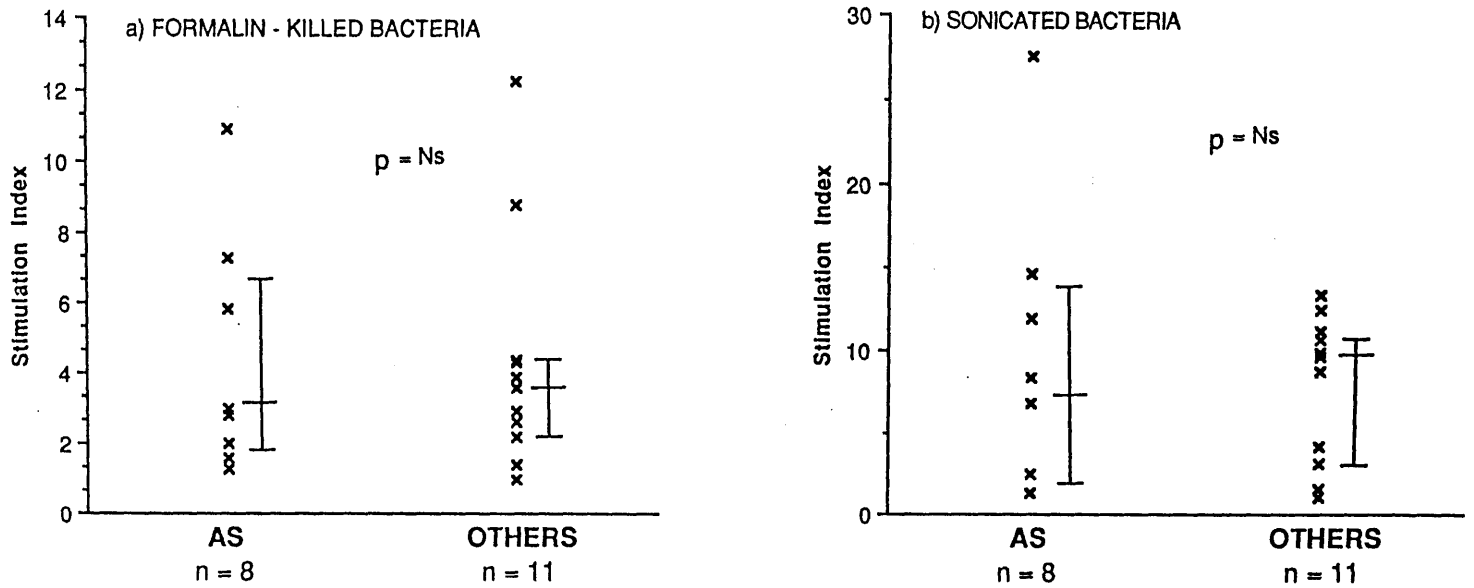
Synovial lymphocytes	Conc. of PHA (ug/ml) 2.5*	5	25	50	125
(a)	18.8 (2385)	29.8 (3783)	28.9 (3668)	21.3 (2702)	13.8 (1760)
(b)	16.5 (9162)	44.5 (24705)	50.0 (27185)	42.6 (23639)	16.6 (9248)
(c)	38.6 (17351)	246 (11835)	333 (149671)	213 (95637)	69.2 (31070)

* results expressed as : Stimulation index (cpm)

Stimulation index = $\frac{\text{cpm of stimulated cells}}{\text{cpm of unstimulated cells}}$

Synovial lymphocytes from 3 non-AS patients were studied.

Figure XV : Proliferative response to Klebsiella of synovial lymphocytes from AS and other patients



OTHERS = 4 Psoriatic Arthritis and 7 Rheumatoid Arthritis patients.

Stimulation Index = $\frac{\text{cpm of stimulated cells}}{\text{cpm of unstimulated cells}}$

Ns = not significant ; Medians and interquartile ranges shown.

Comparing lymphocytes from synovial fluid and peripheral blood

We had, unfortunately, matched PBLs for only three of the AS synovial samples. The stimulation indices of these three matched pairs are shown below.

Stimulation Index			
Formalin-killed Klebsiella		Sonicated Klebsiella	
Synovial cells	PBL	Synovial cells	PBL
7.3	5.1	27.5	16.4
3.0	6.3	14.6	32.5
2.8	2.1	6.7	4.2

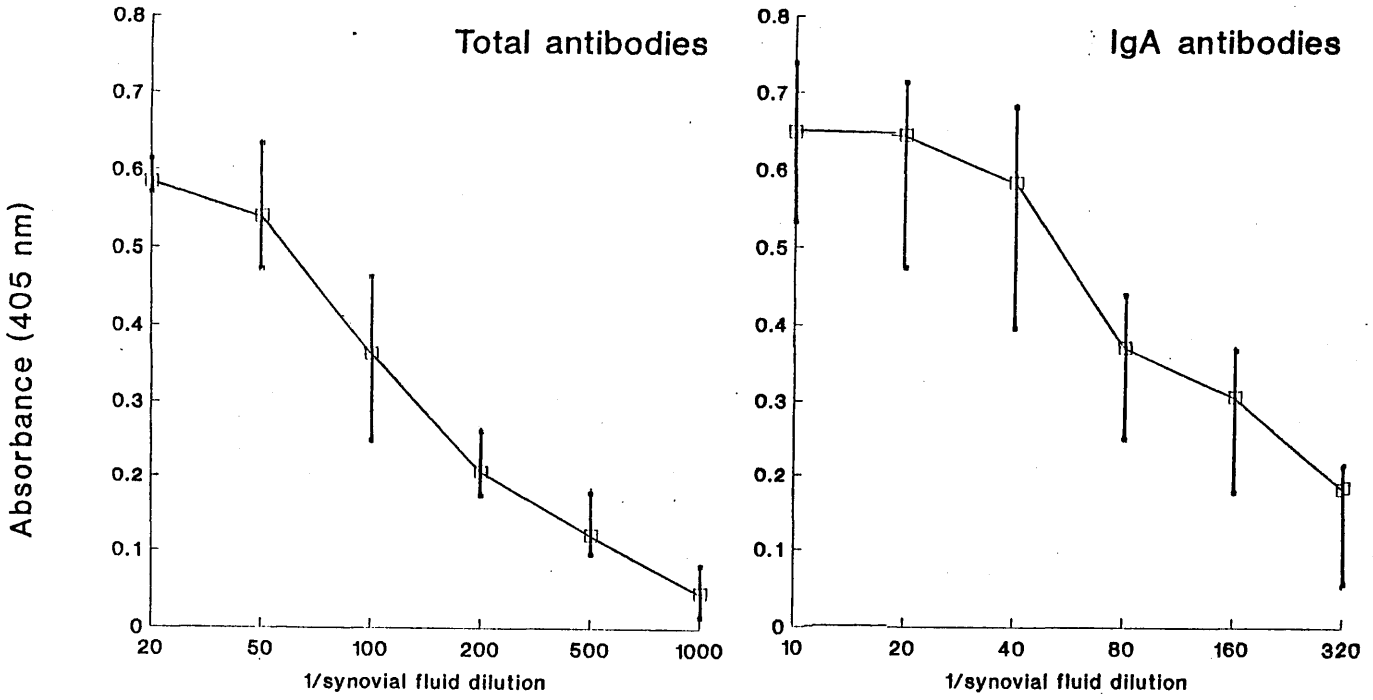
Clearly, no pattern emerges when these three synovial and blood samples are compared.

4.4.2 Measurements of anti-bacterial antibodies in synovial fluids

Effect of synovial fluid concentration

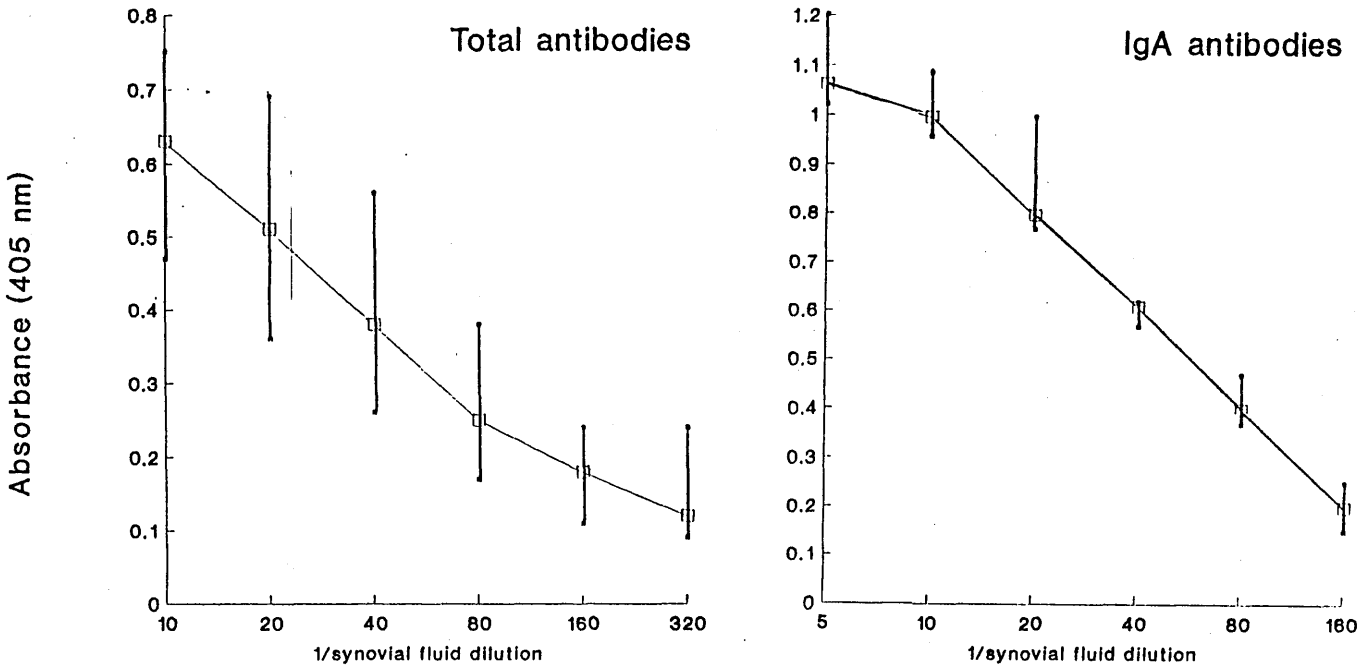
A range of dilutions of four synovial fluids were tested against sonicated and formalin-killed bacteria. The results for Klebsiella are shown in Figures XVI and XVII. Similar dilution curves were obtained with the other bacteria Salmonella, Shigella and Yersinia. Dilutions of synovial fluid which lay within the most linear part of the curve were chosen for the assay. In the case of sonicated bacteria, a 1:100 dilution was used in the total antibody ELISA and a 1:80 dilution for the IgA antibody ELISA. A 1:40 dilution was used for testing both total and IgA antibodies to formalin-killed bacteria.

Figure XVI. Effect of synovial fluid dilution on measurement of antibodies to sonicated Klebsiella by ELISA



Medians and interquartile ranges of four synovial fluids shown

Figure XVII. Effect of synovial fluid dilution on measurement of antibodies to formalin-killed Klebsiella by ELISA



Medians and interquartile ranges of four synovial fluids shown

Comparing synovial fluids from AS and other patients

Using the dilutions outlined above, both total and IgA antibodies to Klebsiella, Salmonella, Shigella and Yersinia were measured in the synovial fluid from 13 AS patients, 15 RA patients and 12 patients with a variety of other diseases including PsA, SLE, Osteoarthritis and Gout. The AS patient group did not have higher levels of antibody to any of the bacteria than either of the other two patient groups (Table 10 and Table 11). In addition, when tested in one experiment, the absorbance values of the AS samples in response to these four bacteria were not significantly different from those found in response to E.coli.

4.4.3 Immunoblotting of Klebsiella proteins with synovial fluids

Synovial fluids were studied for the presence of total antibodies to separated Klebsiella proteins by immunoblotting. A 1:100 dilution of the fluids was used as in the ELISA (Section 4.4.2). The resulting immunoblots were similar to those found with patient sera (Section 3.3.4). Table 12 shows the frequency with which each protein band occurs in the AS, RA and " Others " patient groups. Clearly, no band can be said to be associated - by its presence or absence - with the AS patient group.

4.5 Discussion

In AS, no consistent abnormality has been demonstrated in the cell-mediated immunity to Enterobacteriaceae. However, all but one (137) of the reported studies have looked at lymphocytes from the peripheral blood. In ReA, marked proliferative responses to the causative organism have been demonstrated in lymphocytes from the synovial joints

Table 10 Antibodies to sonicated bacteria in synovial fluids of AS and other patients. (Absorbance$_{405nm}$ sample/Absorbance$_{405nm}$ standard.

	Total antibodies			IgA antibodies		
	AS	RA	Others*	AS	RA	Others
n	13	15	12	13	15	12
Klebsiella	0.45 (0.34-0.69)	0.44 (0.24-0.61)	0.56 (0.38-0.67)	0.14 (0.07-0.37)	0.25 (0.12-0.44)	0.17 (0.12-0.35)
Salmonella	0.85 (0.55-0.95)	0.62 (0.49-0.96)	0.83 (0.69-0.91)	0.30 (0.20-0.66)	0.51 (0.23-0.71)	0.40 (0.32-0.71)
Shigella	0.73 (0.42-1.0)	0.74 (0.46-1.0)	0.86 (0.62-1.1)	0.28 (0.24-0.70)	0.41 (0.23-0.73)	0.36 (0.26-0.70)
Yersinia	0.89 (0.53-1.2)	0.76 (0.60-1.1)	0.90 (0.71-1.2)	0.39 (0.20-0.57)	0.40 (0.26-0.58)	0.40 (0.24-0.64)

* Included patients with Psoriatic arthritis, Systemic Lupus Erythematosus, Osteoarthritis and Adult Still's Disease.

Medians and interquartile ranges shown.

For each bacteria, AS samples were compared with the other two patient groups. No significant differences were found.

Table 11 Antibodies to formalin-killed bacteria in synovial fluids of AS and other patients Absorbance$_{405nm}$ sample/Absorbance$_{405nm}$ standard.

	Total antibodies			IgA antibodies		
	AS	RA	Others*	AS	RA	Others
n	13	15	12	13	15	12
Klebsiella	0.38 (0.32-0.55)	0.33 (0.20-0.50)	0.47 (0.32-0.50)	0.68 (0.37-0.62)	0.52 (0.36-0.59)	0.46 (0.31-0.56)
Salmonella	0.61 (0.42-0.66)	0.42 (0.24-0.55)	0.35 (0.27-0.55)	0.42 (0.25-0.69)	0.35 (0.20-0.47)	0.40 (0.27-0.54)
Shigella	0.44 (0.32-0.58)	0.51 (0.38-0.63)	0.38 (0.26-0.50)	0.41 (0.31-0.70)	0.46 (0.29-0.58)	0.44 (0.30-0.59)
Yersinia	0.47 (0.28-0.48)	0.25 (0.16-0.40)	0.31 (0.25-0.52)	0.45 (0.18-0.49)	0.21 (0.15-0.36)	0.26 (0.23-0.42)

* Included patients with Psoriatic arthritis, Systemic Lupus Erythematosus, Osteoarthritis and Adult Still's Disease.

Medians and interquartile ranges shown.

For each bacteria, AS samples were compared with the other two patient groups. No significant differences were found.

Table 12 Immunoblotting of Klebsiella proteins with synovial fluids from AS and other patients.

Protein band No.	Approx mol. wt.	AS n=12	RA n=10	Others* n=10
1		1	1	0
2		0	0	0
3	96kD	1	1	1
4		1	2	2
5		7	5	4
6	66kD	6	5	3
7		3	4	1
8		3	4	3
9		2	2	4
10		4	7	3
11		3	3	2
12	45kD	1	2	2
13		5	7	6
14		12	7	9
15		11	10	9
16	29kD	9	8	5
17		4	3	2
18		2	3	0
19		3	1	2
20	24kD	5	7	3
21		4	4	4
22		1	2	1
23		2	0	2
24		3	2	0

* Included patients with Psoriatic arthritis, Systemic Lupus Erythematosus, Osteoarthritis and Adult Still's Disease.

The protein bands were labelled consecutively in descending order of molecular weight.

The number of samples in each group in which the band was found is shown.

in the absence of a significant response by PBLs (137). We therefore decided to look for evidence for a local cell-mediated immune response to Klebsiella within the joints of AS patients.

The proliferative response to Klebsiella of AS synovial lymphocytes was found to be comparable with that of synovial cells from patients with other arthritic diseases in which Klebsiella has not been implicated. The stimulation indices for both sonicated and formalin-killed bacterial preparations were generally low. They were considerably lower than those reported (143) for lymphocytes from ReA patients in response to the infecting organism (>200 in some cases). In addition, we found no evidence that the responses of synovial cells were higher than those of PBLs in a study of three synovial-blood lymphocyte matched pairs. This may be too small a number with which to draw any real conclusions but the median stimulation indices of our eight synovial samples were within the range of those reported by others (24, 79, 80) for patients' PBLs. Taken together, these results suggest that there is no significant cellular response to Klebsiella within the joints of AS patients.

The results of this and other studies of cellular immunity must, however, be interpreted with caution. It is known that age and sex can influence cell-mediated immunity (94) but apart from Kinsella et al. (79), none of the studies discussed made an attempt to match subject groups for these variables. Owing to the limited availability of synovial samples, we were unable to control for these factors in our study but there were not large differences between our two patient groups.

Non-steroidal anti-inflammatory drugs (NSAIDs) can have an inhibitory effect on cell-mediated responses (145) and the majority of AS patients receive such drugs as part of their treatment. This could account, in part, for the absence of a significant anti-Klebsiella response.

Although most of the patients in the control group will also have been receiving NSAID treatment, patients are given a variety of drugs and doses and these could influence the immune response to varying degrees.

After we had isolated the synovial cells, they were stored in liquid nitrogen until we had collected a sufficient number of samples to study. This meant that cells were frozen for various lengths of time, some stored for up to 18 months. Although we used a recommended cryopreservation technique which is routinely used in our laboratory and is thought to preserve cell function (146), it is possible that storage of cells in this way affects their functional activity. However, the responses observed to PHA suggest that the cells had retained a high proliferative capacity.

The effect of these various factors on cellular immunity may be particularly important in our study because of the small number of samples that we collected (8 AS and 11 others). AS patients do not often require joint effusions and the yield of synovial cells from those that do is not always sufficient for this type of assay. Moreover, , the data in each of our groups are quite widely scattered : the cellular responses of individuals vary quite considerably. It is possible that a difference between the two groups would emerge if a larger number of samples were studied. However, our study gives no indication that lymphocytes from the joints of AS patients make the strong response to Klebsiella that ReA synovial lymphocytes make to the infecting organism.

It is possible that the cellular response to bacteria as a whole is normal while an abnormal response to individual bacterial antigens occurs. An attempt is underway in our laboratory to study the cellular immune response to individual bacterial proteins such as heat-shock proteins in AS and other arthritic diseases.

In addition to the study of cellular immunity, we investigated the humoral response to Enterobacteriaceae in the joints. By ELISA, total and IgA antibodies to Klebsiella and the three arthritogenic bacteria Salmonella, Shigella and Yersinia were measured in synovial fluids from AS patients, RA patients and patients with a variety of diseases including PsA, SLE and Gout. No significant difference was observed between the three groups for any of the bacteria. In addition, the levels of antibody in AS synovial fluids to each of these four bacteria was no higher than that to E.coli, a Gram-negative organism which has not been implicated in the disease. We also studied anti-Klebsiella antibodies in the synovial fluid by immunoblotting and were unable to detect a difference between the groups with respect to the presence of antibodies to any of the protein bands. (The limitations of the immunoblotting procedure are discussed in Section 3.5.) These results suggest that there is no significant humoral immune response to these Enterobacteriaceae within the joints in AS.

The cell-mediated immune response of synovial cells in AS was studied because of the known high responsiveness of ReA synovial cells to the infecting organism but there is very little data on the **humoral** response in the ReA joint. Keat et al. (163) found that the synovial fluid from patients with sexually-acquired ReA contained antibodies to Chlamydia but these were found in levels comparable to that of the serum, suggesting that they were circulating antibodies, derived from the peripheral blood rather than locally produced. This lack of humoral immunity may seem surprising in view of the marked cellular responses reported in ReA. However, different types of antigen can selectively elicit a particular type of immune response (e.g. cell-mediated immunity is preferentially induced by cell associated antigens such as viral particles). Further studies on humoral immunity in ReA joints are necessary and would help us to interpret our findings in AS. In our out-patient clinic, cases of ReA with a known

bacterial origin are not very common and we were unable to collect a sufficient number of synovial fluids from such patients to carry out any useful study.

Currently, studies by other investigators in our laboratory are involved in looking for the presence of bacterial antigens within the synovial joints. Using an ELISA to measure the reactivity of rabbit anti-Klebsiella antisera to synovial fluids, no evidence was found for the presence of Klebsiella antigens in the synovial fluids of AS patients (147). Immunohistochemical studies using synovial tissue and cells derived from synovial fluid are currently underway. The presence of bacterial antigens within the joint is not, however, necessary for a bacterial-specific immune response to occur there. According to the molecular mimicry theory, antigenic stimulation by Klebsiella could take place in the gut, and specifically activated lymphocytes could circulate to the joint where they are stimulated by the cross-reactive B27 antigen.

It should be borne in mind that these and other studies of the joints in AS use material from **peripheral** joints since this is all that is normally available. However, peripheral arhthritis develops in only about 20% of AS patients (148) and it is characterised by a non-specific synovitis which is histologically different from the classical enthesis of AS. It is therefore possible that the pathogenic mechanisms involved in the peripheral disease are distinct from those of the spinal disease.

In conclusion, this study provided no evidence for an immune response to Enterobacteriaceae in the peripheral joints of AS patients.

CHAPTER 5

IMMUNE COMPLEXES

5.1 Introduction

In a humoral immune response, specific antibodies are elicited which bind non-covalently to the antigen forming immune complexes. These complexes have an important physiological role : by aggregating antigen and interacting with phagocytic cells *via* Fc and complement receptors, they help in the clearance of antigen from the host. They can, however, be harmful : when deposited in tissue, they can activate complement leading to local inflammation and tissue damage. In this way, immune complexes are thought to play an important role in the pathogenesis of several diseases such as glomerulonephritis, SLE (149) and RA (150). In some cases, the levels of circulating immune complexes (CIC) in the sera have been shown to correlate with the activity of the disease (151).

In a search for evidence for the role of the immune system in AS, several groups have measured CIC levels in AS patients. A number of assays have been used and many conflicting results have been reported. Several studies have detected CIC in the sera of over 50% of patients using a variety of methods : a Raji cell radioimmunoassay (70, 71), a conglutinin binding assay (71), a C1q binding assay (70), a polyethylene glycol (PEG) precipitation and single radial immunodiffusion (SRID) assay (72), PEG precipitation laser nephelometry (73), measurement of macromolecular C3 (74) and inhibition of antibody mediated cytotoxicity (75). In each of these studies, a sample was considered positive for CIC when the levels were above the normal range (usually mean + 2SD of control samples). In contrast, there have been several reports of a low frequency of CIC in AS (in some cases, less than 10%).

The assays used in these studies include a C1q binding assay (152, 153, 154), an anti-C3 assay (71) and a fluorescent antibody immunophagocytosis assay (155).

Clearly, there is a lack of agreement between the results of different groups and different assays. CIC were demonstrated by Espinoza et al. (70) in 83% of AS patients using a C1q binding assay but Rosenburg et al. (152) were unable to detect CIC in **any** of their patients with the same type of assay. In the study by Rosenbaum et al. (71), 71% of AS patients were found to have CIC levels above the normal range in the Raji assay while only 14% of the same samples proved positive in an anti-C3 assay.

Perhaps surprisingly, only a few of the studies mentioned have looked at the association between CIC and disease activity. Deicher et al. (73), Panayi et al. (74) and Espinoza et al. (70) found that high levels of CIC were associated with active disease while no such association was found by Corrigal et al. (75). In two of these studies, conclusions were based upon data from a small number of patients (thirteen (74) and eighteen (75)).

It is notable that Deicher et al. (73) who demonstrated an association between CIC and disease activity, used ESR as their only measure of activity. ESR has been shown to correlate better with the peripheral arthritis of AS than with the spondylitis (84). Moreover, in the study of Espinoza et al. (70), the presence of peripheral joint involvement was one of the criteria used in assessing a patient as having active disease, and the highest levels of CIC were seen during periods of peripheral synovitis. In their review, McGuigan et al. (156), suggest that high levels of CIC in AS may not be related to the spondylitis at all but to the peripheral arthritis. It is certainly possible that distinct pathogenetic mechanisms are involved at these two sites. Unlike the spinal disease, the peripheral synovitis of AS is histologically similar to that of RA (157, 158), a disease in which CIC are known

to play an important pathogenetic role (150). However, little attempt has been made to study directly the association between CIC and peripheral joint disease in AS. Panayi et al. (74) and Corrigal et al. (75) have compared patients with and without peripheral joint involvement and found no difference between the two groups but once again, small numbers of patients were studied.

As well as the possibility that they play a role in the disease, CIC could be useful in the identification of any environmental factor associated with AS : where there is an immune complex, there is usually an antigen and it may be possible to isolate the complex and identify the antigen. In some immune complex-mediated diseases caused by infectious organisms such as infective endocarditis (159) and Hepatitis B virus-induced vasculitis (160), components of the infecting organism have been demonstrated within the CIC by techniques such as immunoblotting. The presence of bacterial antigens within CIC have been demonstrated in the reactive arthritides. Lahesmaa-Rantala et al. (161) used an ELISA to show the presence of Yersinia-specific IgM- and IgA- CIC in the sera of patients with Yersinia-arthritis (at levels higher than those of patients with uncomplicated yersiniosis). The same assay was used to demonstrate Yersinia-specific CIC in synovial fluids (135).

Some attempt has been made to look for disease-specific antigens within CIC in AS. Using PEG precipitation and acid dissociation of complexes, Bruneau et al. (76) found that components of CIC from AS patients bound to each other but not to precipitates from RA and SLE patients, suggesting the presence of disease-specific antigens. Rodahl et al. (162) have used immunoblotting to demonstrate a 70kD envelope glycoprotein of a psoriasis-associated retrovirus-like particle in the CIC from some AS patients. By an ELISA of alkaline-dissociated complexes, they demonstrated antibodies to another psoriasis-associated protein (27kD) in CIC from sera and

synovial fluids (163). As far as it is known, there have been no reported studies on the presence of Klebsiella within CIC in AS.

5.2 Aims of Study

1) To measure CIC in AS and investigate their association with disease activity and the presence of peripheral arthritis.

2) To look for the presence of Klebsiella antigens within CIC by immunoblotting.

5.3 Subjects and Methods

5.3.1 Measurement of CIC levels in sera using the Raji assay

Subjects

Patients with active disease (A), patients with inactive disease (IA) and controls (C) were studied. The details are given over the page.

	C	IA	A
Number	39	29	30
Mean age in years (Range)	31 (22-50)	45 (26-68)	36 (24-62)
Male:Female ratio	3:1	7:1	5.2:1

Collection of samples

Sera and synovial fluids were collected as previously described (Sections 3.3.2 and 4.3.1). Samples were not thawed more than once.

Maintenance of Raji cells

Cells from the lymphoblastoid Raji cell line (Flow, UK) were grown under sterile conditions in RPMI 1640 medium containing 10% heat-inactivated FCS and 50IU penicillin / 5µg/ml streptomycin. The cells were placed in 75cm^2 tissue culture flasks (Bibby, UK) at a density of 2x10^5 cells/ml and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Every 3-4 days, the cells were counted in 0.05% trypan blue using an Improved Neubauer Chamber and readjusted to a density of 2x10^5/ml in fresh medium. Cells which had been passaged three days previously were used in the assay.

Iodination of anti-human IgG

The method described by Barkas *et al.* (164) was used. 1mCi (10µl) of carrier-free (^{125}I) sodium iodide (Amersham, UK) was added to 1ml of a 1mg/ml solution of purified goat anti-human IgG (Sigma, UK) followed by 100µl of a 0.2mg/ml dilution of Chloramine T (Sigma, UK). The mixture was gently shaken at room temperature for 5

minutes. 100µl of a 0.2mg/ml solution of sodium metabisulphite (Sigma, UK) was then added and the mixture carefully pipetted on to the top of a 20 x 1cm glass column (Bio-Rad, USA) which had been packed with Sephadex G25 (Pharmacia, Sweden) and equilibrated with 30ml of PBS containing 1% BSA. The column was eluted with PBS containing 1% BSA and 5 minute fractions (approximately 0.5ml) were collected. 5µl aliquots were counted on a gamma counter (Packard, U.K.). The first peak of radioactivity represented the labelled antibody and the fractions making up this peak were pooled, made up to a volume of 3ml and stored in 1ml aliquots at -20°C.

Preparation of aggregated human IgG (AHG) standards.

A 1mg/ml solution of purified human IgG (Sigma, UK) in saline was heated in a water bath at 63°C for 30 minutes to aggregate the protein. The AHG was then stored in 200µl volumes at -70°C. (The aliquots were thawed once only and stored no longer than one month.) On the day of the assay, nine doubling dilutions of AHG in saline were prepared from a starting concentration of 500µg/ml. To each of these, 50µl of a 1:2 dilution of pooled normal human serum (NHS) were added (as a source of complement) and the samples incubated for 30 minutes at 37°C. These were used as the standards.

Assay

The method described by Theofilopolous (165) was used. The test sera were spun at 1000g for 10 minutes to remove large aggregates then diluted 1:2 in saline. 25µl volumes were added in duplicate to $2x10^6$ Raji cells which had been washed and resuspended in 50µl of RPMI 1640 medium. The samples were incubated for 45 minutes at 37°C with gentle shaking every 10 minutes. 1ml of the medium was added to each sample and the cells spun at 800g for 10 minutes at 4°C. The cell pellets were washed twice more in the same way then incubated with 25µl of the optimum dilution of ^{125}I-labelled goat anti-human IgG in RPMI 1640 containing

1% human serum albumin (RPMI-HSA). After a 30 minute incubation at 4°C, the cells were washed three times in RPMI-HSA and the radioactivity of the cell pellet was measured in a gamma counter. The amount of CIC present in the test sera was determined using a standard curve obtained by incubating the cells with 25µl of the AHG standards. The results were expressed as µg/ml AHG equivalents.

5.3.2 Measurement of CIC levels in sera using polyethylene glycol precipitation and single radial immunodiffusion (PEG-SRID)

Subjects

The subjects described in the previous section were used.

Assay

A commercially available kit (" Merrid CIC Kit ", Mercia Diagnostics, UK) was used according to the manufacturer's instructions. Briefly, 100µl of a 12% solution of PEG was added to 500µl of sera and the mixture left for 3 hours at 4°C. Complexed material was precipitated by centrifuging at 1500g for 20 minutes at 4°C and washed twice in washing buffer. The pellet was resuspended in 500µl of buffered saline and incubated at 37°C for 30 minutes. 10µl of each sample were dispensed into the wells of SRID plates containing anti-human IgG or anti-human IgM. The plates were left at room temperature for 48 hours then stained with coomassie blue stain. The diameter of the precipitation rings around the wells was measured. The concentration of immunoglobulin was determined from the ring diameter using the conversion table provided.

5.3.3 Immunoblotting of immune complexes with anti-Klebsiella antisera

Subjects

a) Sera

6 patient and 6 control samples from the subjects described in Section 5.3.1 were studied. The 6 samples in each group which contained the highest levels of CIC were used.

b) Synovial fluids

6 AS and 5 other patients (2 RA, 2 PsA and 1 SLE) were studied.

Anti-Klebsiella antisera

Two rabbit antisera specific for *Klebsiella pneumoniae* K43 were used. One was prepared by another investigator in our laboratory : New Zealand white rabbits were injected with formalin-killed *Klebsiella* on five occasions over a period of ten weeks. The other was kindly provided by Dr. Tyrone Pitt (Central Public Health Laboratory, Colindale). The reactivity of the antisera to *Klebsiella* was tested in an ELISA as described in Section 3.3.2 except that a 1:1000 dilution of biotinylated anti-rabbit immunoglobulin (Amersham, UK) was used as the second antibody.

PEG precipitation and immunoblotting of immune complexes

10ml of sera or 10-30ml of synovial fluid were mixed with a final dilution of 2% PEG 6000 in PBS and left for 3 hours at 4°C. Complexed material was precipitated by centrifuging at 1500g for 20 minutes at 4°C then washed twice in 2% PEG. The pellet was resuspended in 200µl of PBS and the protein concentration determined using a Lowry assay. It was then mixed with gel sample buffer (Section 3.3.3) to give a final dilution of 2mg/ml. 20µl of each sample were run on a 10% SDS-polyacrylamide gel and

electrophoretically transferred to nitrocellulose membranes as described in Section 3.3.3. Immunoblotting was carried out as previously described (Section 3.3.3) except that a 1:1000 dilution of rabbit anti- Klebsiella antisera was used as the first antibody and a 1:500 dilution of biotinylated anti-rabbit immunoglobulin (Amersham, UK) as the second.

5.3.4 Statistical Analysis

Mann-Whitney, Spearman-rank and Chi-squared tests were used (see Appendix B.)

5.4 Results

5.4.1 Measurement of CIC levels in sera using the Raji assay

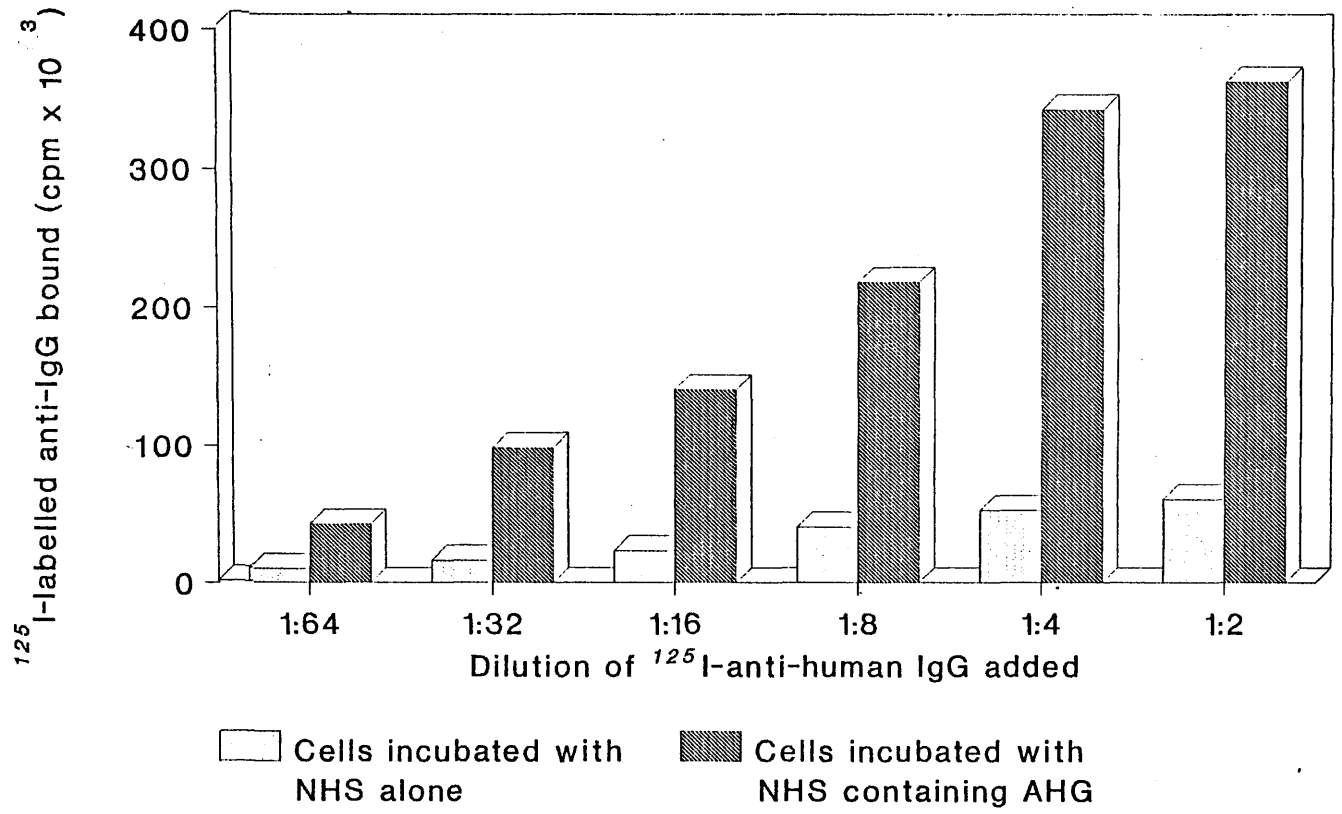
Effect of concentration of iodinated anti-human IgG

To determine the optimum dilution of ^{125}I-anti-human IgG, various dilutions of the antibody were added to $2x10^6$ Raji cells which had been incubated with a 1:2 dilution of NHS containing 500µg/ml of AHG or with a 1:2 dilution of NHS alone. The resulting uptake of ^{125}I is shown in Figure XVIII. The optimum antibody dilution is the dilution which produces the highest difference between cells incubated with and without AHG. At a 1:4 dilution, the difference is equivalent to 290 x 10^3 cpm and this is only slightly increased (302 x 10^3 cpm) when a 1:2 dilution is used. This experiment was repeated and a similar result was obtained. A 1:4 dilution of the antibody was therefore used in the assay.

Standard curves

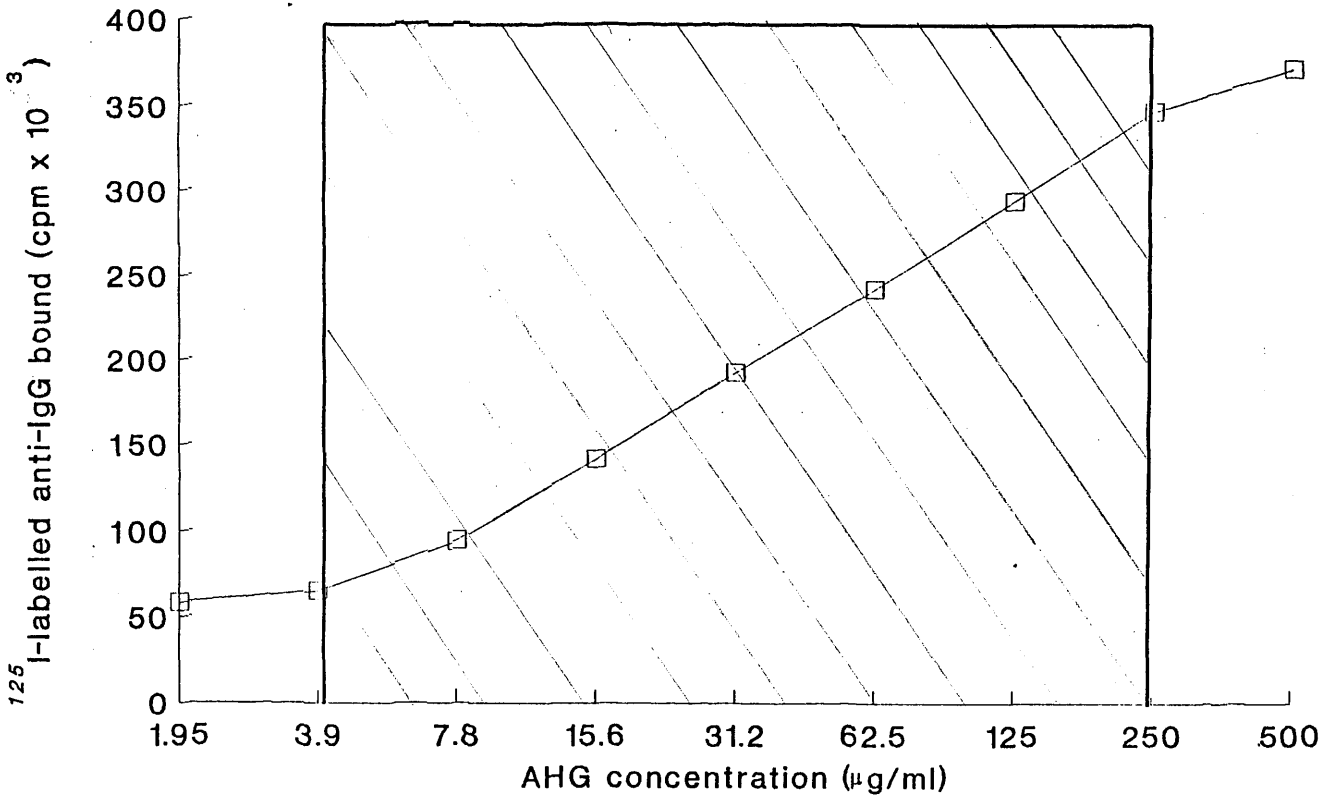
An example of a standard curve is shown in Figure XIX. The linear part of the curve lies within the range 4-

Figure XVIII. Effect of ^{125}I-labelled anti-human IgG dilution on the detection of AHG by the Raji assay



AHG = aggregated human IgG

Figure XIX. Raji assay standard curve



Shaded area shows the part of the curve used AHG = aggregated human IgG

250µg/ml AHG. Any sample lying to the right of this was diluted and re-tested and any sample lying to the left was recorded as 4µg/ml.

Comparing patient and control sera

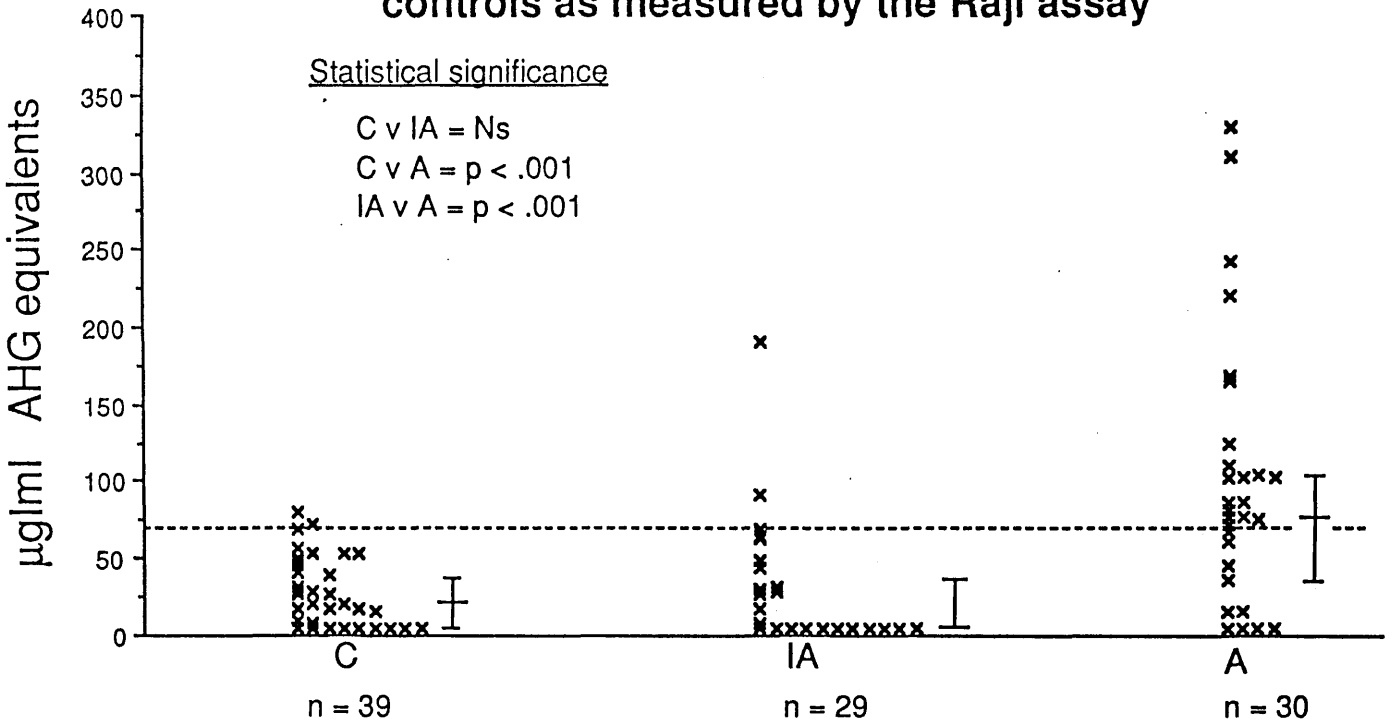
CIC levels were measured in the sera of 30 patients with active disease, 29 patients with inactive disease and 39 controls. The levels found in the total patient group (median = 44 µg/ml AHG equivalents; interquartile range = 4-87 µg/ml AHG equivalents) were significantly higher ($p < 0.05$) than those of the control group (median = 20 µg/ml AHG equivalents; interquartile range = 4-46 µg/ml AHG equivalents). A much greater difference was found when the CIC levels of patients with active disease were compared to those of controls ($p < 0.001$) or patients with inactive disease ($p < 0.001$) as shown in Figure XX.

In order to compare our results with those of others, the results were also expressed as the number of patients who were " positive " for CIC - i.e. those with CIC levels above the normal range. The 95th percentile was taken as the upper limit of normal values. 36% (21/59) of all patients were found to be positive for CIC : 63% (19/30) of patients with active disease and 7% (2/29) of patients with inactive disease. The difference between these two groups was highly significant ($chi^2 = 18.1$; $p < 0.001$).

Comparing sera from patients with and without peripheral joint disease

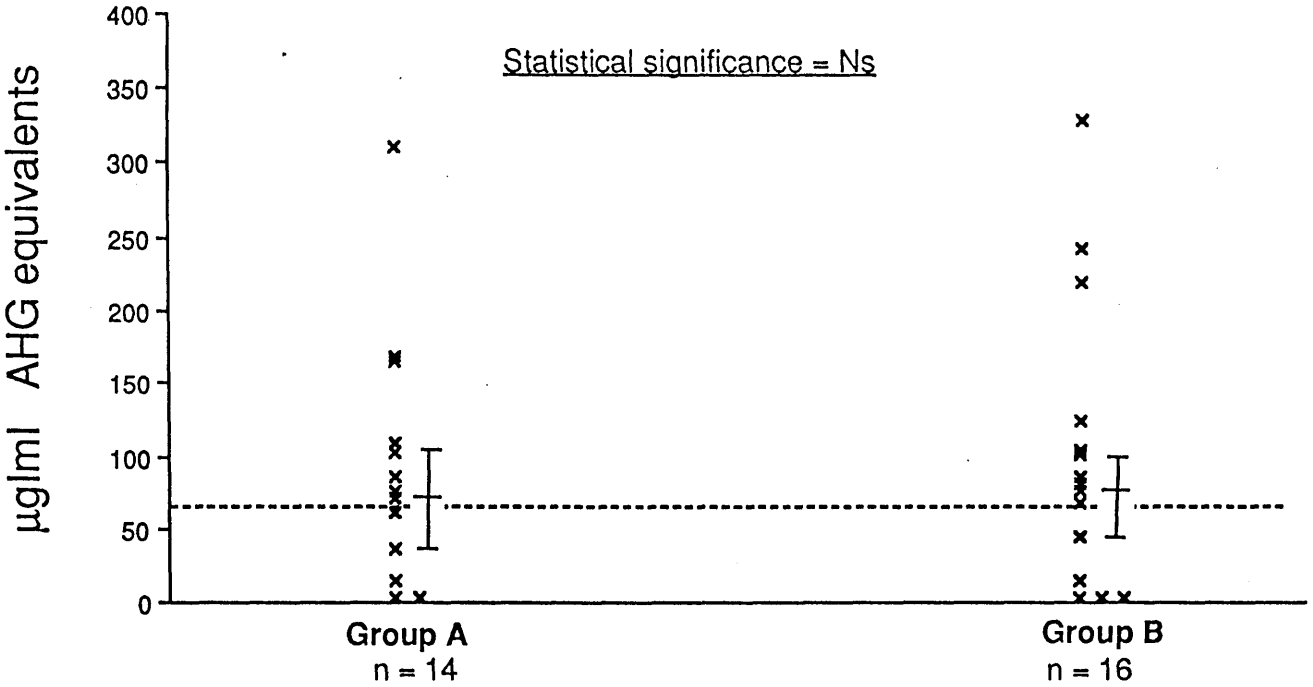
The possibility of an association between CIC and peripheral joint disease was studied. The presence of peripheral joint disease was one of the factors considered in our assessment of AS activity and all patients with peripheral joint disease studied were in the active disease group. We therefore compared only patients with ($n = 14$) and patients without ($n = 16$) peripheral involvement within this active group. The CIC levels are shown in Figure XXI.

Figure XX : CIC levels in the sera of patients and controls as measured by the Raji assay



C = controls; IA = patients with inactive disease; A = patients with active disease.
AHG = aggregated human Ig G ; Ns = not significant
CIC = circulating immune complexes
Medians and interquartile ranges shown.
The horizontal broken line represents the upper limit (95th percentile) of control samples

Figure XXI : CIC levels in the sera of patients with and without peripheral joint disease as measured by the Raji assay.



Group A = "active" patients without peripheral joint disease.
Group B = "active" patients with peripheral joint disease.
AHG = aggregated human IgG Ns = not significant.
CIC = circulating immune complexes
The horizontal broken line represents the 95th percentile upper level of control samples

There was no significant difference between the two groups when the median levels were compared. In addition, 62% (10/16) of patients with and 57% (8/14) of patients without peripheral joint disease had CIC levels above the normal range and the difference between the two groups was not significant in a chi-squared test.

5.4.2 Measurement of CIC levels in sera and synovial fluids using the PEG-SRID assay

a) Sera

Comparing patients and controls

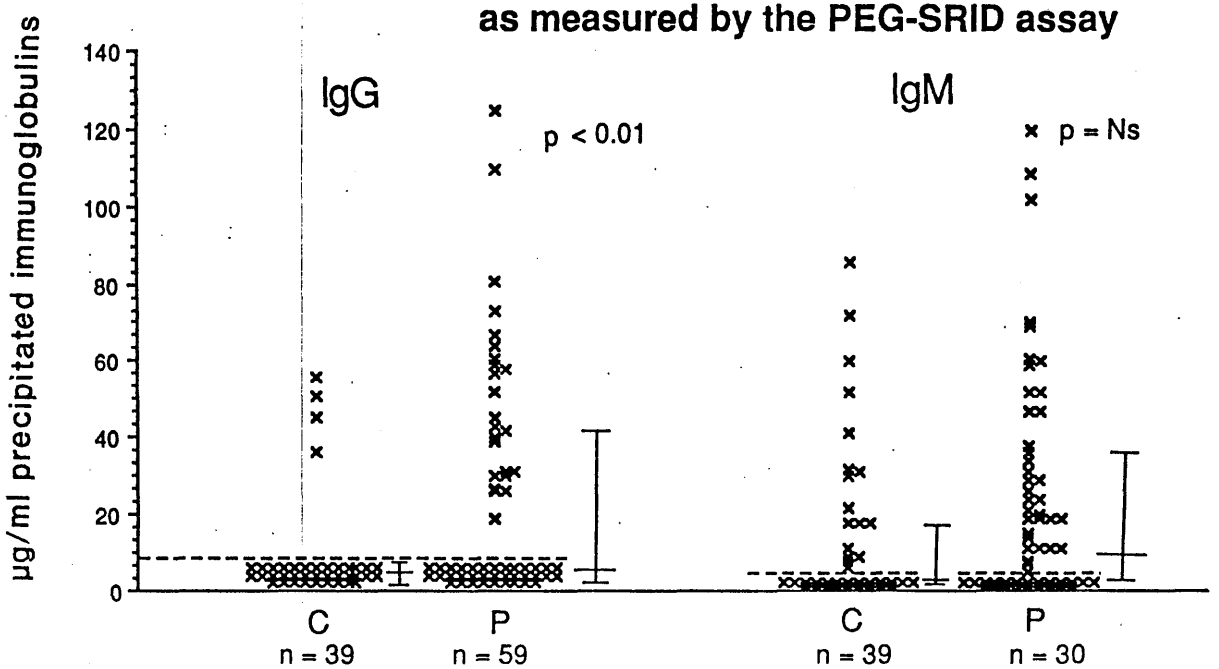
The serum samples which had been tested in the Raji assay were also studied for the levels of IgG- and IgM- CIC using a PEG-SRID assay. The lower limit of detection of the assay was 6μg/ml. IgG-CIC were detected in 10% (4/39) of control samples and 41% (24/59) of patient samples and the difference between the two groups was significant in a chi^2 test ($chi^2 = 9.2$; $p < 0.01$). When the median levels of CIC were compared in a Mann-Whitney test, a significant difference was also found ($p < 0.01$) (See Figure XXII). The high CIC levels in patients were not dependent on disease activity since the number of positive samples in the active disease group (14/30) was not significantly different from that of the inactive disease group (10/29) and the median levels in the two groups were the same.

In the case of IgM-CIC, there was no difference between the patient and control groups or the the active and inactive disease groups when either the number of samples with detectable CIC or the median levels were compared.

Comparing patients with and without peripheral joint disease

Patients in the active disease group who had peripheral joint disease were compared to those who had no peripheral

Figure XXII : CIC levels in the sera of patients and controls as measured by the PEG-SRID assay



C = controls ; P = patients.
PEG - SRID = polyethelyne glycol precipitation and single radial immunodiffusion
Ns = not significant ; CIC = circulating immune complexes
Medians and interquartile ranges shown.
All samples below the horizontal broken line have undetectable levels of precipitated immunoglobulins.

involvement. The results are shown in Figure XXIII. No significant differences were found.

b) Synovial fluids

IgG-CIC and IgM-CIC were measured in the synovial fluids of 7 AS and 15 RA patients. IgG-CIC were detected in 6/7 AS patients and the median level was 94μg/ml (interquartile range = 12-257μg/ml). This was not significantly different from the levels found in RA patients (median = 20μg/ml; interquartile range = 6-71μg/ml). IgM-CIC were detected in 3/7 AS patients and the median level (8μg/ml; interquartile range = 2-32μg/ml) was not significantly different from that of RA patients.

5.4.3 Correlation between the Raji and PEG-SRID assays

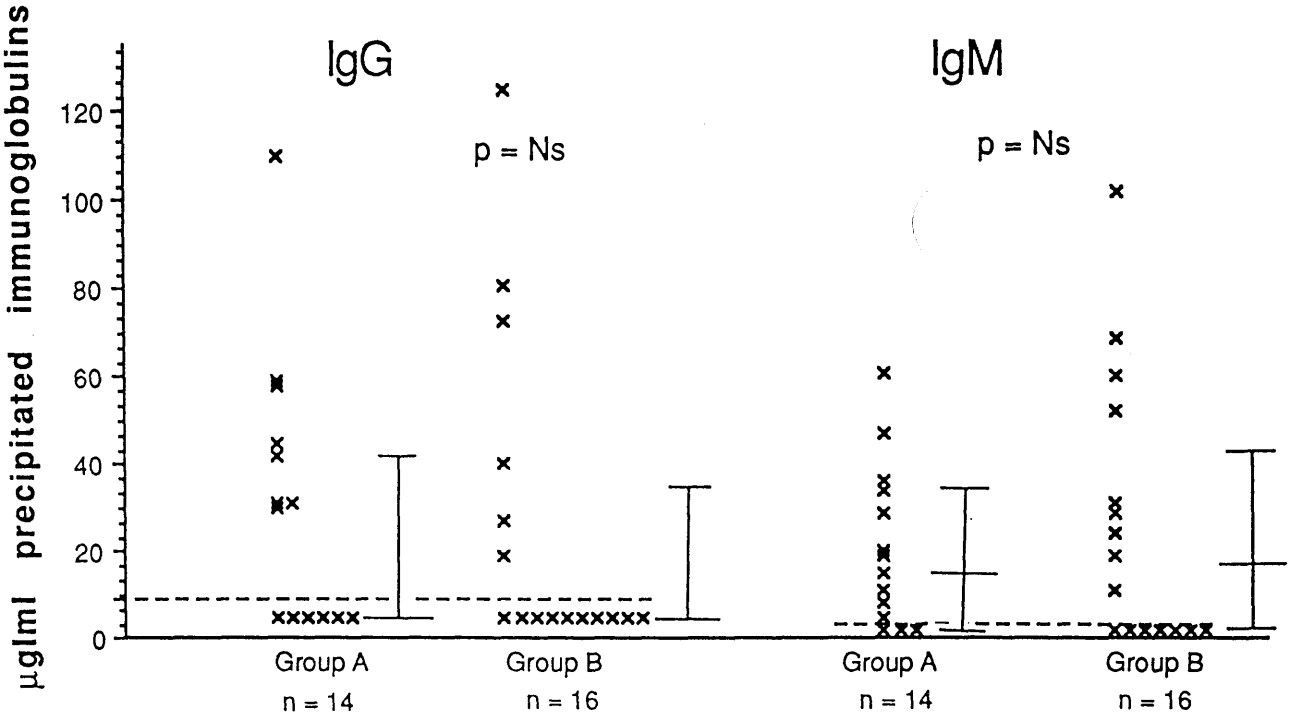
The correlation between the results of the Raji assay and the PEG-SRID assay was studied using a Spearman-rank test. A co-efficient of 0.43 ($p < 0.05$) was obtained indicating that there was a statistically significant but low correlation between the two assays.

5.4.4 Immunoblotting of immune complexes with anti-Klebsiella antisera

Reactivity of antisera to Klebsiella

The reactivity of the two antisera to formalin-killed Klebsiella (K43) was studied by an ELISA. 1:1000 dilutions gave absorbance (405nm) readings of 0.86 and 0.78, suggesting that the sera contained high levels of anti-Klebsiella antibodies. The equivalent absorbance reading for the pre-immune serum was 0.02. When used in immunoblotting of sonicated Klebsiella, the antisera dilution showed dense staining of a large number of bands.

Figure XXIII : CIC levels in the sera of patients with and without peripheral joint disease as measured by the PEG-SRID assay



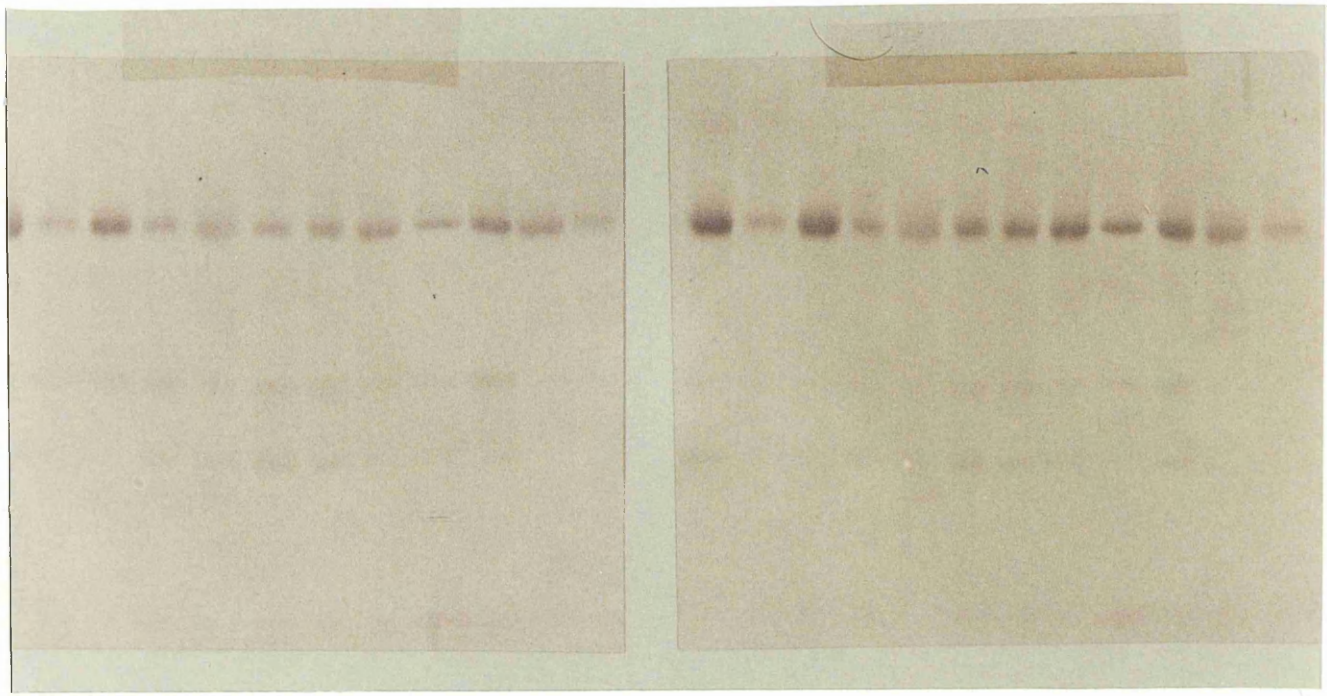
Group A = "active" patients without peripheral joint disease.
Group B = "active" patients with peripheral joint disease.
PEG-SRID = polythene glycol precipitation and single radial immunodiffusion
CIC = circulating immune complexes
Medians and interquartile ranges shown
All samples below the horizontal broken line have undetectable levels of precipatated immunoglobulin

Immunoblotting of immune complexes

An attempt was made to look for the presence of Klebsiella antigens within immune complexes from AS patients. PEG precipitates of sera from 6 AS and 6 control subjects were separated on SDS-polyacrylamide gels and immunoblotted with an anti-Klebsiella rabbit antiserum or the corresponding pre-immune serum. Figure XXIV shows the very similar blotting pattern obtained with the two sera. The dense band of around 50kD was also found in the absence of either rabbit sera : this is the heavy chain of IgG which is recognised by the biotinylated anti-rabbit immunoglobulin. In addition to this, there are six bands which are obtained with the pre-immune serum as well as with the anti-Klebsiella serum. This reactivity can therefore be explained by the previous antigenic exposure of the rabbit. Two bands with molecular weights of around 70-80kD were found in some of the samples with the immune serum only, suggesting that this response is due to anti-Klebsiella antibodies. However, these bands were found in the controls as well as the AS samples. This suggests that they are simply commonly found "Klebsiella-like" antigens. No bands of molecular weights above 80kD were seen in any of the samples. The same PEG precipitates were immunoblotted with another anti-Klebsiella antiserum. A different banding pattern was obtained but again, there was no difference between AS and control samples. (There was no corresponding pre-immune sample available for this antiserum.)

We also studied immune complexes from synovial fluids. PEG precipitates from the synovial fluid of 6 AS and 5 control patients (2 RA, 2 PsA and 1 SLE) were immunoblotted with an anti-Klebsiella antiserum. A different blotting pattern from that obtained with the serum samples was found. Again, there were bands which were found with both the immune and the pre-immune sera. Three bands with molecular weights of around 30-40kD appeared only or more strongly with the immune serum, but

Figure XXIV. Immunoblotting of serum immune complexes with anti-Klebsiella antisera



pre-immune serum

anti-Klebsiella antiserum

this occurred in the controls as well as the AS samples. One band (around 80kD) was present only in the AS group but it was found in only one of the six samples. (The photograph taken of these blots was unclear and is not shown.)

Instead of transferring the proteins for immunoblotting, some gels were stained and up to 15 distinct bands could be seen. There were no bands found in the AS group which were absent from the control group.

5.5 Discussion

This study sought to determine whether AS patients have high levels of circulating immune complexes as has been previously reported (70, 71, 73) and to see whether these CIC are associated with active disease or peripheral joint involvement. Two methods were used - a radioimmunoassay based on the ability of immune complexes to bind to a human lymphoblastoid cell line (Raji) via complement receptors, and a PEG-SRID assay which is based on the ability of 2% PEG to selectively insolubilise complexed immunoglobulin. The Raji assay was designed to measure IgG-CIC and the PEG-SRID assay could detect CIC containing different immunoglobulin isotypes.

The results of the Raji assay suggest that, compared to controls, AS patients have high levels of IgG-CIC and this is associated with active disease. Measurement of the same samples in the PEG-SRID assay also revealed a significant difference between IgG-CIC levels in the sera of patients and controls but this was independent of disease activity.

In the PEG-SRID assay, we compared absolute levels of complexed immunoglobulin (μg/ml) but some authors (151) express their results as a percentage of the total concentration of the immunoglobulin in the serum. It is

possible that a sample has a high level of complexed immunoglobulin because the level of **total** immunoglobulin is high. We were unable to express our results in this way because of the large number of samples in which undectable levels of CIC were found but evidence that the CIC levels measured in this assay were not dependent on serum immunoglobulin levels comes from the finding that while high levels of serum immunoglobulins are associated with active disease (see Section 1.4.1), high concentrations of CIC were not.

IgM-CIC were not found at a higher frequency in AS patients than controls and there was no difference between patients with active and inactive disease. This finding of high concentrations of IgG-CIC but not IgM-CIC is as one might expect in a chronic disease : IgM is an antibody class normally associated with a primary immune response.

We also attempted to measure CIC containing IgA : none were detected in any of the samples. However, we suspect that this is due to an inability of the technique to detect such complexes rather than their absence in AS. Serum samples from ten patients with IgA nephropathy - a disease associated with IgA-CIC (166) - also gave negative results. It could be that IgA-complexes have properties which make them unsuitable for detection in this type of assay - e.g. they may be less soluble because of their inability to fix complement and may not diffuse well in agar. It seems unlikely that IgG- and IgM- but not IgA-CIC are present in AS since it is IgA immunoglobulins and antibodies which are most commonly raised in AS (see Chapters 2 and 3). Moreover, the presence of IgA within CIC of AS patients has been demonstrated by other investigators (72, 155). In any further studies, another assay for measuring IgA-CIC should be used - e.g. PEG precipitation coupled with an ELISA.

Our results differ somewhat from those of other studies in which the same assays have been used. Using the Raji

assay, we found higher than normal levels of CIC in 36% of patients when taken as a whole while Espinoza et al. (70) and Rosenbaum et al. (71) reported high levels in 73% and 69% of patients respectively. This cannot be explained by a lower percentage of patients with active disease in our study since we had the highest percentage (51%) of such patients. It is probably because of the relatively high levels of CIC found in our control population. In both of the studies described, the upper limit of the normal range was below 25µg/ml AHG equivalents while we found this to be 74µg/ml. (It should be pointed out that most studies of CIC use mean + 2SD of the control samples as the upper limit of the normal range; we used the 95th percentile which is more appropriate to the non-normal distribution of the data.) The reason for these relatively high control CIC levels is not clear although sample selection may play a significant part. It is known that many factors such as diet and exercise can influence CIC levels (167). Moreover, although subjects who had a known or recent infection were excluded from our study, we cannot rule out the possibility of sub-clinical infections.

There was not a good correlation between the results of the two assays used in our study. The high IgG-CIC levels found in AS by the Raji assay were strongly associated with active disease while PEG-SRID revealed a high concentration which was independant of disease activity. In addition, a low correlation co-efficient was obtained in a correlation test.

A lack of correlation between CIC assays is quite commonly found. Immune complexes are a very heterogenous population. They can differ from each other in several ways including size, antigen composition, antibody class, antigen : antibody ratio and solubility. Over thirty methods have been developed for the detection of CIC (168). Each assay is based on one of several properties of immune complexes - e.g. their size, their ability to

bind to complement components, their ablilty to interact with Fc or complement receptors on cells or with rheumatoid factors. Assays based on different principles can often give very different results. In an inter-laboratory study (169) in which seven methods were compared, only two pairs of tests were found to correlate significantly with each other and the highest correlation co-efficient was under 0.5. A WHO collaborative study of eighteen methods demonstrated that some techniques are more suitable than others for detecting CIC in certain diseases (170).

In view of this, the WHO report recommended that two or more assays based on different principles should be used in any study of CIC. We chose the Raji assay since this was reported to be one of the most sensitive and reliable methods by a joint IUIS/WHO working group (168). The PEG-SRID assay was used because of its ability to detect CIC of different immunoglobulin classes.

Neither of the two assays can detect all CIC. The Raji assay only detects complement-fixing antibodies and not all IgG antibodies can fix complement (although most do). The PEG-SRID assay measures both complement-fixing and non-complement-fixing antibodies but some low molecular complexes may not be insolubilised in 2% PEG (151). It seems, therefore, that IgG-CIC which are measurable by the PEG-SRID assay are elevated in AS patients regardless of disease activity while those measured in the Raji assay are only found at high levels in patients with active disease.

The results of neither assay on serum immune complexes provided evidence that CIC are involved in peripheral joint disease : patients with and without peripheral arthritis had similar levels of serum IgG-CIC in both assays and similar IgM-CIC levels were found by PEG-SRID. However, if immune complexes are involved in peripheral disease, one might expect to find them concentrated in the

joints themselves. By PEG-SRID, we measured the levels of IgG and IgM immune complexes in AS synovial fluids and found them to be comparable to that of synovial fluids from patients with RA, a disease associated with high levels of immune complexes in the joints. Moreover, although we were unable to study matched synovial fluid - serum pairs, the median level of IgG complexes in sera from patients with peripheral joint disease was only 6μg/ml (the lower limit of detection of the assay) while that of the synovial fluids studied was 92μg/ml.

These high levels of immune complexes could be due to the predominance of some disease related-antigen within the joint. However, immune complexes may simply be deposited in the joints from the circulation because of the increased vascular permeabliity of inflamed tissue. Moreover, the inflamed joint is a site of tissue damage and many of the antigens within synovial fluid immune complexes may be components of damaged tissue.

Their presence in the circulation or within the joints does not necessarily mean that immune complexes play any pathogenic role in AS, and there is no evidence that they do. The usual manifestations of immune-complex-mediated diseases such as vasculitis and glomerulonephritis are not found in AS (171) except for a few reported cases of IgA nephropathy (172, 173). Not all immune complexes induce inflammation and it has been reported that CIC in AS contain high concentrations of C3 and C4, making them more soluble and therefore less likely to cause tissue damage (73).

Although they may not be pathogenic, the presence of immune complexes suggests that there is an ongoing immune response in AS. By studying these immune complexes, we may find evidence for the involvement of some environmental factor, such as Klebsiella, in the disease. Using immunoblotting, we looked for the presence of Klebsiella antigens within immune complexes (PEG

precipitates) from sera and synovial fluids. Anti-Klebsiella antisera had similar reactivity to PEG precipitates from AS and control samples, providing no evidence for the presence of Klebsiella in AS immune complexes.

This was, however, only a preliminary study using a very crude preparation of immune complexes. Although PEG can separate complexed immunoglobulins from free immunoglobulins, it will also insolubilise other large proteins. In the immunoblots obtained with the serum precipitates, many of the bands found may represent normal serum proteins and the different blotting pattern seen with the synovial fluid samples may be largely due to the different protein composition of synovial fluid.

Removal of irrelevant proteins and concentration of immune complexes may be necessary. Several methods have been used for the purification of immune complexes in various diseases : e.g. PEG precipitation followed by sucrose density ultracentrifugation (174), gel filtration and affinity chromatography with Protein A (175), and affinity chromatography on conglutinin columns (176). We attempted to isolate immune complexes from PEG precipitates using gel filtration but were unable to obtain a sharp peak of immunoglobulin in the high molecular weight fractions (as measured by ELISA).

As discussed in Chapter 3, one of the problems with the immunoblotting technique used is the possibility that the proteins lose their antigenicity after denaturation. An ELISA might be useful in looking for intact Klebsiella antigens within immune complexes. Moreover, complexes can be dissociated by alterations in pH and the antigen and antibody fractions can be studied separately for the presence of Klebsiella antigens or Klebsiella-specific antibodies.

It should, however, be borne in mind that CIC do not necessarily contain exogenous antigen. For example,

immune complexes can be formed through immunoglobulin-anti-immunoglobulin interactions : by definition, seronegative spondyloarthropathies are not assocoated with rheumatoid factor, but some immune complexes may be made up of idiotype-anti-idiotype antibodies. As already discussed, damaged tissue and dietary antigens can be components of immune complexes. Moreover, it is possible that any complexes of interest are not found free in the circulation or the synovial fluid, but are deposited in tissue such as the synovium.

In conclusion, AS patients have high levels of circulating immune complexes and an understanding of the aetiology of the disease may come from identification of the antigenic components of these complexes. We found no evidence for the presence of Klebsiella antigens but a more extensive search for this and other suspected agents is required.

CHAPTER 6

CROSS-REACTIVITY STUDIES

6.1 Introduction

Molecular mimicry or cross-reactivity occurs when two different proteins have similar antigenic determinants such that an immune response raised against one protein also reacts with the other. Cross-reactivity between microbial antigens and host tissue is thought to play a role in several autoimmune diseases in man : an immune response to the infecting organism is elicited, breaking normal tolerance to the cross-reactive host tissue. The best known example is Rheumatic Fever which is preceeded by an infection with Group A Streptococcus pyogenes. Streptococcal cell walls cross-react with human heart tissue (26) and patients with the disease have high levels of heart-reactive antibodies which can be absorbed out by the bacteria (177). Similarly, Coxsackie B virus is thought to produce myocarditis in humans and experimental animals by cross-reacting with heart tissue (178), and cross-reaction between agliadin and an adenovirus may play a role in coeliac disease (179).

Since the discovery of the strong B27 association in AS (13, 14) and the reports that patients have both high faecal carriage of Klebsiella (41, 53, 107) and high levels of antibody to this bacterium (67, 105), the possibility of cross-rection between B27 and Klebsiella has been extensively studied.

Ebringer's group (180) investigated this by studying the reactivity of anti-Klebsiella antisera to B27-positive (B27+) and B27-negative (B27-) lymphocytes using an ELISA. They found that B27+ cells, either from AS patients or

healthy controls, were more reactive with anti-Klebsiella antisera than were B27- cells. Similar results were obtained when a complement-dependent microcytotoxicity assay was used (40). In a reverse experiment, antisera were raised in rabbits against B27+ cells and their ability to bind to various bacterial preparations was studied by immunodiffusion, agglutination and radiobinding experiments (25, 40). These anti-B27 antisera showed significantly higher reactivity to certain strains of Klebsiella, Yersinia and Enterobacter than sera from unimmunised rabbits. Similar studies were carried out using tissue-typing sera which are obtained from pregnant women immunised with foetal lymphocytes carrying paternal HLA antigens. By haemagglutination, radiobinding and competitive radioimmunoassays, anti-B27 tissue-typing sera were shown to be more reactive to Klebsiella than were antisera specific for other HLA-B locus products or HLA-A-specific sera (42).

While Ebringer's group propose that cross-reaction occurs between Klebsiella and the B27 molecule itself, Geczy and his co-workers (46) maintain that anti-KLebsiella antisera show reactivity to the cells of B27+ AS patients because a Klebsiella-derived component is found on the surface of these cells in close association with the B27 antigen. Using a chromium-release assay, they showed that antisera raised against certain strains of Klebsiella specifically lysed approximately 80% of lymphocytes from B27+ patients (B27+ AS+) but none of the lymphocytes from B27- patients (B27- AS+) or, most interestingly, B27+ normal individuals (B27+ AS-) (181). Using these antisera, Geczy's group were able to distinguish between B27+ AS+ and B27+ AS- cells in two blind trials using cells supplied by a British (182) and a Dutch (115) group. Geczy et al. (183) showed that B27+ AS- cells could be made susceptible to lysis by anti-Klebsiella antisera by incubating them with the filtrates of culture supernatants from K43 but not other non "cross-reactive" serotypes of Klebsiella. The active factor, which they called "modifying factor", was

found to be associated with Klebsiella outer membranes and had a molecular weight of approximately 30kD (184). Other enteric organisms including certain isolates of Salmonella, Shigella and E.coli were found to possess a similar activity (185) and the authors propose (46) that this modifying factor is encoded by plasmid genes.

Several investigators, however, have failed to find evidence to support the findings of either Geczy's or Ebringer's group. As far as it is known, only one other group has managed (after many unsuccesful attempts) to produce an anti-Klebsiella antiserum which can distinguish between B27+ and B27- cells (186). Beaulieu et al. (47) looked at 98 different antisera to various strains of Klebsiella including some raised against faecal isolates from AS patients and two raised against K43 and K21 isolates provided by Geczy (which his group had found to give rise to antibodies cytotoxic for B27+ cells). The antisera were tested using a chromium-release microcytotoxicity assay similar to that of Geczy's but none of them were found to be reactive with B27+ AS+ or B27+ AS- cells. Other groups, including our laboratory, have reported negative results with anti-Klebsiella antisera in the microcytotoxic assay (48, 49, 50). Inman et al. (187) studied anti-Yersinia and anti-Chlamydia antisera from both immunised rabbits and recently infected patients. Anti-bacterial antibodies from neither source were specifically cytotoxic for B27+ lymphocytes.

This cytotoxic assay only measures antibodies which fix complement and some cross-reactive antibodies may therefore go undetected. Georgopolous et al. (188) used an ELISA to study several anti-Klebsiella antisera including some raised against strains identical to Geczy's and "positive" antisera provided by both Geczy and Ebringer. They failed to find any evidence for cross-reactivity. Similar ELISA results were reported by Singh et al. (189).

A more recent approach to the study of cross-reactivity between Klebsiella and B27 has involved the use of monoclonal antibodies. By immunoblotting, Ogasawara et al. (43) demonstrated that an anti-B27 monoclonal antibody reacted with 60kD and 80kD proteins of Klebsiella pneumoniae K43. No reactivity was observed when several other Gram-negative bacteria or a series of other monoclonal antibodies were used. An antiserum was raised to the 80kD protein and its reactivity to Klebsiella envelopes was partially inhibited by absorption with B27+ cells although it did not react with B27+ cells in a microcytotoxic assay. van Bohemen et al. (190) reported different results with the same monoclonal antibody - it was found to recognise a 20kD protein from Shigella but showed no reactivity to Klebsiella. Other monoclonal antibodies have been shown to have high reactivity to strains of Klebsiella (44), Yersinia (191) and Shigella (192).

As well as the use of anti-B27 monoclonal antibodies, monoclonal antibodies have been raised to arthritogenic bacteria and their reactivity to B27+ cells studied. Kono et al. (193) demonstrated that a Yersinia-specific monoclonal antibody reacted with 12/12 B27+ lymphoblastoid cell lines and only 4/31 B27- cell lines. (Three of the B27- lines which reacted with the monoclonal antibody were positive for B7, an HLA antigen which cross-reacts with B27.) A cell line which lost its expression of B27 through mutation no longer reacted with the antibody.

These studies have investigated cross-reactions using purely immunological techniques. A more recent approach, made possible by the complete amino acid sequencing of the B27 antigen (194), has involved looking for amino acid sequence homology between B27 and bacterial proteins. As well as providing support for the molecular mimicry theory of AS, this approach could provide valuable information on which bacterial proteins might be involved.

Schwimmbeck et al. (45) used a computer search programme to screen a major protein database (Dayhoff Database (195)) for amino acid sequences shared between B27 and any other sequenced protein. They identified a stretch of six amino acids of the B27 allotype B27.1 - residues 72-77 - that matched residues 188-193 of the nitrogenase enzyme of Klebsiella pneumoniae : Glutamine-Threonine-Aspartic acid-Arginine-Glutamic acid-Aspartic acid (QTDRED) The authors claim that the probability of a hexamer occurring at random is 1:64 million. This sequence is localised in the hypervariable region of the B27.1 antigen and was not found in any of the other HLA antigens. Hydrophobicity plots revealed that the hexamer is hydrophilic and therefore likely to be expressed on the surface of a molecule where it would be accessible to the immune system.

To determine whether this sequence homology reflected an immunological cross-reaction, the same group (196) made a series of synthetic peptides representing the hexamers together with flanking regions of various lengths : e.g. AKAQTDREDLRTLLRY, representing residues 69-84 of B27.1, and NSRQTDREDELIGGC representing residues 185-196 of Klebsiella nitrogenase. Affinity purified antibodies to B27-peptides were shown to be reactive in an ELISA to Klebsiella-derived peptides.

Shwimmbeck et al. (45) then looked for the presence of antibodies to these synthetic peptides in the sera of patients with AS and RD. By ELISA, they found that 29% of AS patients and 53% of RD patients had significant levels of antibodies to a B27-derived peptide, and over 40% of both groups were found to possess antibodies reactive to a Klebsiella-peptide. Less than 5% of normal individuals had antibodies to either peptide.

Amino acid sequence homology with B27 is not confined to Klebsiella. In a recent study, Steiglitz et al. (197) screened a panel of Shigella strains and identified a 2

megadalton plasmid specific for arthritogenic strains of the bacteria. The complete nucleotide sequence of the plasmid was determined and the amino acid sequence inferred from it was found to contain a pentamer homologous to residues 71-75 of B27.1 (AQTDR). This stretch of the B27 antigen clearly overlaps with the QTDRED hexamer shared with Klebsiella nitrogenase. It is not known whether patients with Shigella-arthritis possess antibodies to this pentamer.

6.2 Aims of Study

1) To measure the binding of anti-Klebsiella antisera to B27+ cells in an immunofluorescence assay.

2) To measure the binding of anti-B27 tissue-typing sera to Klebsiella in an ELISA.

3) To look for the presence of antibodies in patients' sera to a synthetic peptide representing a hexamer (QTDRED) shared by the B27 antigen and a Klebsiella protein.

6.3 Subjects and Methods

6.3.1 Measurement of anti-B27 reactivity of anti-Klebsiella antisera.

Anti-Klebsiella antisera

Three rabbit antisera specific for Klebsiella pneumoniae K43 were used - the two described in Section 5.3.3 and another previously prepared in our laboratory. An antiserum specific for Klebsiella pneumoniae K21 was provided by Tyrone Pitt (Central Public Health Laboratory, Colindale) and a non-immune rabbit serum was also studied. The reactivity of the antisera to Klebsiella was tested in an ELISA as described in Section 3.3.2 except that a

1:1000 dilution of biotinylated anti-rabbit immunoglobulin was used as the second antibody.

Immunofluorescence assay

Lymphocytes were isolated from peripheral blood and stored in liquid nitrogen as previously described (Section 2.3.2 and 4.3.1). On the day of the assay, the cells were thawed and washed three times in RPMI 1640 medium by spinning at 250g for 10 minutes. They were counted in 0.05% trypan blue in an Improved Neubauer Chamber and adjusted to a concentration of $5x10^7$ cells/ml in RPMI containing 0.1% sodium azide and 0.1% HSA (incubation medium). 10µl of cells were added to the wells of a 96-well round-bottom plate (Corning, UK). 100µl of the rabbit test sera or 50µl of a 1:25 dilution of an anti-B27 monoclonal antibody (Serotec, UK) in incubation buffer were added and the cell suspension was carefully mixed. The plate was left at room temperature for 45 minutes. 100µl of cold incubation medium were added to each well and the plate spun at 250g for 10 minutes at 4°C. The supernatant was aspirated and the pellets were resuspended in 200µl medium and washed twice more in the same way. The cells were then resuspended in 50µl of a 1:30 dilution of fluorescein-labelled swine anti-rabbit immunoglobulins (Dakopatts, Denmark) or a 1:50 dilution of fluorescein-labelled sheep anti-mouse IgG (SAPU, Scotland) in incubation buffer and left at room temperature for 45 minutes. 150µl of cold incubation medium were added to each well and the cells washed as before. The pellets were resuspended in 10µl of cold PBS then transferred to pre-washed glass microscope slides (Chance Propper, UK) and covered with a glass coverslip (Chance Propper, UK).

The cells were viewed under a fluorescence microscope (Leitz Wetzlar, Germany). The total number of cells in one field of view was counted under normal light and the number of these cells which were peripherally stained with fluorescence was counted under U.V. light. Several fields

of view were selected until at least 100 cells had been counted. The number of fluorescently stained cells was expressed as a percentage of the total number of cells after the background had been subtracted (the percentage of cells stained in the presence of fluorescein-labelled second antibody only).

6.3.2 Measurement of anti-Klebsiella reactivity of anti-B27 tissue-typing sera

Sera

Human tissue-typing sera were kindly donated by the UK Transplant Service (Bristol). Three anti-B27 antisera, two anti-B14 antisera and one antiserum of each of the following HLA specificities were supplied : B8, B13, B16, B17, A1, A2 and A3. Each of these had been obtained from a single pregnant woman. In addition, two commercially available anti-B27 sera and an anti-B5 antiserum (Behring, UK) were used. Each antiserum was known to be reactive to their corresponding HLA antigen in the microcytotoxicity assay.

ELISA

An ELISA of antibodies to formalin-killed and sonicated Klebsiella and E.coli was carried out as previously described for patient sera (Section 3.3.2).

6.3.3 Measurement of antibodies to the peptide QTDRED in patients' sera

Subjects

Patients with inactive disease (IA), patients with active disease (A) and controls (C) were studied. The details are given over the page.

	C	IA	A
Number	19	15	15
Mean age in years (Range)	33 (23-50)	49 (26-71)	45 (26-64)
Male:Female ratio	3.2:1	6.5:1	6.5:1

Peptide Synthesis

The amino acid hexamer QTDRED (Glutamine-Threonine-Aspartic acid-Arginine-Glutamic acid-Aspartic acid) was synthesised for us by BioMac, UK using a Biosearch 9500 automatic synthesiser and purified using reverse-phase HPLC. The molecular weight of 762g/mol was confirmed by FAB-mass spectrometry.

Preparation of peptide-HSA conjugate

The peptide was conjugated to HSA at a peptide:protein ratio of 40:1 using the gluteraldehyde coupling method (198). A 1mg/ml solution of the peptide and a 2.2mg/ml solution of HSA were made up in PBS and 1ml of each solution were mixed. 2ml of 1% gluteraldehyde in PBS were added and the mixture stirred overnight at 4°C. The solution was then dialysed against PBS to remove the gluteraldehyde.

ELISA

a) Unconjugated peptide

The ELISA plates were pre-coated with poly-L-lysine (PLL) to enhance the binding of the peptide : 100µl of a 20mg/ml solution of PLL (Sigma, U.K.) were added to the wells and the plates incubated at 37°C for 1 hour. The plates were washed twice in PBS then coated with 100µl of a 200µg/ml solution of the peptide in PBS. The assay was carried out

as described for total antibodies to bacteria (Section 3.4.1).

b) Peptide-HSA conjugate

Wells were coated with 100µl of an 8.9µg/ml solution of HSA conjugated to peptide (4µg/ml peptide) or 8.9µg/ml HSA alone in 50mM carbonate buffer (pH9.6). The assay was carried out as described for total antibodies to bacteria (Section 3.4.1). The absorbance (405nm) readings obtained with HSA alone were subtracted from the readings obtained with the peptide-HSA conjugate.

6.3.4 Statistical Analysis

The Mann-Whitney test was used (see Appendix B).

6.4 Results

6.4.1 Measurement of anti-B27 reactivity of anti-Klebsiella antisera

Demonstration of B27 on surface of cells using a monoclonal antibody

The reactivity of an anti-B27 monoclonal antibody to lymphocytes from three B27+ and three B27- individuals was studied. The mean (+ SEM) percentage of B27+ and B27- cells which were fluorescently stained was 97.3 (+1.3) % and 12.3 (+2.3) % respectively. This demonstrates that anti-B27 reactivity can be detected using this immunofluorescence assay.

Reactivity of rabbit anti-Klebsiella antisera to Klebsiella

The reactivity of the rabbit anti-Klebsiella antisera to Klebsiella was confirmed by an ELISA. When tested against the K43 serotype, 1:1000 dilutions of the three anti-K43 antisera gave absorbance (405nm) readings of 0.86, 0.78

and 0.69. The anti-K21 antiserum gave an absorbance (405nm) reading of 0.85 in an ELISA with K21. This shows that the four antisera contain high levels of anti-Klebsiella antibodies. The non-immune rabbit serum did not contain significant levels of such antibodies : a 1:1000 dilution gave an absorbance (405nm) reading of < 0.02 in response to Klebsiella of either serotype.

Binding of anti-Klebsiella antisera to B27+ cells

a) Effect of antiserum dilution

Figure XXV shows the level of immunofluorescent staining found when lymphocytes from a B27+ individual are incubated with various concentrations of anti-Klebsiella antisera : 1:2, 1:4, 1:8, 1:16 and 1:64 dilutions. There is clearly significant binding of antibodies to the cells at high serum concentrations, with a negligible response at a 1:64 dilution. This was repeated with cells from two other B27+ individuals and a similar pattern was obtained. A 1:4 dilution of each antiserum was used for comparing B27+ and B27- cells since this dilution lay within the linear part of the curve.

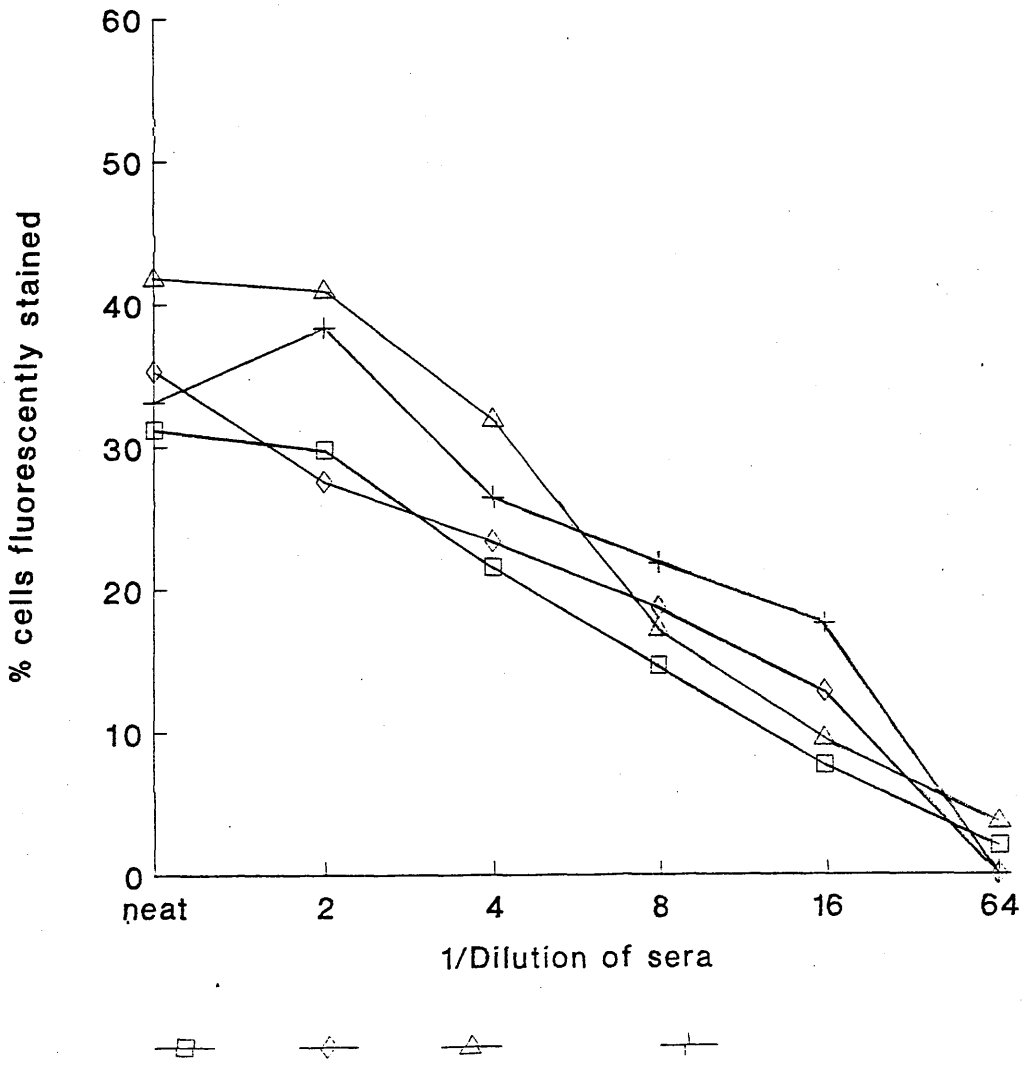
b) Comparing B27+ and B27- cells

To see if the reactivity of the anti-Klebsiella antisera to B27+ cells was any higher than that to B27- cells, a 1:4 dilution of each antiserum was tested against cells from 10 B27+ individuals (7 B27+ AS+ and 3 B27+ AS-) and 10 B27- individuals. The results are shown in Figure XXVI. There was no significant difference in the binding to B27+ and B27- cells for any of the four antisera.

c) Comparing immune and non-immune sera

The binding of a non-immune serum to cells from five B27+ individuals was measured. The median percentage of fluorescently stained cells was 22.5% (interquartile range = 18.4 - 27.4%) and this was similar to that found with

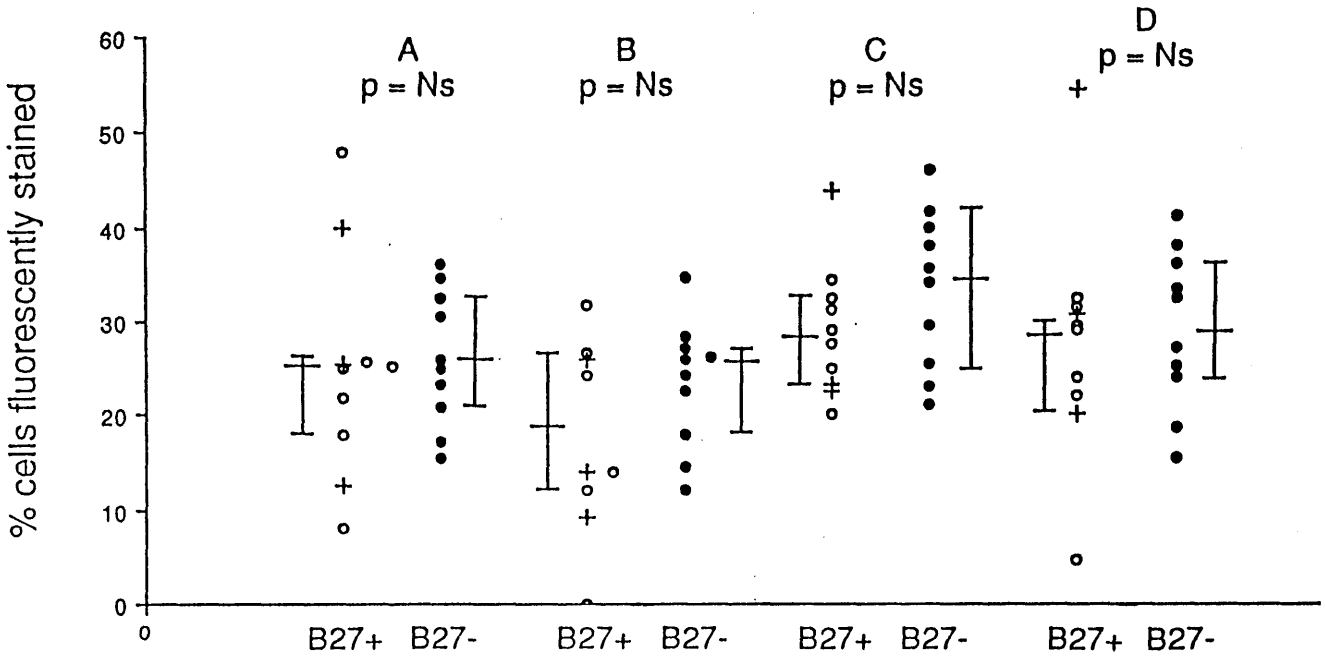
Figure XXV. Effect of antiserum dilution on the reactivity of anti-Klebsiella antisera to B27+ lymphocytes as measured by immunofluorescence



▪ antisera raised against *Klebsiella pneumoniae* K43

▪ antisera raised against *Klebsiella pneumoniae* K21

Figure XXVI: **Reactivity of anti-Klebsiella antisera to B27+ and B27- lymphocytes as measured by immunofluorescence.**



A,B and C = antisera raised against **Klebsiella pnuemoniae** K43

D = antiserum raised against **Klebsiella pnuemoniae** K21

\+ Cells from B27+As patients: ° Cells from B27+ controls

Ns = not significant

Medians and interquartile ranges shown.

the corresponding immune serum (median = 19.2%, interquartile range = 12 - 26.6%).

6.4.2 Measurement of anti-Klebsiella reactivity of anti-B27 tissue-typing sera

Comparing anti-B27 and other tissue-typing sera

The binding of five anti-B27 tissue-typing sera and ten antisera of other HLA specificities to formalin-killed and sonicated Klebsiella was studied in an ELISA. 1:10, 1:100 and 1:500 dilutions of each antiserum were tested and the results are shown in Figures XVII and XVIII. The anti-B27 antisera did not show higher binding than the non-B27 antisera to either preparation of Klebsiella at any of the dilutions studied.

Comparing Klebsiella and E.coli

The binding of each anti-B27 antiserum to E.coli was also studied and was found not to be significantly different from the level of binding to Klebsiella. This was true for each antiserum dilution studied and when either formalin-killed or sonicated bacteria were used.

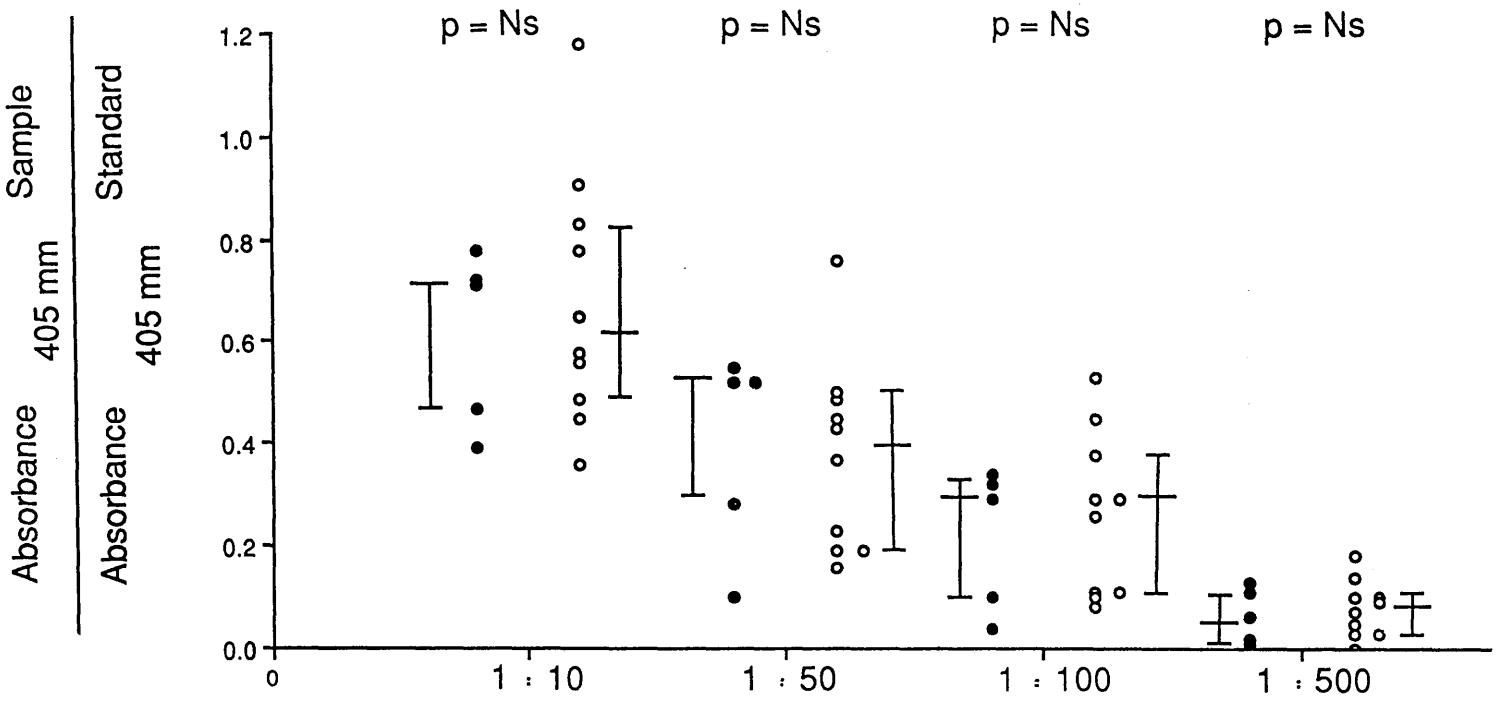
6.4.3 Measurement of antibodies to the peptide QTDRED in patients' sera

Unconjugated peptide

An ELISA was used to look for antibodies to the unconjugated peptide QTDRED in the sera of ten patients and ten controls. A 1:10 serum dilution was used. All absorbance (405nm) readings were below 0.1, indicating a negligible response.

An antiserum to the peptide which had been raised in our laboratory and which was known to contain high levels of peptide-specific antibodies (see next section) also gave absorbance readings of below 0.1. This suggests that this

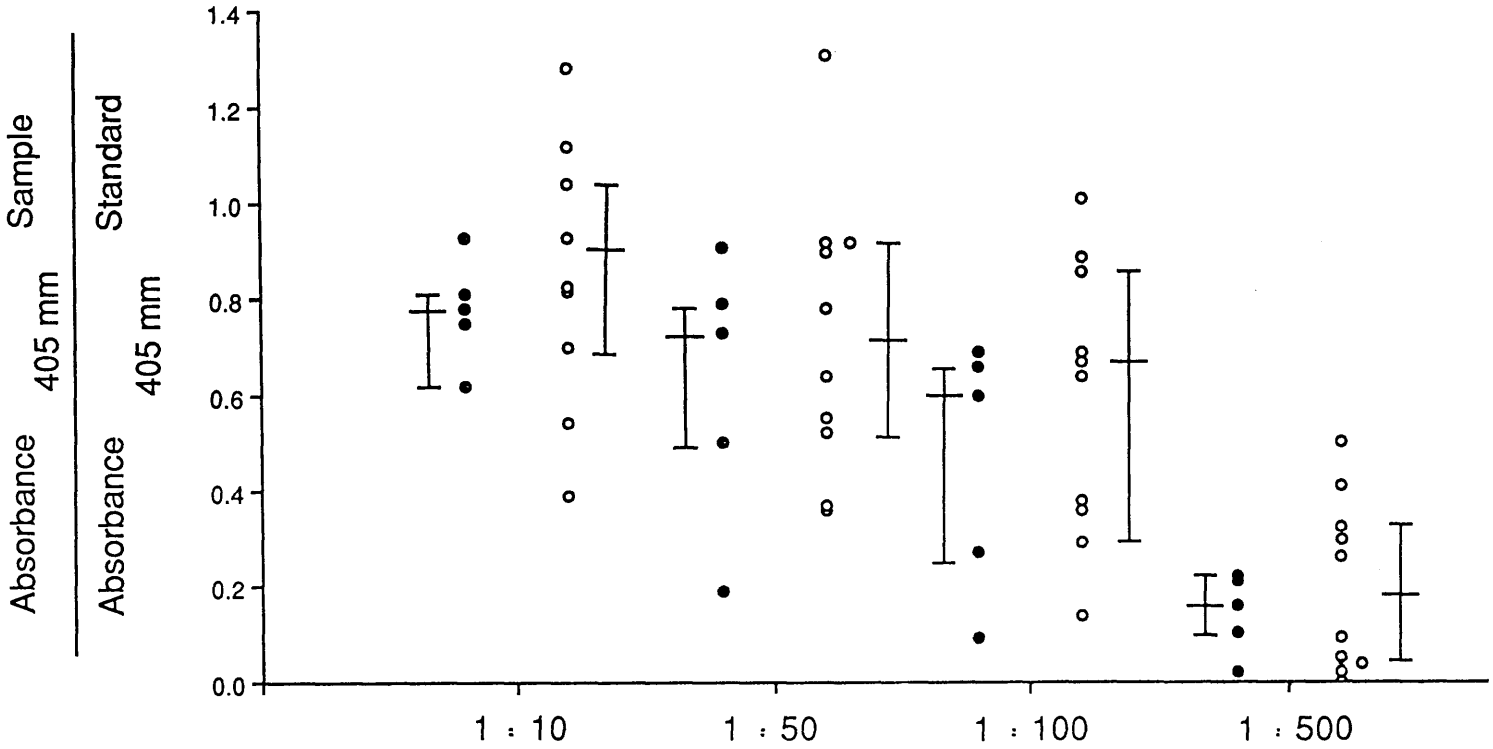
Figure XXVII: Reactivity of tissue-typing sera to formalin-killed Klebsiella as measured by ELISA.



• anti-B27 antisera; ° antisera of other HLA specificities.

Medians and interquartile ranges shown.

Figure XXVIII: Reactivity of tissue-typing sera to sonicated Klebsiella as measured by ELISA



• anti-B27 antisera; ° antisera of other HLA specificities.

Medians and interquartile ranges shown.

ELISA system with unconjugated peptide is inefficient at detecting anti-peptide antibodies.

Peptide-HSA conjugate

With HSA alone as the blank, the reactivity of the anti-peptide rabbit antiserum to the peptide-HSA conjugate was measured by ELISA. A 1:1000 dilution gave an absorbance (405nm) reading of around 1.0 (compared to < 0.1 for the pre-immune serum). This indicates that peptide-specific antibodies can be detected using this conjugated peptide ELISA.

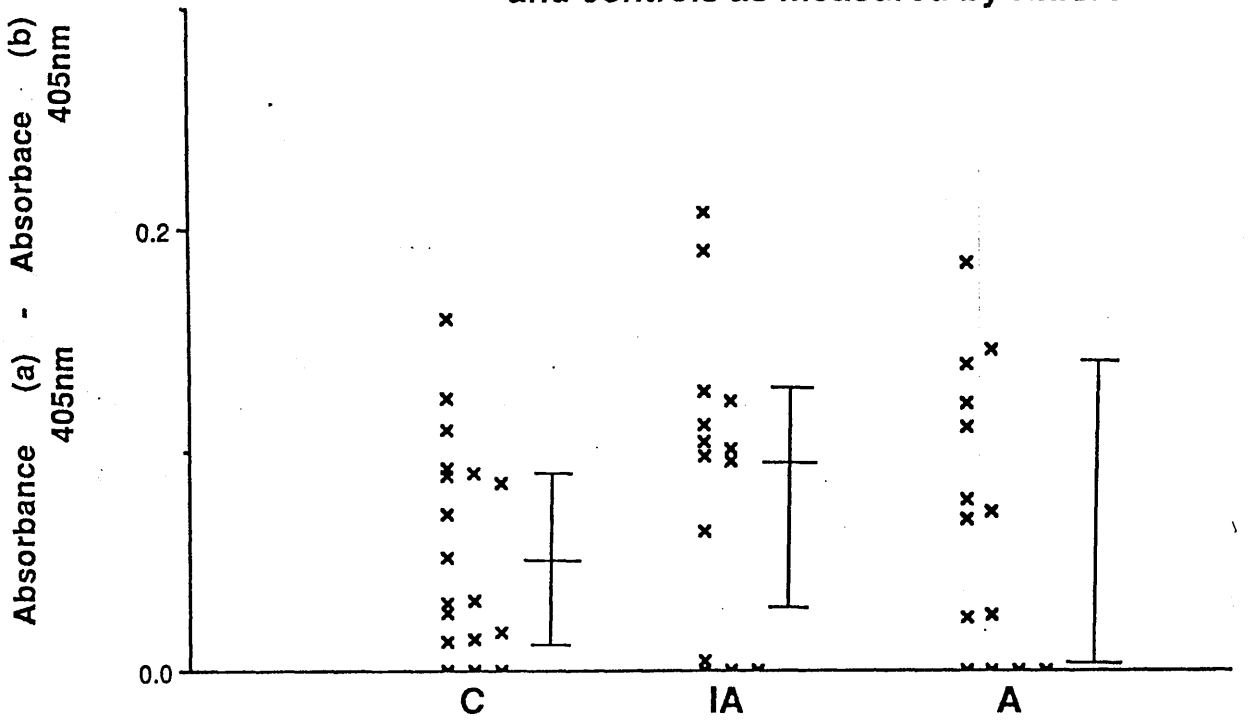
Using this system, the presence of antibodies to the peptide was studied in the sera of 19 controls, 15 patients with inactive disease and 15 patients with active disease. As expected, the absorbance (405nm) values obtained when wells were coated with the human protein HSA alone were very similar to that of uncoated wells. Figure XXIX shows the absorbance (405nm) readings obtained with the peptide-HSA conjugate and a 1:10 dilution of sera. In each subject group, the median absorbance value is below 0.1, indicating a very low level of antibodies to the peptide. When the three groups were compared, no significant difference was found. 1:50 and 1:100 serum dilutions were also studied and there was again no difference between the groups.

Discussion

This study investigated the possibility of cross-reaction between the HLA-B27 protein and *Klebsiella* antigens by measuring the reactivity of anti-Klebsiella antisera to B27+ cells and the ability of anti-B27 antisera to bind to *Klebsiella*.

Using an immunofluorescence assay, we measured the anti-B27 reactivity of four rabbit antisera raised against *Klebsiella pneumoniae* K43 or K21, bacteria which have been

Figure XXIX : Antibodies to the peptide QTDRED In sera of patients and controls as measured by ELISA



C = controls; IA = patients with inactive disease; A = patients with active disease
(a) = sera in wells coated with peptide - HSA conjugate
(b) = sera in wells coated with HSA alone
A 1:10 dilution of sera was used.
Medians and interquartile ranges shown.

implicated in AS. As far as we know, this is the only reported study which has used immunofluorescence to look at this. Each antiserum demonstrated significant binding to B27+ lymphocytes but this was no higher than that found to B27- cells. Moreover, a non-immune rabbit serum showed a similar level of binding. The observed reactivity seems, therefore, to be independent of the B27 antigen and anti-Klebsiella antibodies. It may be attributable to an antibody response to any number of cell-surface antigens. This is a problem with studies such as this : rabbit serum antibodies may recognise commonly found cell-associated antigens which could mask any specific response to B27. Nevertheless, Geczy and Ebringer have both been able to demonstrate a selective binding of rabbit anti-Klebsiella antisera to B27+ cells. Like us, several groups have failed to confirm their findings (47, 48, 49, 188).

Most of the cross-rectivity studies have employed microcytotoxic assays. It is not clear why such conflicting results have been obtained with this assay. Beukelman et al. (186) suggest that the use of different bacterial strains, experimental animals and immunisation procedures may account for the inability of other investigators to reproduce Geczy's or Ebringer's results but the negative findings reported with Geczy's bacterial isolates (47) and with antisera from Geczy and Ebringer (188) do not support this explanation.

Even if the findings of Geczy's or Ebringer's group were confirmed by other investigators, to convince us that anti-Klebsiella antibodies are reactive to the B27 antigen requires more than simply demonstrating that anti-Klebsiella antisera bind to B27+ cells more readily than B27- cells. For example, neither of the two groups have described whether this binding is blocked by polyclonal or monoclonal anti-B27 antibodies.

We also studied cross-reactivity between B27 and Klebsiella by measuring the ability of human tissue-typing sera to bind to Klebsiella. Using an ELISA, we found no evidence that anti-B27 tissue-typing sera were more reactive than non-B27 antisera to either formalin-killed or sonicated Klebsiella. Moreover, the anti-B27 antisera showed no more reactivity to Klebsiella than to E.coli, a bacterium which is not thought to cross-react with B27. This contrasts with the findings of Avakian et al. (42) who used three assay systems to show that anti-B27 tissue-typing sera were more reactive than non-B27 antisera to Klebsiella. The use of a different assay or a smaller number of antisera in our study may, in part, account for these different findings. However, in Avakian's study, the differences observed in the reactivity of anti-B27 and non-B27 sera to Klebsiella were not large. In their competition assay, they measured the ability of tissue-typing sera to inhibit the binding of anti-Klebsiella antisera to Klebsiella. The mean level of inhibition shown by the anti-B27 antisera was around 85% and the non-B27 antisera produced 70% inhibition. While this difference may be statistically significant, it does not provide convincing evidence that anti-B27 antibodies bind to Klebsiella. In the direct binding experiments, the difference in the percentage of radiolabelled Klebsiella sonicates bound by anti-B27 and non-B27 antisera was an equally unimpressive 5%.

One of the problems with studies like these is the high background level of antibodies to Klebsiella found : most individuals possess a significant level of antibodies which bind to Enterobacteriaceae (see Chapter 3) and it is possible that any response mediated by anti-B27 antibodies is masked. This is why many recent studies have employed monoclonal antibodies and these have provided some evidence for cross-reaction between B27 and certain Enterobacteriaceae. However, conflicting data have been reported on the reactivity of anti-B27 monoclonal antibodies to Klebsiella (43, 44) and two studies (44,

191) demonstrated high reactivity of such antibodies to strains of E.coli, a bacterium which has not been associated with the seronegative spondyloarthropathies. Moreover, monoclonal antibodies react with a single epitope and any cross-reactive epitope identified may be of little importance in the normal immune response to the antigen. The molecular mimicry theory of AS is dependent on the ability of the immune response to the bacteria **induced in patients** to cross-react with the B27 antigen.

Recent studies have been involved in looking for a **molecular basis** for cross-reaction between B27 and Klebsiella. Schwimmbeck et al. (196) identified a stretch of six amino acids (QTDRED) found in both the B27.1 protein and a Klebsiella nitrogenase and they were able to demonstrate high levels of antibodies to peptides containing this hexamer in the sera of patients with AS. In an attempt to confirm their findings, we used an ELISA to measure serum antibodies to a synthetic peptide representing this six amino acid sequence.

Initially, unconjugated peptide was used as in Schwimmbeck's study but this ELISA was unable to detect specific antibodies in an anti-peptide antiserum. This is most likely because we used a much smaller peptide and small peptides do not bind well to ELISA plates (198). We then conjugated the peptide to HSA, a protein which human serum antibodies do not recognise. Using this in the ELISA, we were able to demonstrate high levels of anti-peptide antibodies in the rabbit antiserum and this system was therefore used to study sera from patients. Very low levels of anti-peptide antibodies were found and there was no difference between patients and controls. This contrasts with the high ELISA readings found by Schwimmbeck et al. (196) (median absorbance values of around 1.0 compared to < 0.1 for the same serum dilution in our study). This difference is probably due to the size of the peptides used. They used peptides of 13-16 residues representing the homologous hexamer together with

flanking residues normally found in the B27 or Klebsiella protein. It is clear that our peptide, which consisted of the hexamer only, is immunogenic since we were able to raise an antiserum to it, but the epitope formed may be very different from that found when the peptide is part of a large stretch of amino acids. The flanking residues can strongly influence the three-dimensional structure of the peptide. It is probable that the shape of the hexamer used in Schwimmbeck's study resembles the epitope of the native protein more than does our peptide and this could explain our inability to detect antibodies in patients' sera. A larger peptide should perhaps have been used in our study.

The findings of Schwimmbeck's group have stimulated a great deal of interest but their proposal that the antibody response to this homologous sequence has a pathogenic role in AS is questioned for a number of reasons. (1) The evidence that peptides derived from B27 cross-react with Klebsiella-derived peptides is not convincing : while two of Schwimmbeck's anti-B27 peptide antisera bound well to a Klebsiella-derived peptide (196, 199), another showed very little response and both antisera raised to Klebsiella-peptides had little or no reactivity to a B27-peptide. (2) There is no reported evidence that cross-reactivity between these peptides occurs at the protein level : in a 1988 review, Schwimmbeck et al. (200) claim that anti-peptide antibodies are cytolytic for B27+ cells and bind to isolated Klebsiella nitrogenase but as far as we know, the details of this study have never been published. (3) The QTDRED hexamer is found in the B27.1 allotype only but all six variants of B27 are associated with AS (30). (4) In their search for shared sequences, Schwimmbeck et al. (196) found " several homologies between HLA-B27 and microbes ". The " best fit " was with Klebsiella nitrogenase but this may be no more relevant than, say, a five amino acid sequence shared with some virus. Peptides representing some of the other homologous regions would

serve as useful controls in studies of anti-peptide antibodies in patients. No peptide controls were used in Schwimmbeck's study. (5) Klebsiella nitrogenase is unlikely to be of importance in AS : the gene for this enzyme is normally only expressed in nitrogen-free environments and should therefore be suppressed in a human host.

This sequence homology between B27 and a Klebsiella protein may be purely coincidental. Many such homologies exist between disparate proteins (201). An eight amino acid sequence common to IgG and HIV has been identified and there has been no claim that this has any pathogenic significance.

Numerous studies have investigated the possibility that B27 cross-reacts with Klebsiella or other Enterobacteriaceae but one question which is central to the molecular mimicry theory of AS is often ignored : do patients show any evidence of an autoimmune response to the B27 antigen? Two groups (202, 203) have looked for antibodies directed against B27+ cells in the sera of patients and none were found. It is of course possible that anti-B27 autoimmunity is mediated, not by antibodies, but by T-cells, but no evidence for this has been reported.

In conclusion, although the molecular mimicry theory of AS is an attractive theory which has stimulated much interest over the last decade, the evidence for it is not convincing and the findings of ours and many other studies fail to support it.

CHAPTER 7

DISCUSSION

For more than a decade, there has been much interest in the theory that Klebsiella or other Enterobacteriaceae play a pathogenic role in AS through molecular mimicry with the B27 antigen. So, what is the evidence to support such a proposal?

1) Reports that patients have a high faecal carriage rate of Klebsiella

These reports are controversial : several investigators (54, 55, 56) have failed to support the findings of Ebringer's group (41, 51). Using rectal swabs, we isolated Klebsiella from only 17% of patients with AS and found no difference between patients with active disease and those with inactive disease. Moreover, none of the Klebsiella isolated were K43 - the serotype most strongly implicated in AS (43, 46). The molecular mimicry theory of AS does not, however, rely on the constant presence of Klebsiella in the bowel : the bacterium could stimulate an immune response which is continued because of the presence of the cross-reactive B27 antigen.

2) Reports that patients have elevated levels of IgA antibodies to Klebsiella or other Enterobacteriaceae

Although higher than normal levels of bacterial-specific IgA antibodies in patients' sera have been demonstrated in this and other studies (39, 67, 69), their significance is far from clear. In many cases, the differences observed between patients and contols, although statistically significant, are not marked. Most individuals already possess significant levels of antibodies which react with Enterobacteriaceae and it is not clear how meaningful small increases in these levels are. Moreover, there is much

controversy over exactly which anti-bacterial antibodies are raised. Some studies have demonstrated elevated IgA antibodies to Klebsiella only (39,67), another found raised IgA antibodies to Yersinia only (69), and our study demonstrated high levels of antibodies to Yersinia and Shigella which were associated with active disease and raised antibodies to Klebsiella and Salmonella which were independent of disease activity. A varied assortment of "control" organisms have been used in different studies - even in studies by the same group.

It is by no means clear that these raised anti-bacterial antibodies are unique to AS patients. Although Ebringer's group have found a normal anti-Klebsiella antibody response in patients with other inflammatory diseases such as RA and PsA (52), one study has demonstrated high serum levels of antibodies to Klebsiella in patients with RA (123) and, most interestingly, such antibodies have been found to be raised in inflammatory bowel disease (103, 123). It is suggested that this could be due to an abnormal bowel permeability in such patients. Increased bowel permebility has been reported in AS (124) and this was supported by a recent study of 17 of our patients (207). Studies by Bjarnasson et al. (125) and Jenkins et al. (126) suggest that NSAID treatment may be responsible for this. It is possible that increased bowel permeability allows the transfer of normally excluded bacterial antigens across the intestinal mucosa into the circulation where they induce a systemic immune response. Thus, the high anti-bacterial antibody levels found in AS may have no pathogenic significance but could simply be a consequence of therapy. In a study soon to be carried out in our labaratory, the level of anti-bacterial antibodies in healthy volunteers receiving NSAIDs will be compared to those of healthy controls.

Although AS patients have elevated antibodies to gut bacteria in their serum, there is little evidence of antigenic stimulation of the gut itself. Trull et al. (65)

demonstrated high levels of anti-Klebsiella antibodies in patients' saliva but our study and that of Pease et al. (69) showed no difference between patients and controls in the level of salivary antibodies to either Klebsiella or Yersinia. It could be argued that salivary antibodies do not provide a good indication of the intestinal immune response (97, 102) but in a recent study, anti-Klebsiella antibodies were measured in gut lavage fluid and the levels found in AS patients were no higher than in contols (103).

If antibodies to Klebsiella or other Enterobacteriaceae played any pathogenic role in AS, we would expect to find them at the site of disease - the joints. We found that the antibody levels to several bacteria in the synovial fluids of AS patients were no higher than those found in synovial fluids from patients with RA or one of a variety of other inflammatory joint diseases. Moreover, in reactive arthritis, the **cellular** immune response to the causative organism is markedly elevated within the joint (137, 140) but we found no evidence that synovial lymphocytes from AS patients make a high proliferative response to Klebsiella.

3) Reports of cross-reaction between Klebsiella and the B27 antigen

The main theory proposed to explain the involvement of bacteria in AS is that of molecular mimicry with the B27 antigen but the subject of cross-reaction between Klebsiella and B27 is one of much controversy. Several investigators (47, 48, 49, 50) have failed to support the findings of either Ebringer's (25) or Geczy's (46) groups. A large number of these studies have relied on measuring the reactivity of anti-Klebsiella antisera to B27+ cells in a microcytotoxic assay. Using an immunofluorescence method, we found that anti-Klebsiella antisera were no more reactive to B27+ lymphocytes than to B27- cells. We also measured the ability of several human tissue-typing sera to bind to Klebsiella and other Enterobacteriaceae in an

ELISA. Anti-B27 sera showed no more reactivity than tissue-typing sera of other specificities. These studies which involve comparing B27+ and B27- cells or B27 and non-B27 antisera have obvious problems with high backgrounds and more sophisticated methods are clearly required. Ideally, purified B27 antigen and isolated Klebsiella proteins should be studied but these would be difficult to prepare. More practical approaches are possible : e.g. if, by running B27+ cells on a gel and immunoblotting with anti-B27 monoclonal antibodies, the bands representing the B27 antigen could be identified, we could study the reactivity of purified polyclonal or monoclonal anti-Klebsiella antibodies to these bands.

One group sought a possible molecular basis for cross-reactivity between B27 and Klebsiella and identified a six amino acid sequence shared by the B27 antigen and a Klebsiella nitrogenase (45). However, there is no evidence that this sequence homology reflects an immunological cross-reaction at the protein level.

Even if cross-reaction between Klebsiella and B27 could be proven beyond doubt, it might have no pathogenic significance. Other examples of cross-reactivity between gut bacteria and human cell surface antigens have been demonstrated - e.g. between E.coli and ABO blood group antigens of human erythrocytes (204) and as far as it is known, these cross-reactions have no pathogenic consequences.

Thus, the evidence supporting the proposal that Enterobacteriaceae play a role in AS is, at best, unconvincing. In addition, there are certain fundamental problems with the theory that molecular mimicry between bacteria and the B27 antigen leads to tissue damage. Firstly, the B27 antigen is found on all nucleated cells, so why should the inflammation in AS be restricted to the joints and a few other isolated sites? Secondly, there is

no evidence that patients express an anti-B27 response. As far as we know, only two reported studies (202, 203) have looked for anti-B27 antibodies in patients' sera (where none were found), and the presence of such antibodies in the joints has not been investigated.

It is possible that Enterobacteriaceae could play a more direct role in the disease - e.g. bacterial antigens may be present in the joint where they induce an immune response which causes damage to surrounding tissue. This could be the case in the known reactive arthritides where there is increasing evidence for the presence of bacterial antigens (36, 64) and a bacterial-specific cellular immune response (137, 140 in the joints. However, we failed to detect a humoral or cellular response to Klebsiella in the joints of patients with AS. A search for bacterial antigens within AS joints is needed and immunohistochemical studies of synovial tissue are underway in our laboratory. (The availability of such tissue from AS patients is, however, limited.) A useful approach to the identification of any environmental agents in AS may be the study of antigens within patients' immune complexes. By running crude preparations of immune complexes (PEG precipitates) on SDS-polyacrylamide gels and immunoblotting with anti-Klebsiella antisera, we found no evidence for the presence of Klebsiella antigens within immune complexes from patients' sera or synovial fluid. However, further studies are required - e.g. purified immune complexes could be dissociated into antigen and antibody components and the reactivity of these components studied by ELISA and immunoblotting.

At present, the evidence that Enterobacteriaceae play a role in AS is far from convincing and the results of this study have failed to support such a proposal.

Appendix A

POINT SYSTEM FOR ASSESSMENT OF DISEASE ACTIVITY

Use of NSAIDs		Morning Stiffness	
None	= 0	None	= 0
Regular	= 1	< 1 hour	= 1
		> 1 hour	= 2

Peripheral arthritis		CRP levels	
None	= 0	< 10	= 0
Previous	= 1	10-50	= 1
Current	= 2	> 50	= 2

ESR

< 30	= 0
30-60	= 1
> 60	= 2

Immunoglobulin levels

Normal (IgG = ≤ 15mg/ml; IgA = ≤ 4.5mg/ml) = 0

Elevated (IgG = > 15mg/ml or IgA = > 4.5mg/ml) = 1

NSAIDs = Non-steroidal anti-inflammatory drugs

CRP = C-reactive protein

ESR = Erythrocyte sedimentation rate

Patients with < 5 points are considered to have **inactive disease**

Patients with ≥ 5 points are considered to have **active disease**

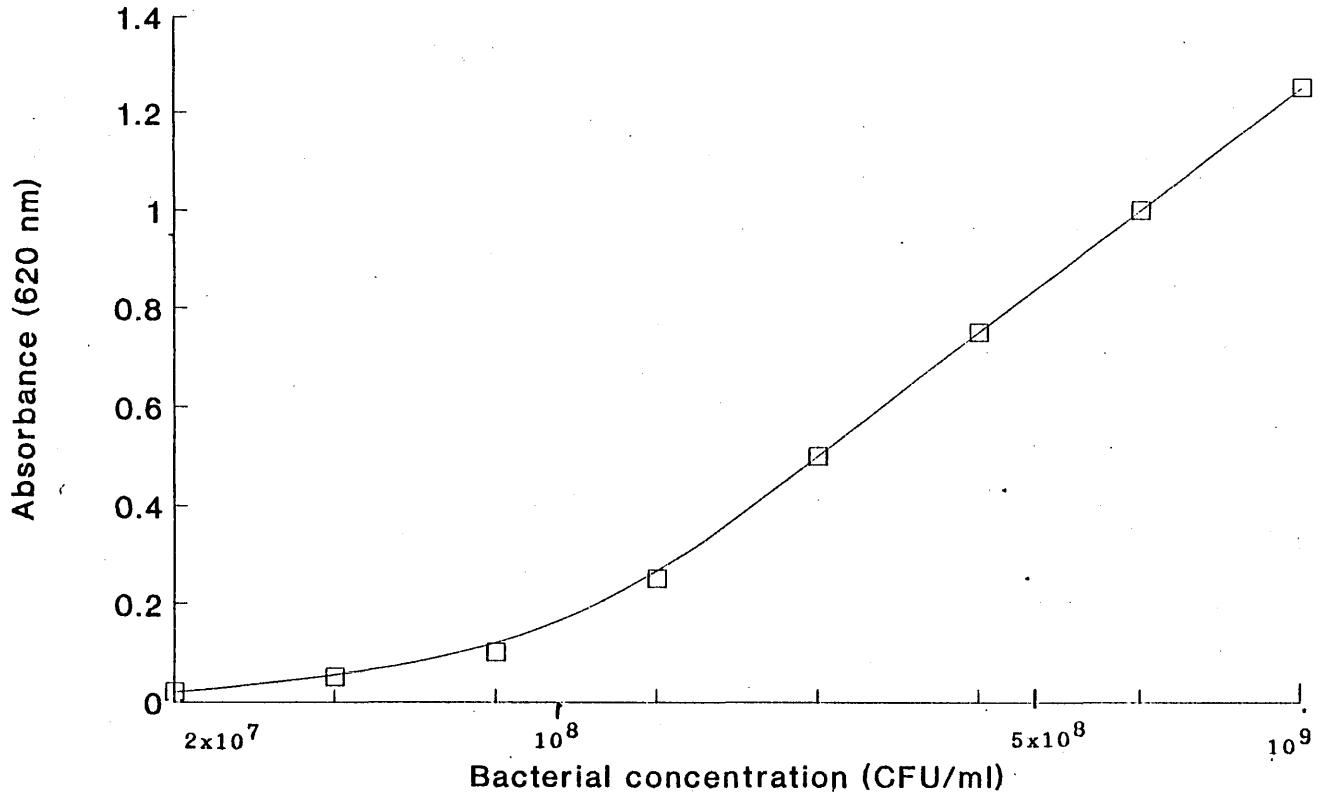
Appendix B

STATISTICAL ANALYSIS

The main statistical test used in this study was the Mann-Whitney U-test for the comparison of two samples. This is a non-parametric method which can be used to analyse data which is not normally distributed. Spearmann-rank and chi-squared tests were also used. All tests were performed on an Amstrad PC 1640 computer using a Statgraphics package (version 2.6). For formulae and detailed descriptions of each test, see Bailey (208).

APPENDIX C

Figure XXX. Correlation between absorbance (620 nm) and concentration of *Shigella flexneri*



CFU = colony forming units

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