# The Role of Enterobacteriaceae as Possible Immunological Triggers in Ankylosing Spondylitis and Other Inflammatory Disorders

by

Rona Cooper

being a thesis submitted for the degree of

Doctor of Philosophy

in the

University of Glasgow

University Department of Medicine

Royal Infirmary

Glasgow

September 1987

**To: Callum**

Table of Contents

Index of Tables

Index of Figures

XVI Lymphocytotoxicity of HLA-B27 antiserum

XVII Lymphocyte transformation responses to PHA in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XVIII Lymphocyte transformation responses to PWM in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XIX Lymphocyte transformation responses to Con A in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XX Serum IgA antibody levels to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXI Serum IgA antibody levels to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXII Serum IgA antibody levels to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXIII Serum IgG antibody levels to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXIV Serum IgG antibody levels to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXV Serum IgG antibody levels to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXVI Serum IgM antibody levels to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXVII Serum IgM antibody levels to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXVIII Serum IgM antibody levels to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis and normal controls

XXIX Anti-E.coli IgA antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXX Anti-E.coli IgG antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXI Anti-E.coli IgM antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXII Anti-Yersinia IgA antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXIII Anty-Yersinia IgG antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXIV Anti-Yersinia IgM antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXV Anti-Campylobacter IgA antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXVI Anti-Campylobacter IgG antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXVII Anti-Campylobacter IgM antibody levels in patients with ankylosing spondylitis, Crohn's disease, rheumatoid arthritis, ulcerative colitis and normal controls

XXXVIII Correlation between absorbance at 620nm and concentration of Klebsiella pneumoniae

XXXIX Correlation between absorbance at 620nm and concentration of Escherichia coli

XL Correlation between absorbance at 620nm and concentration of Yersinia enterocolitica

XLI Correlation between absorbance at 620nm and concentration of Salmonella typhimurium

XLII Correlation between absorbance at 620nm and concentration of Campylobacter jejuni

XLIII Correlation between absorbance at 620nm and concentration of Pseudomonas aeruginosa

Acknowledgements

I would like to thank the following for their help during the compilation of this thesis.

Dr C G Gemmell and Dr R D Sturrock for their advice and guidance.

The Medical Staff of the Centre for Rheumatic Diseases for the collection of patients' samples.

Dr R Park, Ms Jean Hunter and Staff of the Gastroenterology Unit for the collection of samples and their patience with my persistent enquiries.

Dougie Bell and Staff for their help in the Animal House.

The Department of Clinical Immunology for tissue typing.

Miss Debbie Crawford for her time and expertise in the typing of this thesis.

My friends and colleagues in the Departments of Medicine and Bacteriology at the Royal Infirmary, for their support and encouragement.

And, to Callum, for his patience and understanding.

SUMMARY

Previous research has found the association between ankylosing spondylitis (AS) and the human leukocyte antigen B27 to be the strongest known association of a human leukocyte antigen and disease. Genes in linkage disequilibrium, molecular mimicry and interaction of B27 with foreign antigens have all been proposed as possible mechanisms to account for the association. Reports of a high incidence of Klebsiella carriage in patients with AS directed investigation towards cross-reactivity between Klebsiella and B27 antigens. Sera to certain Klebsiella isolates have been found to be cytotoxic for B27 positive peripheral blood lymphocytes (PBL) from patients with AS. Cross-reactivity has also been demonstrated, between serum raised to B27 positive lymphocytes and various enteric bacteria and recently raised levels of anti-Klebsiella IgA antibodies have been found in patients with active AS. It has therefore been suggested that AS may occur as a result of immunological damage following infection by gram negative bacteria carrying antigens that are stereochemically similar to self antigens.

The contribution made by Klebsiella and other enteric bacteria, in AS and three other disease population controls - rheumatoid arthritis (RA), Crohn's disease (CD) and ulcerative colitis (UC) - was therefore determined using various immunological techniques.

The association of Klebsiella and AS was investigated by measuring the frequency of faecal carriage of Klebsiella in patients with active, probably active and inactive disease. No increase in carriage was

found in any of the patient groups when compared to community controls. Erythrocyte sedimentation rate and C-reactive protein levels correlated with disease activity but serum IgA, IgG and IgM levels did not. Peripheral joint involvement did not affect any of these parameters except serum IgM levels which were raised in patients with peripheral joint involvement. This could suggest a recent or ongoing infection in these patients.

The 'cross-tolerance' theory to explain the pathogenesis of AS was investigated using a lymphocytotoxicity assay. There was no reaction between anti-Klebsiella antibodies and B27 positive peripheral blood lymphocytes from AS patients. Therefore no similarities exist between Klebsiella antigens and antigens on peripheral blood lymphocytes including the B27 antigens.

The cell mediated immune response was measured in patients with AS, CD and RA. Transformation by phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) was normal in the AS and CD groups but the response to concanavalin A (Con A) was lowered. PHA, Con A and PWM responsiveness was decreased in patients with RA. Patients with AS and CD may therefore have an abnormal T suppressor cell activity whereas RA patients have an overall abnormal T cell response.

Lymphocyte responsiveness to bacterial antigens in patients with AS and CD was generally normal but in the RA group again the response was lower confirming the above depression of T cell activities.

The humoral immune response to bacterial antigens was found to be

activated in patients with AS, CD, UC and RA. IgA antibodies to Klebsiella pneumoniae and Yersinia enterocolitica were raised in all patient groups. Anti-E.coli IgA antibodies were increased in the AS and CD groups whereas anti-Campylobacter jejuni IgA antibody levels were increased in patients with RA and UC. Anti-Klebsiella IgM antibody levels were lowered in AS patients, therefore Klebsiella antigens could not be responsible for the raised levels of total serum IgM. The increased IgA levels may be due to an increased permeability of the gut to bacterial antigens leading to an increased gut mucosal IgA response allowing the release of IgA into the circulation.

In conclusion, the pathogenic role of gram negative bacterial antigens in ankylosing spondylitis still remains unresolved but the role of Klebsiella is not specific for ankylosing spondylitis.

## Abbreviations

| | |
|---|---|
| A | active |
| A405nm | absorption at 405nm |
| AAU | acute anterior uveitis |
| AS | ankylosing spondylitis |
| BSA | bovine serum albumin |
| CD | Crohn's disease |
| CF | culture filtrate |
| CIC | circulating immune complexes |
| C.jejuni | Campylobacter jejuni |
| CMI | cell mediated immunity |
| Con A | concanavalin A |
| cpm | counts per minute |
| $^{51}Cr$ | radioactive chromium |
| CRP | C-reactive protein |
| CTL | cytotoxic T lymphocytes |
| DNA | deoxyribonucleic acid |
| ECA | enterobacterial common antigen |
| E.coli | Escherichia coli |
| EDTA | ethylenediaminetetracetic acid |
| ELISA | enzyme linked immunosorbent assay |
| ESR | erythrocyte sedimentation rate |
| FT | formalin treated |
| GI | gastrointestinal |
| GC | glucocorticoid |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HLA | human leukocyte antigen |
| HT | heat treated |
| HTS | supernatant from heat treated bacteria |
| IBD | inflammatory bowel disease |
| IN | inactive |
| K.pneumoniae | Klebsiella pneumoniae |
| LPS | lipopolysaccharide |
| MHC | major histocompatibility complex |
| MN | monocytes |
| MOMPs | major outer membrane proteins |
| NCTC | National Collection of Type Cultures |
| NSAIDs | non-steroidal anti-inflammatory drugs |
| OA | osteoarthritis |
| PA | probably active |
| PBL | peripheral blood lymphocytes |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PG | prostaglandin |
| PHA | phytohaemagglutinin |
| PJI | peripheral joint involvement |
| PS | polysaccharide |
| Ps.aeruginosa | Pseudomonas aeruginosa |
| PWM | pokeweed mitogen |
| RA | rheumatoid arthritis |
| ReA | reactive arthritis |
| RMPI | Rosewell Park Memorial Institute |
| RS | Reiter's syndrome |

| | |
|---|---|
| SEM | standard error of the mean |
| SI | stimulation index |
| SLE | systemic lupus erythematosus |
| SON | sonicated |
| *S.typhimurium* | *Salmonella typhimurium* |
| $T_H$ | T helper |
| $T_S$ | T suppressor |
| UC | ulcerative colitis |
| *Y.enterocolitica* | *Yersinia enterocolitica* |

## Objectives

1. To investigate the isolation frequency of Klebsiella in patients with ankylosing spondylitis and whether it is associated with disease activity.

2. To investigate the association between clinical disease assessment and laboratory parameters in patients with ankylosing spondylitis.

3. To investigate the hypothesis that the HLA-B27 antigen on lymphocytes of patients with ankylosing spondylitis stereochemically resembles an antigen(s) from Klebsiella pneumoniae.

4. To measure the cell mediated immunity in patients with ankylosing spondylitis and other inflammatory disorders by following their lymphocytic response to mitogens and bacterial antigens.

5. To investigate the humoral immune response in patients with ankylosing spondylitis and other inflammatory disorders by measuring antibody levels to Klebsiella and other enterobacteria.

6. To establish whether enterobacterial common antigen has a role in the disease pathogenesis of ankylosing spondylitis and other inflammatory diseases.

# INTRODUCTION

## Historical and clinical features of ankylosing spondylitis

Ankylosing spondylitis (AS), an inflammatory disorder of the synovial joints, is characterised by inflammatory lesions at entheses (junctional zones between tendon or ligament and bone) both within and at a distance from the spine. The term is derived from the Greek words "ankylos" meaning bent or crooked and "spondylos" referring to the vertebra. In modern terms ankylosing refers to the coalescence of two bones originally distinct and spondylitis coming from spondyle - one or other of the joints of the spine.

It is a chronic but fluctuating inflammatory condition and may involve other sites such as peripheral joints, acute inflammatory eye disease (anterior uveitis), aortic valve disease, bursitis and tendonitis. Pathologic changes involve the synovium, capsular ligamentous attachments to the bone, non articular sites of the aortic root, apex of the lung and anterior uvea. The changes observed are of nonspecific chronic inflammation.

The pathology of the disease is dominated by restricted spinal movement and ultimately ossification in the thoracolumbar region. A sequence of events has been proposed by McGuigan for this enthesopathy (McGuigan, Geczy and Edmonds 1985). Inflammatory lesions consist of infiltrates of lymphocytes, plasma cells and neutrophils. Bone at bone-ligament junctions is eroded and contains cellular infiltrates.

As the inflammation process abates there is a loss of these infiltrates and an increase in fibrous tissue with repair of the lesion by deposition of woven bone. This bone deposition eventually makes another bone ligament junction. Remodelling of the bone occurs and is replaced by mature cancellous bone. This series of events is thought to occur across intervertebral joints, sacroiliac joints and around apophyseal synovial joints. In effect these joints become immobilised by thin pieces of bone laid down by repeated bouts of inflammation at the entheses. Once immobilised, endochondral ossification of the previously normal intervertebral disc or hyaline joint cartilage occurs just as it does in other conditions in which joints are immobilised.

Genetics and HLA status

It was first recognised that AS was more predominant in males than females (Hill, Hill and Bodmer 1976) and that there was a tendency to familial clustering (Wright 1978 and Calin 1985) suggesting that the disease was genetically inherited. When, in 1973, Brewerton et al (1973b) and Schlosstein et al simultaneously reported the association of the genetic marker HLA-B27 with AS it seemed possible that the disease pathogenesis might be understood.

Over 90% of AS patients are HLA-B27 positive whereas only 8% of the normal caucasian population possess this antigen on their cells. The presence of the antigen and AS varies from population to population (Khan 1985). 80% of Mexicans (Arellano et al 1984) and Japanese (Ryder, Andersson and Svejgaard 1979) with AS are B27 positive and 50%

of North American blacks with the disease have the antigen (Khan et al 1977). The B27 antigen is also found in many racial groups (Brewerton 1976) where it is associated with AS. The highest known prevalence of B27 in any human race is in the Haida Indians of British Columbia who have a 50% prevalence of B27 (Gofton et al 1975). The frequency of B27 in the Navajo Indians is 36% (Rate et al 1980) and in both populations 10% of the adult males develop AS. Where the antigen is absent or extremely rare, as in Australian Aborigines and South American Indians, AS is rarely seen (Cleland, Hay and Milazzo 1975 and Khan 1985).

Research into the pathogenesis of AS has followed two main directions - the relationship between AS and HLA-B27 related diseases within families and ethnic groups to delineate their epidemiology and inter-relationships; and studies to show any immunological abnormalities that could link the genetic contribution of the major histo-compatibility complex (MHC) with the pathology underlying the clinical manifestations of the disease. An understanding of the MHC would therefore be fundamental in trying to understand the immunology of AS.

The MHC of humans, known as the human leukocyte antigen (HLA) system, is a multigene family that controls several important immunological functions (Wake 1986). The genes are located on the short arm of chromosome 6 and encode for three classes of molecules (Fig I). The class I antigens, HLA-A, -B and -C are polymorphic, cell surface glycoproteins. They are encoded by a 44K dalton transmembrane polypeptide which is associated non-covalently on the cell surface with $B_2$ microglobulin (a non HLA encoded 12K dalton protein). These

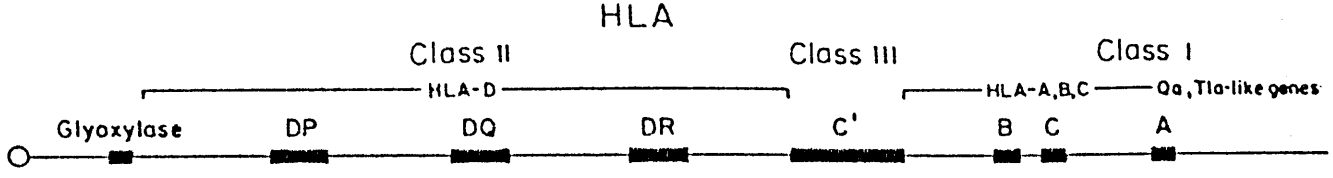



Fig I Molecular map of the HLA complex (taken from Wake 1986).

antigens are found on virtually all nucleated cells and are involved in the presentation of foreign antigens to cytotoxic T lymphocytes. There are also two remaining genes Qa and Tla whose functions are unknown. The class II antigens are encoded within the HLA-D region and are sub-divided into three regions DR, DQ and DP. These antigens (also called Ia antigens) are expressed primarily on B lymphocytes and antigen-presenting cells, such as macrophages, and present antigens to helper T lymphocytes. The class III proteins are the serum components of the complement cascade.

The high association of AS with HLA-B27 is also increased in many other forms of seronegative inflammatory disease (Ebringer 1980) particularly those with a predilection for the sacroiliac joint and an association with acute anterior uveitis (AAU). 60% of patients with Reiter's syndrome and 90% of those who develop arthritis after enteric bacterial infections are B27 positive. 50% of those with AAU alone and 100% with AAU plus AS also possess this antigen.

Theories of aetiopathogenesis

No-one has yet managed to determine the relevance of the association between AS and HLA-B27. Why are AS and other related seronegative diseases linked by their common association of an inflammatory arthritis of the sacroiliac joints and with the presence of a particular histocompatibility antigen?

Several reports have indicated that B27 positive relatives of probands with spondylitis are significantly more likely to have spondylitis

than B27 positive relatives of normal B27 positive individuals (Calin et al 1983 and Van der Linden, Valkenburg and Cats 1985). This implies that the B27 antigen in spondylitis is different from that in disease free individuals or that additional factors are present in spondylitic families. The association of AS and B27 does not explain the male predominance and why some individuals are affected more severely than others and why most B27 positive individuals do not have the disease. Since a small proportion of AS sufferers are not B27 positive this suggests that the disease susceptibility gene is not that of B27 but a closely associated gene. However, the genetic susceptibility could not alone account for the pathogenesis of AS as B27 positive monozygotic twins have been seen to be discordant for the disease (Eastmond and Woodrow 1977). This argues strongly in favour of environmental factors being involved.

Some investigations have suggested that there are colo-rectal mucosal abnormalities common in patients with AS (Jayson 1970) and recently Stodell reported an abnormal increase in absolute numbers of IgG plasma cells in the rectal lamina propria of individuals who have spondylitis and clinically normal bowel flora (Stodell, Butler and Zemelman 1984) implying that there is a local immune response to microbial antigens within the gut lumen.

There have been various theories put forward to explain the possible role of an HLA antigen in disease predisposition. The HLA antigen could act as a specific receptor for infectious agents, hormones etc, which could affect the disease process (Svejgaard and Ryder 1976) or the HLA molecule could be altered causing it to be recognised as

foreign (Zinkernagel 1974). The primary structure of the antigen itself may be very important to the disease susceptibility. A variant or subtype, perhaps differing in one or two amino acids or a sugar moiety, could be strongly associated with a particular disease, perhaps by mimicking a foreign antigen more closely than other HLA molecules of the same serological specificity (Upfold et al 1985). However, the two main theories that have been proposed to explain the association between B27 and AS have been extensively reviewed in recent years by Ebringer (Ebringer and Ebringer 1981; Ebringer 1982; Ebringer, Baines and Ptaszynska 1985b) and Woodrow (1985). They are the two gene theory (McDevitt and Bodmer 1974) and the one gene theory (Ebringer 1978a).

The two gene or 'linkage disequilibrium' theory suggests that on chromosome 6 next to the B27 gene is another putative gene whose product is responsible for the development of AS. The two genes have not been separated from one another because of recent phylogenetic origin of the linked gene, or, 'linkage disequilibrium' provides a selective advantage which maintains the genetic association. Those carrying the B27 and AS gene will develop the disease but those without the AS gene will not. The other B locus antigen may play a role in increasing the susceptibility to AS particularly in the absence of B27 but this is not easy to demonstrate because of the rarity of B27 negative cases. One would need to show its frequency in B27 negative patients was significantly higher than in B27 negative healthy individuals. However, no second gene has been found and as the disease gene cannot be defined by its structural properties, only its functional properties of being associated with the disease, no

investigation to test for the presence of the gene product defined by the AS gene has been designed. Also, the theory does not explain AS patients who do not have the B27 gene. Perhaps other HLA antigens that are cross-reactive with B27 such B7, BW22 or BW42 are involved but this would require the AS gene to be linked to at least three other genes which would seem unlikely. More evidence against this hypothesis is from the fact that the association of B27 and AS occurs in many ethnic groups throughout the World. If this theory held true it would imply the persistence of an extremely marked degree of linkage disequilibrium being maintained over a considerable period of time and under varied selective conditions. Also, the theory does not explain the way in which the AS gene product would lead to an increased susceptibility to AS. Therefore this model is limited and does not provide a theoretical or experimental framework in which to study the pathogenesis of AS and explain the B27 association.

The second of the two hypotheses is the one gene, cross tolerance or molecular mimicry theory. It suggests that the B27 gene is the major AS gene. There is no 'disease susceptibility' locus and the increased incidence of the disease is attributed directly to the marker HLA gene. It suggests that the HLA molecule stereochemically resembles an antigen(s) found on some external agent such as a micro-organism (Fig II). Either the cell surface determinant functions as a specific receptor for important mediators in the inflammatory process or an antigenic similarity exists between the B27 determinant and genes of a particular micro-organism. Infection with the cross-reacting antigen would initially produce a low immune response and the antibodies produced would bind to the foreign antigen as well as the HLA antigen,

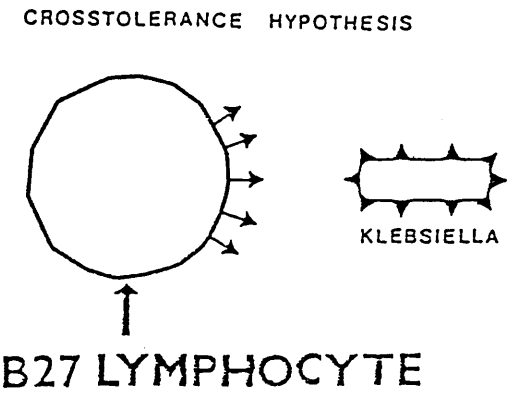



Fig II The cross-tolerance hypothesis (taken from Ebringer 1983).

ie, would be auto-immune. Continuing proliferation of the infectious agent would then stimulate the immune system until eventually a high immune respone is induced (Young, Ebringer and Archer 1978). The auto-immune antibodies binding to self antigen could activate the complement cascade producing tissue damage in a different part of the body from the original infection. The cross-reactive antibodies would react not only with the B27 antigen but also tissue specific antigens found in the spine, sacroiliac joints and other sites. Binding of such an auto-antibody to cross-reactive antigens near the presacral and pava-aortic lymph nodes would lead to chronic inflammation and inadequate elimination of the micro-organism leading to a prolonged chronic disorder such as AS.

A proposed mechanism could be -

INFECTION — occuring first in the gut leading to production of antibodies.

↓

ANTIBODIES — are produced in the lymph nodes draining the colon and rectum and will bind to cross-reacting self-antigens and activate the complement cascade.

↓

INFLAMMATION — occurs as a result of complement activation and will release free radicals and hydrolytic enzymes. Tissue damage will occur at a site different from the original site of infection and circulating antibodies will bind to other cross-reacting self-antigens, eg, in the uvea.

↓

CHRONIC DISEASE — will follow several episodes of inflammation. Recurrent waves of infection will continue the process producing a chronic disorder such as AS.

It is interesting to note that the lymph nodes draining the colon and rectum are the presacral and pava-aortic lymph nodes which are closely related to the sacroiliac joints and the lumbar spine which are the main sites of pathological activity in AS.

Supporting the argument that HLA molecules cross-react with a micro-organism thus leading to disease, Mann et al (1983) presented evidence of a human T-cell lymphoma virus coat protein that mimicks class I HLA determinants. Here the lack of recognition by the host's immune system may result in uncontrolled viral replication and tumorogenesis.

However, in AS there is no conclusive evidence that there is any cross-reactivity between the B27 antigen and a factor(s) expressed on the surface of certain micro-organisms. There is also little indication of a strong immunological reactivity in this disease - inflammation being confined to a very localised area of the body. The hypothesis again does not explain those with AS who are B27 negative although cross-reactivity may occur in patients who have an antigen resembling B27 such as B7, BW22 or BW42.

## The role of bacteriological triggers in ankylosing spondylitis and reactive arthritis

Many other conditions are known to be associated with one HLA antigen or another (Svejgaard et al 1981) though the association between AS and HLA-B27 remains the strongest. Other conditions related to AS such as reactive arthritis, Reiter's syndrome, arthropathies associated with inflammatory bowel disease (IBD) and acute anterior uveitis also occur among those who possess the antigen (Table 1) and have been well reviewed elsewhere (Keat 1982, 1983; Edmonds 1984; Aho 1985).

| Condition | Investigator | Year |
|---|---|---|
| Reactive arthritis | Aho et al | 1974 |
| | Kosunen et al | 1980 |
| | Leirisalo et al | 1982 |
| Reiter's syndrome | Brewerton et al | 1973c |
| | Leirisalo et al | 1982 |
| Arthropathies associated with IBD | Morris et al | 1974 |
| Acute anterior uveitis | Brewerton et al | 1973a |

Table 1 Association between HLA-B27 and disease

No association between an HLA antigen and disease is absolute so other factors must also be important in the initiation of these disorders. Reactive arthritis, an arthritis following sexually acquired non-specific urethritis or enteric infections, and Reiter's syndrome - a triad of non-specific urethritis, conjunctivitis and arthritis - have been implicated after infection with bacteria such as Yersinia, Shigella, Salmonella, Campylobacter and Chlamydia (Table 2).

The relationship and probable association of Reiter's syndrome and reactive arthritis with infection and their association with B27 is important as it may provide the key to the understanding of other seronegative arthropathies such as AS. It may well be that there is an underlying process contributing to the pathogenesis of a wide group of clinical disorders.

The potential explanation of the association between certain enteric organisms and Reiter's syndrome could be that they are antigenically similar to host antigens and that the antibody response to infection results in an auto-immune disease. One should therefore be able to demonstrate an antigen present in bacteria associated with Reiter's

| Study | Year | Bacterial Infection |
|---|---|---|
| Ahronen, Sievers and Aho | 1969 | Yersinia |
| Winblad | 1975 | Yersinia |
| Foley and Mathews | 1984 | Yersinia |
| Sampson and Cope | 1984 | Yersinia |
| Noer | 1969 | Shigella |
| Calin and Fries | 1976 | Shigella |
| Simon | 1981 | Shigella |
| Vartiainen and Hurri | 1964 | Salmonella |
| Warren | 1970 | Salmonella |
| Stein et al | 1980 | Salmonella |
| Urmann, Zurrier and Rothfield | 1977 | Campylobacter |
| Weir et al | 1979 | Campylobacter |
| van de Putte | 1980 | Campylobacter |
| Keat et al | 1980 | Chlamydia |
| Vilppula | 1981 | Chlamydia |

Table 2 Infections associated with reactive arthritis

syndrome, is absent in strains not associated with the disease (Cohen 1985). Also because Reiter's syndrome is associated with HLA-B27, the

bacteria might possess an antigen that cross-reacts with specific B27 antisera. Using radio-immunoassay Cohen was unable to demonstrate such antigens present in arthritis associated strains that were absent in strains not associated with the arthritis. However, an 88K molecule from Yersinia enterocolitica 3 has been found to cross-react with sera from patients with Reiter's syndrome and AS (Yu 1985) and antibodies to this antigen have been found to be higher in the sera of patients who develop arthritis after Yersinia infection than those who do not develop arthritis. Robinson et al (1983b) have also found that Klebsiella pneumoniae K43 bound more frequently to buccal epithelial cells from patients with Yersinia reactive arthritis and more frequently to B27 positive patients than B27 negative patients.

Secretory IgA is also increased in those with Yersinia arthritis compared to those with yersiniosis and no arthritis (Granfors and Toivanen 1986) and van Bohemen et al (1986b) have found titres to Yersinia and Salmonella typhimurium in those with reactive arthritis due to Campylobacter jejuni.

It has also been suggested that circulating immune complexes (CIC) may play a role in the pathogenesis of reactive arthritis and AS. CIC have been implicated in the pathogenesis of tissue injury associated with glomerulonephritis, hepatitis, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Manicourt and Orloff 1981). In a patient with polyarthritis after Salmonella infection Manicourt found CIC were only detectable during the arthritic phase of the disease and that they disappeared with clinical resolution of the joint symptoms. There is also evidence that there are certain similarities between reactive arthritis and IgA mediated glomerulonephritis. Jenette et al

(1982) reported IgA nephropathy in two patients with AS and in one with Reiter's disease and suggested that IgA nephropathy may be associated with the seronegative spondylarthropathies in general rather than specifically for one syndrome. CIC have also been measured in patients with yersiniosis and Yersinia arthritis. Kekomäki et al (1983) found CIC frequently in patients with both the arthritis and non-arthritic groups. Lahesmaa-Rantala et al (1987) have shown that patients developing arthritis after Yersinia infection have significantly more Yersinia specific IgM complexes than patients without arthritis.

CIC have been measured in AS with varying degrees of success (reviewed by McGuigan et al 1985). Recently Bruneau and Bonin (1983) found that the CIC in AS share antigenic and antibody specificities that are not found in other chronic inflammatory disorders such as SLE and RA. Therefore suggesting that there is a disease specific antibody in AS immune complexes. This could indicate that in AS an antigen is present that is found specifically in this disease.

Rødahl and Iversen (1986) have reported that an antiserum towards the envelope glycoprotein, gp 70, of a psoriasis associated-retrovirus like particle reacted with a 70K dalton and a 40-45K dalton component in AS immune complexes. Therefore antigens related to this virus particle may precipitate immunological reactions in AS and may be implicated in inflammatory reactions in affected tissues. The characterization of IC antigens would therefore seem desirable in order to identify antigens that may elicit inflammatory reactions.
The true interaction between cell-surface structures and micro-

organisms is still not understood but studies by two groups have indicated that certain bacteria may be antigenically related to HLA-B27.

Initial observations by Geczy et al in Australia have shown that an antiserum to a certain isolate of Klebsiella pneumoniae is cytotoxic for lymphocytes from patients with HLA-B27 positive AS ($AS^+B27^+$) but not from HLA-B27 negative patients ($AS^+B27^-$) or HLA-B27 positive or negative normal individuals ($AS^-B27^+$, $AS^-B27^-$) (Seager et al 1979; Geczy et al 1980b). 60% of lymphocytes from patients with Reiter's syndrome were also lysed by this antiserum (Edmonds et al 1981). Further studies showed that those cells not lysed may be rendered susceptible to lysis following incubation in a cell-free culture filtrate of Klebsiella or lymphoblastoid B27 cell lines (ie, the supernatant from a Klebsiella culture or the supernatant from Epstein-Barr virus transformed lymphoblastoid cell lines). This 'modifying factor' was found to have a molecular weight of 30K daltons (Geczy, Alexander and Bashir 1980a; Sullivan et al 1982; Orban et al 1983) and that this factor which was responsible for the cross-reactivity may be produced in response to a plasmid (Cameron et al 1983). Recently Geczy et al (1986) have shown that it is possible to induce from patients' lymphocytes, cytotoxic T-lymphocytes (CTL) specific for $AS^+B27^+$ lymphoid cells or $AS^-B27^+$ cells that have been exposed to this Klebsiella culture filtrate. They have further characterised the nature of their modifying factor to be a bacterial protein (Upfold et al 1986). It is therefore possible that CTL specific for this protein, or a degraded product, and restricted to HLA-B27 may contribute to the disease process.

This cross-reactivity with $AS^{+}B27^{+}$ cells is not just restricted to Klebsiella but occurs with other enteric micro-organisms (Prendergast et al 1983, 1984; McGuigan et al 1986b). These cross-reactivity experiments have also been validated by blind studies whereby lymphocytes were investigated for their ability to be lysed without knowing whether they were HLA-B27 positive or negative. Cells from the United Kingdom, the Netherlands and New Zealand have been tested in Sydney using Geczy's Klebsiella antisera and were correctly identified as being spondylitic (Archer et al 1985; van Rood et al 1985; McGuigan et al 1986).

From their results Geczy suggested that several enteric bacteria cross-react with a specific HLA-B27 associated cell surface structure on the cells of patients with AS. Therefore a number of different species of enteric bacteria share a common factor that is related to a "modified" HLA-B27 on the cells of AS patients. Thus supporting the cross-tolerance theory of disease pathogenesis.

Also substantiating this theory are Ebringer and his colleagues who have found that serum raised to B27 positive lymphocytes cross-reacted with antigen(s) on various enteric bacteria (Welsh et al 1980) and that Klebsiella extracts had increased binding activity for mono-specific HLA-B27 tissue typing sera (Avakian et al 1980). They suggested that AS may occur as a result of immunological damage following infection by gram negative bacteria carrying antigens that are stereochemically similar to self antigens. Van Bohemen, Grumet and Zanen (1984) have found an anti-B27 monoclonal antibody, anti-B27 M1, that reacted with a protein component from Klebsiella pneumoniae

K21, K43 and Yersinia enterocolitica 9. Kono et al (1985) have produced an anti-Yersinia monoclonal antibody that reacted in a micro-lymphocytotoxicity assay with B27 positive lymphocytes. Only recently, Ogasawara, Kono and Yu (1986) observed positive reactions with 80K and 60K dalton antigens from Klebsiella and a monoclonal antibody, HLA-B27 M2. Thus, these researchers have again supported evidence for mimicry between enteric bacteria and HLA-B27 antigens.

The evidence provided by Geczy and Ebringer for the involvement of enteric bacteria in AS is of major importance but there has been difficulty, by other groups, in attempting to reproduce their experimental findings. Many have used the $^{51}Cr$ chromium lymphocytotoxicity assay, using serum raised to Klebsiella and E.coli, with negative results (Table 3).

Because of the lack of reproducibility in the $^{51}Cr$ release assay Trapani and McKenzie (1985) attempted to demonstrate the binding of Klebsiella products to B27 positive lymphocytes by other means, such as radioactive binding and sodium dodecylsulphate-polyacrylamide gel electrophoresis and were still unable to show any specific binding. Robinson and Panayi (1983a) could also find no differences in the binding of several bacteria to AS or normal lymphocytes. In 1985 Georgopolous et al simultaneously used micro-lymphocytotoxicity, chromium release and enzyme linked immunosorbent assays and again no interaction was found.

| Study | Year | Serum Tested | Result |
|---|---|---|---|
| Archer | 1981 | Anti-Klebsiella | negative |
| Shinebaum et al | 1981 | 49 anti-Klebisella | negative |
| Beaulieu et al | 1983 | 98 anti-Klebsiella | negative |
| Singh, Milton & Woodrow | 1986 | 23 anti-Klebsiella | negative |
| Kinsella, Fritzler and Lewkonia | 1986 | Anti-Klebsiella, anti-E.coli | negative |

Table 3 $^{51}Cr$ lymphocytotoxicity in ankylosing spondylitis

If an immunologic cross-reaction between HLA-B27 and gram-negative bacterial antigens is important in the pathogenesis of the B27-associated spondylarthropathies it would be expected that antibodies formed by patients in response to such infections would have anti-B27 reactivity. Kono et al (1984) and Cavender and Ziff (1986) have rarely found lymphocyte antibodies in patients with AS, Yersinia induced arthritis, Reiter's syndrome or gram-negative bacterial infections.

Though an association between AS and enteric bacteria may be hypothetically attractive it remains to be demonstrated by consistent and reproducible results and until the original observations are independently confirmed it remains difficult to suggest that particular micro-organisms play a key role in the pathogenesis of AS. However, the possibility that antigenic cross-reactivity occurs between HLA-B27 cells and certain microbes becomes an attractive hypothesis to explain these forms of 'reactive' arthritis. Any

hypothesis seeking to explain the pathogenesis of spondylitis must take into consideration the predominance of AS in males in the 2nd-3rd decade of life, the high prevalence of HLA-B27, that not all AS patients are B27-positive and that although the B27 antigen is present on nearly all nucleated cells the main tissues affected by the spondylitic process are those connected with the spinal column. To date no pathogenic consequences of such cross-reactivity have been demonstrated and the possibility remains that the cross-reactivity between B27 and bacterial antigens may be a genuine phenomenon but of no pathogenic significance.

Bacterial cell wall structure

The study of the role that enterobacteriaceae may play in the pathogenesis of the seronegative spondarthritides should not be considered without some knowledge of the structure and antigenicity of these bacteria. By definition they are gram-negative, non-sporing rods, often motile usually by peritrichate flagella. They include bacteria that inhabit the gastrointestinal tract of man either as bowel commensals or pathogens and have many properties in common such as sharing antigens and being interrelated genetically. The bacteria may be capsulated or non-capsulated. Species, such as Klebsiella, that are capsulated form mucoid colonies. The bacterial cell is a rich source of antigenic determinants and the major antigens comprise of the macromolecules that are found on the bacterial cell surface and those that are excreted into the environment. In the few cases where the equilibrium between man and his bacterial flora is disturbed, or where man encounters a true pathogen with the consequent initiation of

infection, the nature of the bacterial surface often determines the initial event in pathogenesis.

The cell wall of gram-negative bacteria is much more complex than that of gram-positive bacteria. It comprises a thin layer of peptidoglycan (PG) next to the cytoplasmic membrane (CM). Further away is the outer membrane (OM) comprised of protein, phospholipids and lipopolysaccharide (LPS). This OM is anchored to the PG by covalently linked low molecular lipoproteins (Fig III). These molecular structures help to give each bacterial species its own antigenic determinants.

Motile bacteria such as Escherichia coli and Salmonella species possess flagellar or H antigens. These consist of protein subunits of polymers of flagellin and are immunogenic only in the polymerised form. They predominantly induce a T-independent IgM antibody response which may be long lasting.

The main gram-negative surface antigen that forms the basis of serological classification is the O-somatic antigen also known as endotoxin. This lipopolysaccharide (LPS) is located on the outer part of the OM and is present in high concentrations ($3 \times 10^6$ molecules/cell). It is heat stable and therefore retains its antigenicity and immunogenicity at high temperatures. Its structure comprises three distinct regions connected covalently - a hydrophobic lipid A region and two hydrophilic areas called the core oligosacharide and the O specific PS (or O-antigen) regions. The lipid A moiety remains fairly constant from species to species whereas

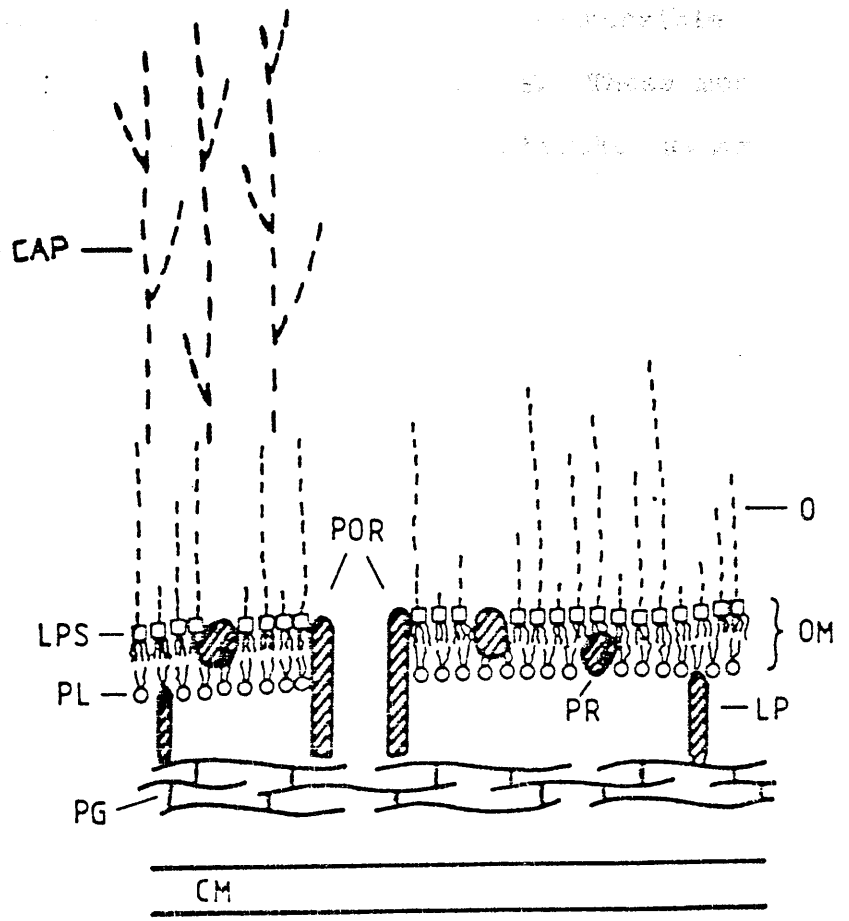



Fig III Cross-section of the envelope of a typical Gram-negative bacterium (taken from Lambert 1983).

CM, cytoplasmic membrane; PG, peptidoglycan; CAP, capsule; OM, outer membrane; LP, lipoprotein; PR protein; PL, phospholipid; LPS, lipopolysaccharide; 0,0 antigenic polysaccharide of LPS.

the core region shows more structural variability. In contrast the O-specific moiety increases dramatically in its structural variability. This part is constructed of oligosaccharide repeating units which usually consist of a short linear main chain with or without single hexose branch substituents. Therefore the potential for antigenic variability is immense.

Some organisms lack specific side chains responsible for the O specificity and are termed rough O-variants. These mutants have defects in the biosynthesis of the O polysaccharide. Ra mutants lack only the side chains while Rb-Re mutants progressively, have lost sugar constituents from the core (Fig IV).

In rough strains another antigen may be bound to the core region of the LPS and this is the enterobacterial common antigen (ECA). This is an amphiphilic glycophospholipid and is located in the OM of almost all wild type strains of enterobacteriaceae. Its expression on the cell surface varies, from being readily available to homologous antibody in non-encapsulated rough strains, to being poorly available to homologous antibody in smooth strains with complete O-antigen chains. Its structure is that of an acidic linear heteropolysaccharide with alternating 1-4 linked N-acetyl-D-glucosamine and N-acetyl-D-mannosaminuronic acid. There are two forms of the antigen - a commonly occurring and poorly immunogenic free form and a bound form which is highly immunogenic but restricted to a few rough strains where it is covalently bound to the core region of the LPS. Both forms have the same antigenic determinant based on the O-mannosaminuronic acid moiety.

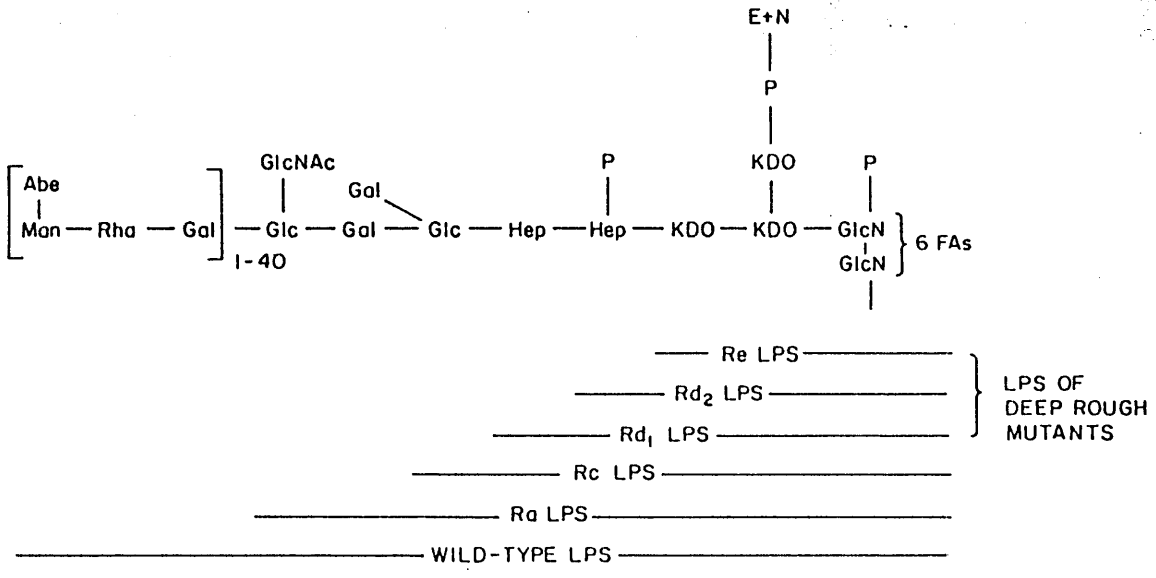



Fig IV Structure of the lipopolysaccharide (LPS) in various mutants of Salmonella typhimurium and the wild type strain (taken from Lambert 1983).

Abe, abequose; Man, D-Mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-mannooctulosonic acid)' GlcN, D-glucosamine; FA, fatty acids; P, phosphate; E+N, ethanolamine.

Enterobacteriaceae also possess K antigens that are used collectively to describe surface or capsular antigens that inhibit O agglutination. These capsular polysaccharides do not seem to be important for cell viability but provide bacteria with defence against dessication, phagocytosis and infection by bacteriophages.

Bacterial PS and LPS stimulate antibody production without any T cell mediation. T cells normally present antigenic determinants in a spatially concentrated form to the B-cell membrane. Bacterial PS have determinants that are repeated at regular intervals and are close together. Presumably this is the reason why bacterial PS do not require T cell assistance. However, large amounts of PS may interfere with the membrane alterations on the B cell, resulting in immune tolerance.

Finally, antigenic outer membrane proteins are expressed on the cell surface of enterobacteriaceae. They vary in number from species to species, eg, E.coli, having more than 24 discrete entities, but may also share common similarities between each species.

Chapter 1

## The relationship between disease activity, Klebsiella, serum immunoglobulins, C-reactive protein and erythrocyte sedimentation rate in ankylosing spondylitis.

### 1.1.1 Introduction

In 1977 Ebringer et al reported that the presence of Klebsiella pneumoniae in the stool specimens from AS patients correlated with clinical disease activity and later went on to demonstrate that patients with inactive disease, but with a positive culture for Klebsiella, subsequently developed active inflammatory disease (Ebringer et al 1978b). Patients with positive Klebsiella cultures have also been found to have higher CRP and ESR values than those with negative cultures (Cowling et al 1980a). It was suggested that the presence of Klebsiella in the gastrointestinal tract might be the stimulus for the production of active spinal inflammation in susceptible individuals. Kuberski et al (1983) have substantiated these results by finding that 54% of active AS patients carried Klebsiella compared to 12% with inactive disease and 13% of controls.

Several other groups have been unable to find an association between Klebsiella carriage and disease activity in AS (Eastmond, Cooke and Wright 1978; Calguneri et al 1981, Warren and Brewerton 1980). Hunter et al (1981) correlated increased levels of CRP in AS patients with a positive stool culture but failed to support the association between active AS and Klebsiella. An attempt to reduce Klebsiella carriage by

dietary manipulation has also failed to eliminate the organism or influence the disease symptoms (Shinebaum et al 1984). Eastmond et al (1980) found no significant increase in Klebisella carriage between active AS and controls but when acute anterior uveitis (AAU) and peripheral synovitis were taken into consideration both were related to the presence of Klebsiella in the gastrointestinal tract. Furthermore, they went on to show that there was a significant increase in the number of clinical events represented by the deterioration in spinal disease activity or peripheral synovitis in patients' faeces that were initially negative but subsequently became positive for Klebsiella (Eastmond et al 1982).

Since the classification of AS patients, according to different disease states, has varied from study to study, Cowling et al (1980a) sought to determine whether laboratory measurements such as CRP and ESR showed any correlation with disease activity in AS. Using only clinical assessment, but deliberately aiming at selecting patients with episodes of AAU, peripheral arthritis, spinal disease or all three, they divided their patients into active, probably active and inactive groups. Their results showed that the active patients had higher CRP and ESR levels than the less active patients.

Both ESR and CRP have been shown to be useful indicators of disease activity in other rheumatic diseases such as rheumatoid arthritis (Amos et al 1977) and a deterioration of disease leads to a significant increase in ESR and CRP. However, ESR and CRP alone should not be used as a disease indicator as sometimes when the ESR is high the CRP level is low and vice versa (McConkey, Crockson and

Crockson 1972).

The ESR is a non-specific test for measuring inflammation and tissue injury and is mainly determined by plasma proteins such as fibrinogen and globulins. The presence of these proteins in the blood increases the rate of red cell rouleaux formation and thus increases their rate of sedimentation (Hall and Malia 1984).

C-reactive protein appears in the blood as a response to tissue destruction and acute inflammation. It has the ability to initiate reactions of precipitation and agglutination, promotes phagocytosis and complement consumption (Pepys 1981). It is implicated to have a role in non-specific resistance to infection as well as in modulation of tissue injury and repair. It may recognise, in the plasma, potentially toxic autogenous materials released from damaged tissues, bind to them and/or facilitate their clearance. It has therefore been suggested that CRP may play a role in the mediation of the inflammatory response.

The CRP and ESR may be a useful guide to prognosis and response to treatment in RA but their importance in AS is less clear. Patients with clinically active AS do tend to have higher levels of acute phase reactants than those with inactive disease but data from Scott, Ring and Bacon (1981) suggest that, in serial studies, these measurements may be highly misleading. Vinje, Møller and Mellbye (1984) showed that CRP and ESR correlated but that CRP was associated with peripheral joint arthropathy. Laurent and Panayi (1983) found CRP was marginally raised in patients with pelvospondylitis whereas the ESR

was not, but in patients with pelvospondylitis and peripheral arthritis both CRP and ESR were significantly raised. Also, they could find no correlation of both these factors with disease activity as measured clinically. Sheehan et al (1986) also, have not been able to demonstrate a relationship between clinical disease activity, ESR and CRP in AS. The main reason for this lack of correlation being the difficulty in assessing clinical disease activity in patients with only pelvospondylitis.

Correlation of disease activity and serum immunoglobulin levels have also been studied in AS. The presence of lymphocytes and plasma cells in the articular and extra-articular lesions of patients with AS suggests that the immune system may be implicated in the pathogenesis of the disease. Several studies have set out to ascertain whether selected parameters of humoral immunity might show evidence of activation in AS. Veys and van Laere (1973) found significant increases in levels of serum IgA, G and M in AS compared to controls, whereas Kinsella, Espinoza and Vasey (1975) could only find an increase in IgA. The findings of Veys and van Laere could not be correlated with peripheral joint involvement or disease activity. Reports by Nikbin et al (1975) showed raised IgA but without an increase in circulating IgA-bearing lymphocytes suggesting that the IgA is being derived from B cells, not in the bone marrow or lymph nodes, but fixed in the tissues in the gut.

Following these reports of raised IgA levels, Cowling, Ebringer and Ebringer (1980b) looked at the effect that inflammation had on serum IgA levels in AS. They divided their patients into active and

inactive groups based on their ESR and CRP levels and found that IgA and IgG were raised in the active patients when compared to the inactive group or controls. They suggested that because inflammation occurs along the axial skeleton, peripheral joints and eye tissue and that IgA is mainly produced within the mucosa of the gastrointestinal (GI) tract, there must be some external triggering factor active across a mucosal surface, such as the GI tract, initiating a rise in IgA. Similar increases in serum immunoglobulins have been found by other workers (Calguneri et al 1981; Hickling, Turnbull and Dixon 1982) and Laurent and Panayi (1983) have shown raised levels of IgA irrespective of whether there was iritis or peripheral arthritis involved whereas IgG levels were only raised in those AS patients with both of these two features. Sanders et al (1987), however, have found raised IgA levels in AS but these did not correlate with CRP levels.

Recently Franssen, van de Putte and Gribnau (1985) investigated the possible effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the association between serum IgA and disease activity. Absolute serum IgA levels correlated positively with changes in disease activity and these levels also decreased during NSAID therapy. Since changes in IgA paralleled changes in disease activity the observations suggest that NSAIDs may have a disease modifying activity. The ESR also decreased during treatment although it has been reported that NSAIDs do not influence acute phase reactants such as ESR and CRP (McConkey et al 1973).

### 1.1.2 Aims of the Study

In the light of conflicting reports of the association of Klebsiella and disease activity in AS this study set out to investigate the isolation frequency of Klebsiella in AS and healthy controls and whether this was affected by the disease state of the patient.

Acute phase reactants and serum immunoglobulins have also been reported to be increased in AS suggesting an activated humoral immune response. In this respect the study sought to investigate the effect that disease activity had on serum immunoglobulins, CRP and ESR.

## 1.2 Materials and Methods

### 1.2.1 Patient groups

For the study of Klebsiella carriage, 64 patients with AS attending the rheumatology clinic were compared to 34 normal controls. The clinical data of patients and controls are shown in Table 4. The controls were friends and/or relatives of hospital staff and community controls from a general practitioners list. None were attending hospital as out-patients.

The patients' disease activity was assessed by a rheumatologist using the following criteria -

| | AS | Controls |
|---|---|---|
| No. patients | 64 | 34 |
| Mean age (years) : (range) | 41 (20-68) | 32 (11-57) |
| Sex* | M = 53 F = 11 | M = 15 F = 19 |
| Duration of disease (years) | 16 (2-45) | NA |
| HLA-B27 positive | 54 (5ND) | ND |
| PJI | 42 | NA |
| UV | 12 | NA |
| PJI + UV | 9 | NA |

Table 4 Clinical data of patients and controls

* M = male F = female
NA - not applicable
ND - not done
PJI - peripheral joint involvement
UV - present or previous uveitis

Active (A) - synovitis of peripheral joints or morning stiffness > 30 mins or uveitis or ESR > 30mm/hr

Probably active (PA) - back pain with stiffness and requiring regular NSAID therapy

Inactive (IN) - requiring occasional analgesic/NSAID therapy

### 1.2.2 Isolation of Klebsiella species

Rectal swabs were obtained from patients and controls using sterile cotton-tipped applicators. Direct cultures were made onto CLED (cysteine lactose electrolyte deficient) agar and Simmond's citrate agar with 1% inositol (SCAI). SCAI is selective for the recovery of Klebsiella species (van Kregten, Westerdaal and Willers 1984). Pure colonies were identified by the API 20E system.

### 1.2.3 Measurement of serum immunoglobulins, C-reactive protein and erythrocyte sedimentation rate

Serum immunoglobulin levels were determined using an immuno-turbidimetry technique on an Encore centrifugal analyser (Baker Instruments).

The CRP levels were measured by fluorescent polarisation on an Abbot TDX Analyser. Both serum imunoglobulin and CRP levels were performed by the Biochemistry Department.

The ESR was measured, using the Westergren method, by the routine haematology service.

### 1.2.4 Statistical analysis

Statistical analysis of the results of this and other chapters was performed using non-parametric methods, detailed in Appendix A.

## 1.3 Results

### 1.3.1 Isolation of Klebsiella species from ankylosing spondylitis patients and controls

Positive cultures for Klebsiella species were found from 2 (9.1%) out of 21 patients with active disease, 1 (4.3%) out of 23 patients with probably active disease and from 1 (5.3%) out of 20 patients with inactive disease. The control rate for Klebsiella isolation was 8.8% and those patients with peripheral joint involvement had Klebsiella isolated on 3 (7.5%) out of 37 occasions (Table 5).

Statistical analysis (Table 6), using the Chi-square test (incorporating Yates's correction) showed no significant difference in the isolation rate of Klebsiella between any of the groups whether there was active disease or peripheral joint involvement.

### 1.3.2 Comparison of serum immunoglobulins, C-reactive protein and erythrocyte sedimentation rate with disease activity in ankylosing spondylitis patients

All results were analysed using the Wilcoxon Rank Sum test.

The ESR was measured in 20 inactive, 22 probably active and 21 active patients. The results are values from individual patients.

The mean ESR in active patients was 42.9 $\pm$ 7.3mm/hr (mean $\pm$ SEM), in probably active patients it was 26.4 $\pm$ 6.5mm/hr and in inactive

| | A | PA | IN | C | PJI | NO PJI |
|---|---|---|---|---|---|---|
| Culture positive | 2 | 1 | 1 | 3 | 3 | 1 |
| (%) | (9.1) | (4.3) | (5.3) | (8.8) | (7.5) | (4.8) |
| Culture negative | 20 | 22 | 18 | 31 | 37 | 20 |
| Total | 22 | 23 | 19 | 34 | 40 | 21 |

Table 5 Isolation rate of Klebsiella species from ankylosing spondylitis patients and controls

PJI - peripheral joint involvement

| | $X^2$ | Significance |
|---|---|---|
| Active vs Inactive | 0.22 | NS |
| Active vs Probably active | 0.406 | NS |
| Active vs Controls | 0.001 | NS |
| Probably active vs Inactive | 0.019 | NS |
| Probably active vs Controls | 0.421 | NS |
| Inactive vs Controls | 0.221 | NS |
| All patients vs Controls | 0.191 | NS |
| Patients with PJI vs patients without PJI | 0.168 | NS |

Table 6 Comparison of disease activity and isolation of Klebsiella

patients it was 15.0 ± 3.2mm/hr (Fig V). The active patients had significantly raised ESR levels compared with the inactive ($p < 0.005$) and probably active groups ($p < 0.05$). There was no significant difference between the inactive and probably active patients.

The CRP was measured in 20 inactive, 20 probably active and 23 active patients. The mean CRP level in inactive patients was 16.0 ± 3.8µg/ml, in probably active patients 25.2 ± 6.0µg/ml and in active patients 27.1 ± 5.1µg/ml (Fig VI). Active patients had significantly raised CRP values when compared to inactive patients ($p < 0.05$) but there was no differences between any of the other groups.

Measurements of IgA were performed on serum samples from 20 inactive, 20 probably active and 25 active patients. The mean levels were, for inactive patients 2.9 ± 0.3g/l, for probably active patients 3.0 ± 0.3g/l and for active patients 3.2 ± 0.2g/l (Fig VII). There was no significant difference between any of the groups.

The IgG and IgM levels were measured in serum samples from 20 inactive, 20 probably active and 24 active patients (Figs VIII and IX). The mean IgG and IgM levels were, respectively, in inactive patients 11.9 ± 0.4g/l and 1.5 ± 0.3g/l, in probably active patients 13.2 ± 0.9g/l and 1.8 ± 0.3g/l and in active patients 14.8 ± 1.1g/l and 1.8 ± 0.3g/l. Again, there was no significant difference between any of the groups.

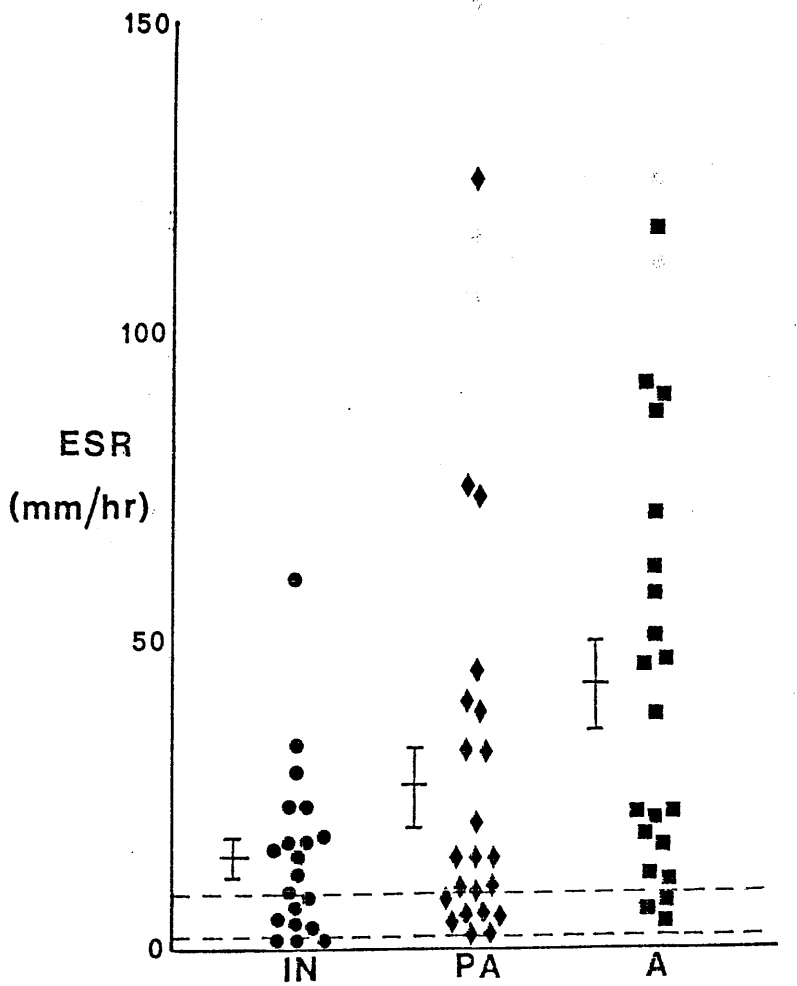



Fig V ESR in ankylosing spondylitis patients with inactive (IN), probably active (PA) and active (A) disease.

Bars indicate mean ± SEM.
Area between dashed lines indicates the normal range.

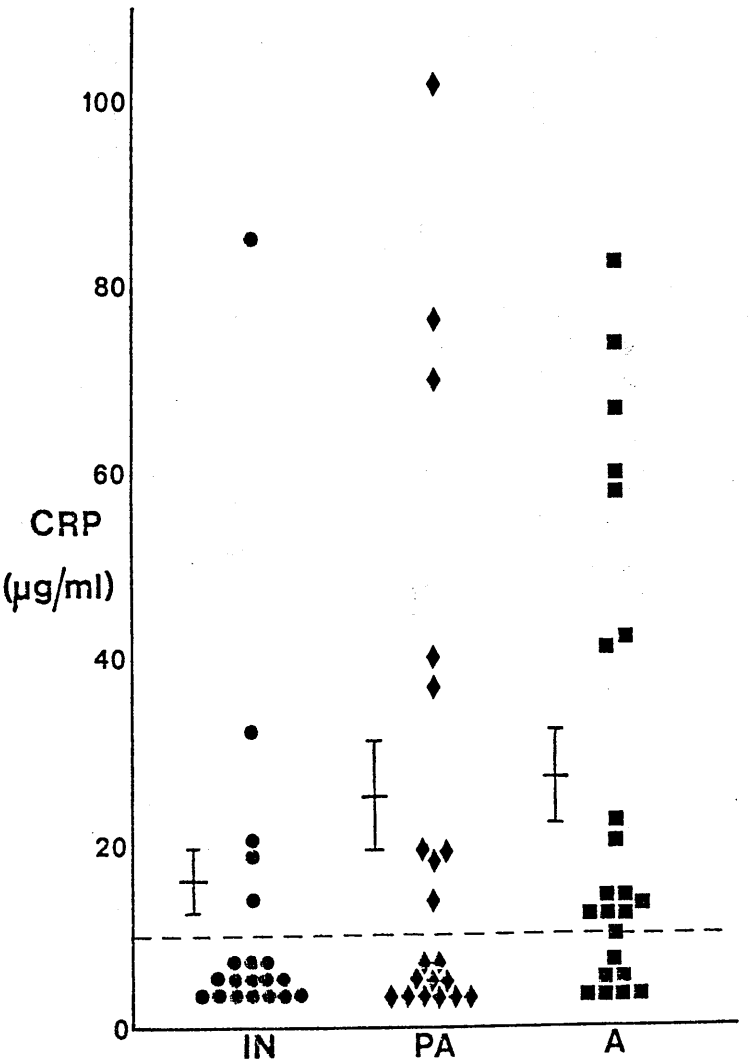



Fig VI Serum CRP levels in ankylosing spondylitis patients with inactive (IN), probably active (PA) and active (A) disease.

Bars indicate mean ± SEM.
Area below the dashed line indicates the normal range.

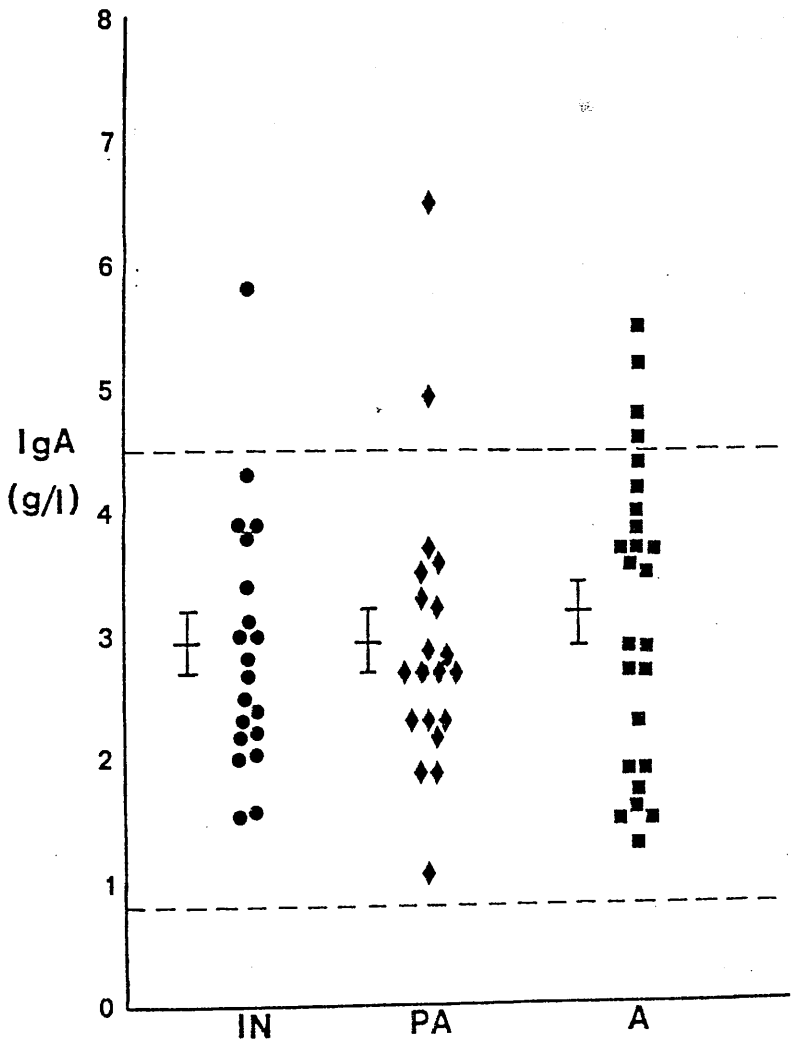



Fig VII Serum IgA levels in ankylosing spondylitis patients with inactive (IN), probably active (PA) and active (A) disease.

Bars indicate mean ± SEM.
Area between the dashed lines indicates the normal range.

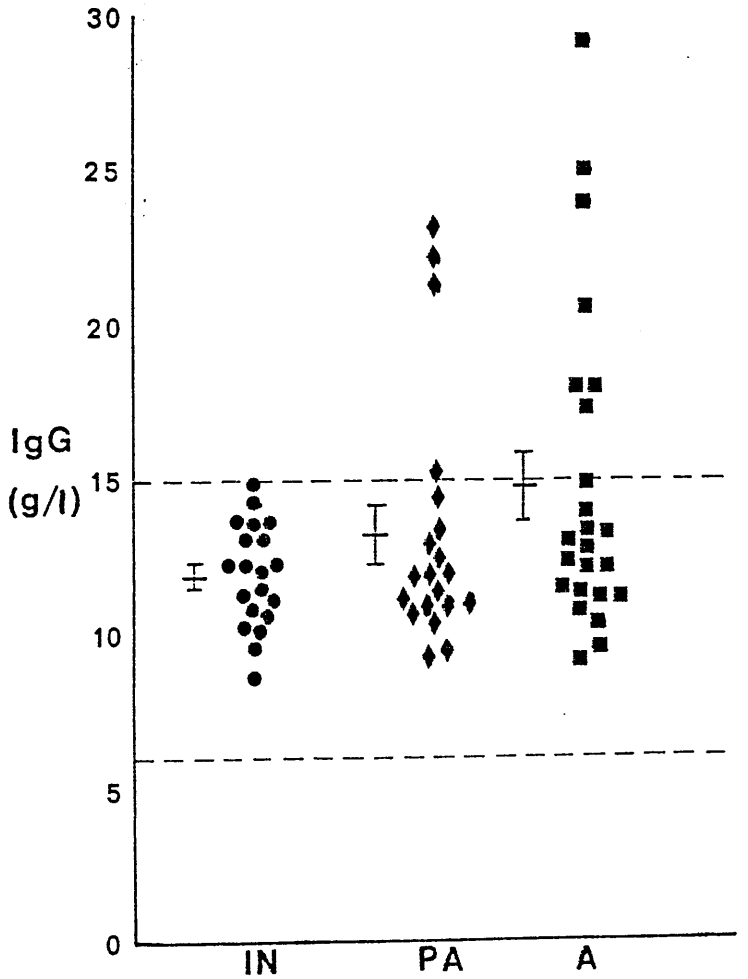



Fig VIII Serum IgG levels in ankylosing spondylitis patients with inactive (IN), probably active (PA) and active (A) disease.

Bars indicate mean ± SEM.
Area between the dashed lines indicates the normal range.

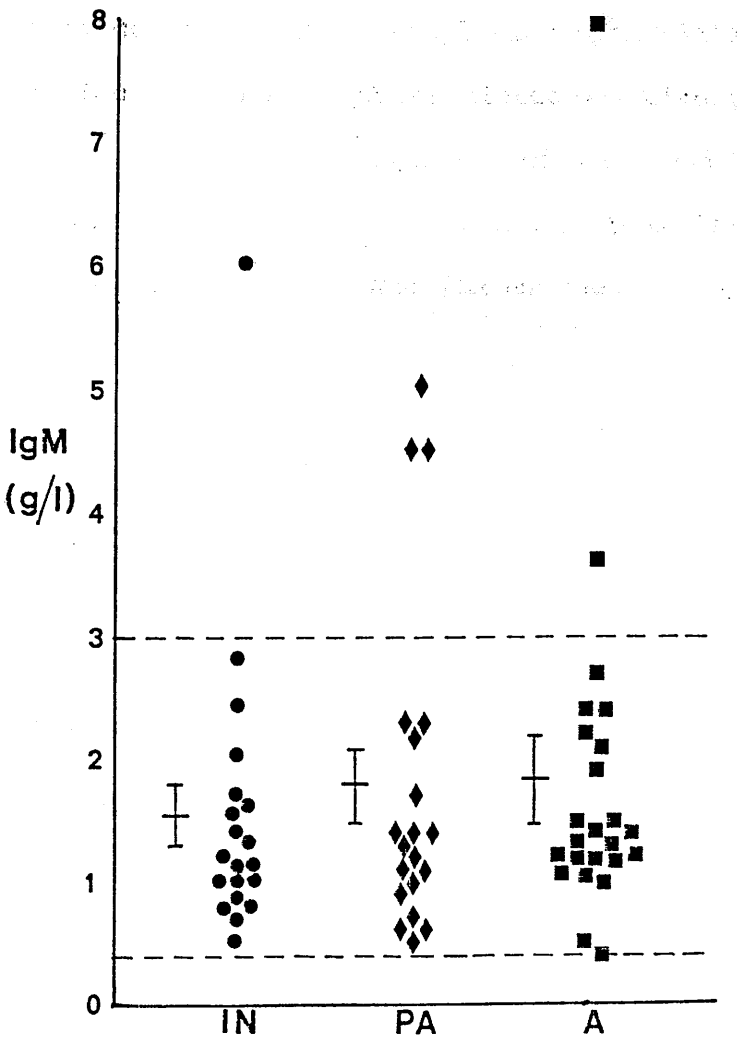



Fig IX Serum IgM levels in ankylosing spondylitis patients with inactive (IN), probably active (PA) and active (A) disease.

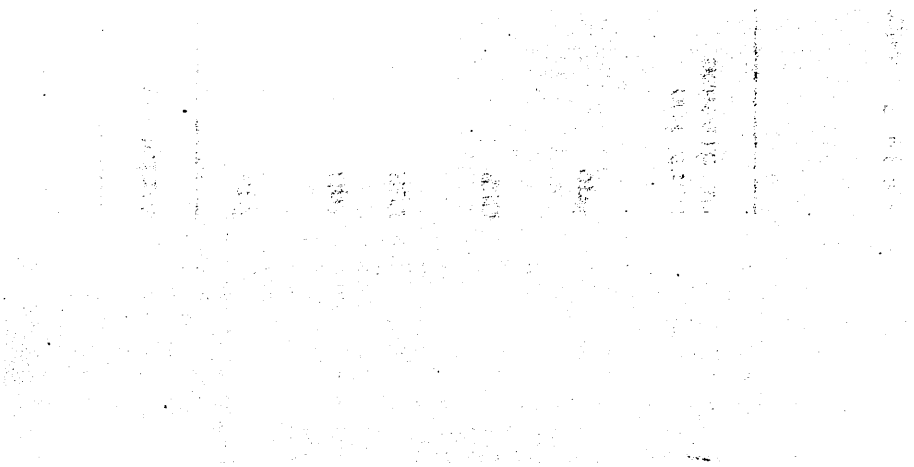
Bars indicate mean ± SEM.
Area between the dashed lines indicates the normal range.

### 1.3.3 Correlations between serum immunoglobulins, erythrocyte sedimentation rate, C-reactive protein, age and duration of disease in ankylosing spondylitis

The matrix of correlations in ankylosing spondylitis patients is presented in Table 7. Serum IgA correlated positively with IgG, ESR and CRP. IgG also correlated positively with ESR and CRP. There was a negative correlation between IgM and the age of the patient and a strong positive correlation between ESR and CRP.

### 1.3.4 Comparison of patients with and without peripheral joint involvement

Serum immunoglobulins, ESR and CRP were measured in patients with and without peripheral joint involvement. The results are expressed in Table 8. Serum IgA and IgG, ESR and CRP levels are not affected by peripheral joint involvement but serum IgM is significantly raised in these patients.



| Variables | IgA | IgG | IgM | ESR | CRP |
|---|---|---|---|---|---|
| IgG | $p < 0.005$ (0.365) | | | | |
| IgM | NC | NC | | | |
| ESR | $p < 0.001$ (0.465) | $p < 0.001$ (0.586) | NC | | |
| CRP | $p < 0.002$ (0.417) | $p < 0.001$ (0.480) | NC | $p < 0.001$ (0.618) | |
| Age | NC | NC | $p < 0.05$ (-0.273) | NC | NC |
| Duration of disease | NC | NC | NC | NC | NC |

Table 7 Matrix of correlations in ankylosing spondylitis patients (r values in parenthesis)

NC - no correlation

| Variable | Patients with peripheral joint involvement | Remaining patients | Significance |
|---|---|---|---|
| IgA (g/l) | 3.1 ± 0.2 (n = 35) | 3.0 ± 0.1 (n = 25) | NS |
| IgG (g/l) | 14.4 ± 0.8 (n = 35) | 12.4 ± 0.6 (n = 24) | NS |
| IgM (g/l) | 2.1 ± 0.3 (n = 35) | 1.3 ± 0.1 (n = 24) | $p < 0.02$ |
| ESR (mm/hr) | 29.3 ± 4.4 (n = 35) | 30.5 ± 7.4 (n = 24) | NS |
| CRP (µg/ml) | 24.1 ± 4.3 (n = 34) | 23.2 ± 4.6 (n = 25) | NS |

Table 8 Serum immunoglobulins, CRP and ESR levels in ankylosing spondylitis patients with and without peripheral joint involvement (mean ± SEM)

NS - no significance

## 1.4 Discussion

The results from this study demonstrate that Klebsiella species are not isolated more frequently from AS patients with or without active disease when compared to normal community controls and that peripheral arthritis does not affect the carriage rate. However, the faecal carriage rates are lower overall than previously reported studies (Ebringer et al 1977; Eastmond et al 1978). As there was no increase in Klebsiella carriage caution should be attributed to the suggestion, from others, that intestinal carriage of Klebsiella might be an aetiological factor in AS. There are many problems in studying the association of Klebsiella with disease activity in AS. Klebsiella carriage is seasonal and is increased by eating fresh uncooked foods. Carriage is also increased in the hospital population so any controls must come from outwith the hospital environment. Klebsiella may also persist in the bowel for months so repeated sampling of one group and not the other may introduce bias, therefore, each patient and control should be sampled only once. In this study patients were only within contact of the hospital environment once every several months and controls were not associated with a hospital environment and samples were taken once from each person in both groups.

No centre has been able to show a statistically significant increase in Klebsiella isolation from the total number of AS patients studied when compared to the controls. The interpretation of their findings depended on the division of AS patients into active, probably active and inactive disease categories. However, there does not seem to be a consensus of opinion amongst clinicians as to how 'active' spondylitis

is defined. Does it include those with acute anterior uveitis and/or peripheral arthritis and does one include laboratory parameters such as CRP and ESR?

This study found raised levels of CRP and ESR in active AS patients, which has been reported by other groups. These increases have been said to correlate with the presence of peripheral arthritis and/or uveitis and are therefore not a true indicator of active pelvospondylitis. This study could not find a correlation of either CRP or ESR with peripheral joint involvement and there were not enough patients with uveitis to make a comparison. The raised levels of CRP and ESR in active patients from this investigation were not due to peripheral arthritis and so CRP and ESR may be a good indicator of disease activity.

The measurement of serum immunoglobulins in AS patients was also included in the study. No differences in levels of IgA, IgG or IgM were found between any of the three disease activity groups suggesting that there is no relationship between the activity of the disease and an increased humoral immune response in these patients. Taking the AS patients as one group, without the division into disease states, research has shown an increase in serum IgA levels but there is a discrepancy as to whether this is due to the presence of peripheral arthritis. Again I found no relationship between peripheral joint involvement and serum IgA or IgG but IgM levels were raised in patients with peripheral arthritis. This could indicate a recent infection in these patients but if the trigger was gut initiated one would expect an increase in IgA levels. Peripheral joint involvement

could therefore be a completely separate phenomenon from the spinal disease in AS.

Some workers have used CRP and ESR alone as a measure of disease activity (Cowling et al 1980a) and by doing so have found raised serum IgA levels in those who have raised ESR and CRP levels. They therefore suggested that serum IgA could be used as another measure of disease activity in AS. Although this study found a positive correlation between IgA, CRP and ESR there was no difference between IgA levels in the disease activity groups as assessed clinically. This suggests that although within each group an increase in IgA corresponds to an increase in CRP and ESR the actual rise in IgA levels is not large enough to make a difference between the groups as seen with CRP and ESR. Dividing the patients into disease activity groups according to ESR and CRP levels, and not clinical assessment, may give a misleading interpretation of increased serum IgA in active patients when in fact IgA does not correlate with disease activity.

In summary these results have not shown a high gastrointestinal carriage rate of Klebsiella species in ankylosing spondylitis patients whether these patients have clinically active disease or peripheral joint involvement. There is therefore no evidence to suggest that these patients are more susceptible to colonisation of the gut by Klebsiella and suggestions that this bacteria may cause the pathogenesis of ankylosing spondylitis should be treated with scepticism. The ESR and CRP, but not serum immunoglobulin levels, may be helpful as indicators of disease activity as they are not affected by peripheral joint involvement and are raised in clinically active

patients. They should, however, only be used along with clinical assessment.

Chapter 2

## Anti-Klebsiella lymphocytotoxicity in ankylosing spondylitis

### 2.1.1 Introduction

The cross tolerance hypothesis, to explain the pathogenesis of ankylosing spondylitis, proposes that the HLA molecule itself stereochemically resembles an antigen(s) found on some external agent such as gram-negative bacteria (Ebringer et al 1985a). If there is a partial cross-reactivity between HLA antigens or cell surface markers and viruses or bacteria, then infections by such micro-organisms may lead to the production of antibodies that will have auto-immune as well as anti-microbial activity.

The pathogenesis of several arthritic disorders that are grouped as the spondyloarthropathies still remains unclear although in the case of Reiter's syndrome the development of arthritis is very often preceded by a symptomatic episode of infection. In contrast, the development of AS is insidious and is not preceded by obvious infective episodes.

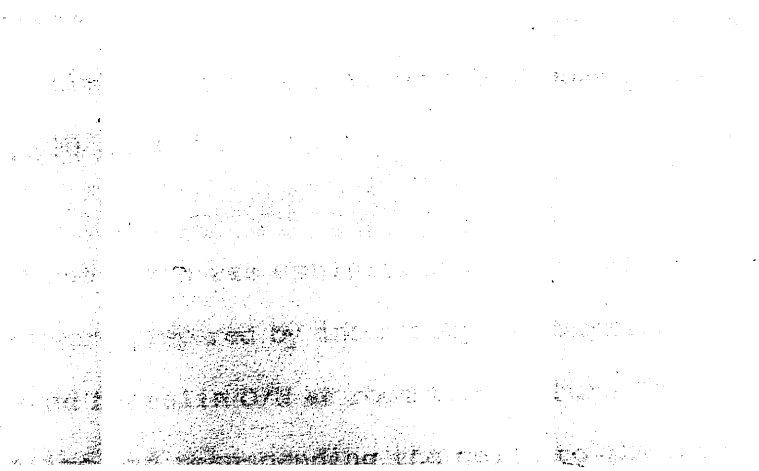
The genus Klebsiella has been strongly implicated in AS (Ebringer et al 1978; Geczy et al 1983) and with more than 90% of AS patients possessing the HLA-B27 phenotype research has endeavoured to find cross-reactivity between Klebsiella antigens and the HLA-B27 antigen. This was done by immunising rabbits with either Klebsiella cell suspensions or B27 positive lymphocytes and the sera obtained allowed

to interact with lymphocytes from AS patients or various bacterial species respectively. Antibodies in the serum, derived from one particular Klebsiella pneumoniae strain, reacted with lymphocytes from B27 positive patients and B27 lymphocytes from normal individuals, if these lymphocytes were first incubated in vitro with supernatants from Klebsiella cultures (Geczy et al 1980). From this data it was concluded that cell from AS patients are modified in situ by bacteria.

A full description of these studies has been reviewed earlier (main introduction).

### 2.1.2 Aims of the study

The aims of this study were (1) to confirm, or otherwise, any cross-reactivity between lymphocytes from AS patients and antisera prepared against Klebsiella species and (2) if there was cross-reactivity, to determine if the cytotoxicity could be transferred in vitro.



## 2.2 Materials and methods

### 2.2.1 Patient groups

20 patients with ankylosing spondylitis and 21 normal healthy laboratory staff were included in the study. Of the AS patients 18 were HLA-B27 positive, 3 of the controls were HLA-B27 positive.

The patients and controls were tissue-typed by the Department of Clinical Immunology.

### 2.2.2 Preparation of bacterial antigens

Klebsiella pneumoniae K43 (NCTC 9163) was used as this was the strain initially reported as being cross-reactive with HLA-B27 PBL (Geczy et al 1980). Three different types of bacterial antigen were prepared - formalin treated whole cells (FT), heat treated whole cells (HT) and the supernatant from heat treated cells (HTS).

Several loopfuls of bacteria were inoculated into 10ml of brain heart infusion broth and incubated for 4 hours at 37°C in an orbital incubator. 0.1ml was then spread onto CLED agar plates and incubated overnight (O/N) at 37°C.

Growth from one plate was emulsified into 5ml of saline. Formalin killed cells were prepared by incubating the bacterial cell suspension in 1% buffered formalin O/N at room temperature (RT), washing three times in saline and resuspending the cells to the original volume.

Heat killed cells were prepared by incubating the bacteria in a Koch steamer (100°C)for 2 hours. After centrifugation (1540g, 15 mins) the supernatant was decanted off and kept as the HTS antigen and the pellet washed and resuspended to the original volume (HT antigen).

### 2.2.3 Antiserum production

New Zealand white rabbits were immunised intravenously with 0.25ml of each antigen preparation on day 0 and thereafter on days 4, 7, 11 and 14 with 0.5ml, 0.75ml, 1.0ml and 1.0ml respectively. The animals were bled at day 21. A pre-immunisation blood sample was also taken on day 0. The presence of anti-Klebsiella antibodies in the sera was confirmed by haemagglutination. Titres for each serum being >1000HU.

HLA-B27 antiserum was obtained from Behring Diagnostics (a division of Hoechst UK Ltd).

### 2.2.4 Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) were obtained from 17 B27 positive and 3 B27 negative ankylosing spondylitis patients ($AS^+B27^+$, $AS^+B27^-$) and from 3 B27 positive and 17 B27 negative normal individuals ($AS^-B27^+$, $AS^-B27^-$). The cells were separated from heparinised whole blood by centrifugation at 358g for 30 minutes on lymphoprep (Nyegaard, Norway). The PBL interface was removed and washed three times with RPMI 1640 medium (Flow, Scotland) containing 10ml HEPES buffer (Sigma), 5ml L-glutamine (200mM) and 5ml penicillin-streptomycin (500iu, 5,000µg/ml; Gibco) per 500ml RPMI medium. The cells were then resuspended in RPMI containing 10% human pooled serum (hps) to a

concentration of $10^7$/ml.

### 2.2.5 Lymphocytoxicity assay

1ml of PBL was labelled with 100µCi (0.5ml) of $^{51}Cr$ sodium chromate (380mCi/mg, Amersham) for 60 minutes at 37°C. The cells were then washed and resuspended in RPMI with 10% hps to a concentration of 3 x $10^6$/ml. 25µl of the $^{51}Cr$-labelled cell suspension was added to round bottomed microtitre plates (Sterilin) in triplicate with 25µl of antiserum and incubated at RT for 30 minutes. Next, 150µl of neat rabbit complement (Buxted Rabbit Company) (see Section 2.3.1) was added to each well and incubation continued for a further 60 minutes. Both antiserum and complement were at room temperature before the addition to the PBL. After centrifugation of the plates at 127g for 5 minutes the supernatants were harvested by a Titertek harvester press and the radioactivity of the samples counted on a Packard gamma counter (A500C).

The amount of radioactivity in the samples was compared to the radioactivity present in the wells containing cells plus complement only and with that in the wells with cells lysed by the addition of 150µl sodium dodecyl sulphate (SDS). The results are expressed as the percentage of maximum $^{51}Cr$ released, which was calculated as follows:

$$\% \ ^{51}Cr \text{ release} = 100 \times \frac{\text{Radioactivity released by antiserum} - \text{Radioactivity released in absence of antiserum}}{\text{Radioactivity released by SDS solubilised cells} - \text{Radioactivity released in absence of antiserum}}$$

## 2.3 Results

### 2.3.1 Effect of complement on the lymphocytotoxicity assay

Initial experiments did not result in a positive reaction between HLA-B27 antiserum and HLA-B27 positive lymphocytes. It was considered that this may be due to the lack of complement in the assay. A dose response assay was therefore set up to find the optimal volume of complement that was required. 25µl and 50µl of neat complement produced no $^{51}Cr$ release. 125µl, 150µl and 175µl gave a $^{51}Cr$ release of 16%, 38% and 38% respectively. A volume of 150µl was subsequently used in the experiments.

### 2.3.2 Lymphocytotoxicity of antibodies to formalin treated Klebsiella

Treating bacteria with formalin will kill the cell, without destroying its antigenicity, by the formation of crosslinks between proteins. This means the cell surface antigens are kept intact and will still be able to evoke an immune response. Antiserum raised to formalin treated bacteria will therefore produce antibodies to the major cell surface components.

In each experiment, the sera were titrated to look for any prozone effect, ie, where higher concentrations of serum produce no reaction and more dilute serum allows the reaction to take place (Figure X). For each separate serum dilution there was no significant difference between AS and control lymphocytotoxicity (Table 9). The serum did, however, show a prozone effect. The highest mean result occurring at a dilution of 1/4 for both groups.

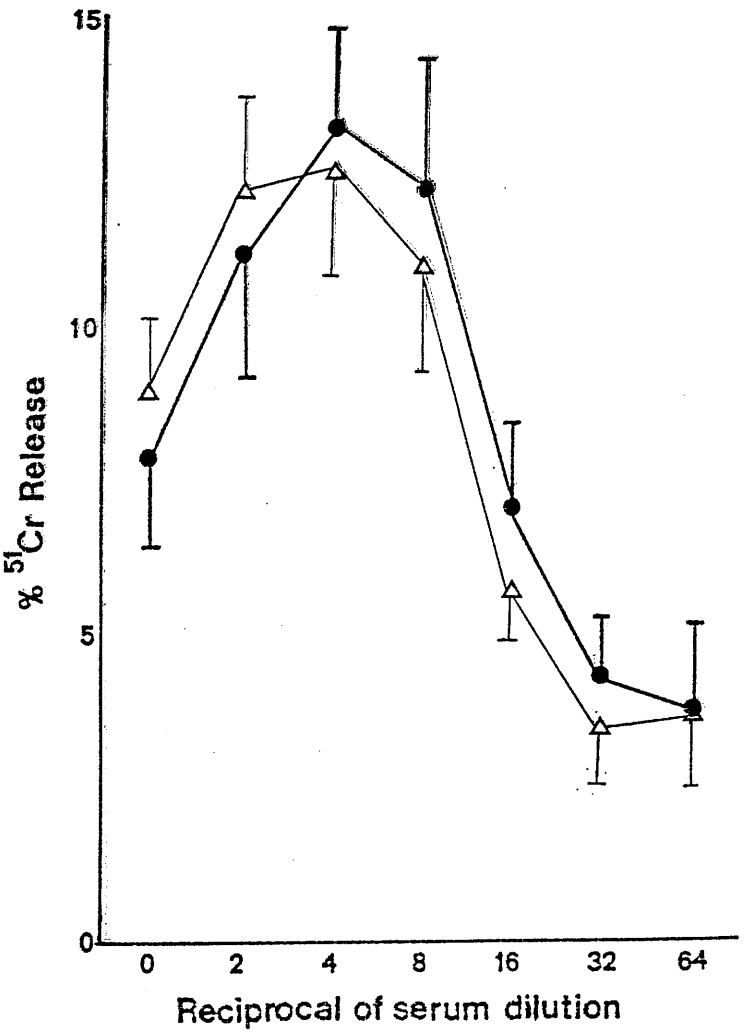



Fig X Mean (+ SEM) lymphocytotoxicity of dilutions of antiserum to formalin treated Klebsiella pneumoniae K43 against peripheral blood lymphcoytes of ankylosing spondylitis (AS) patients and normal controls (C).

● = AS △ = C

| Serum dilution | | Undiluted | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | Highest single result |
|---|---|---|---|---|---|---|---|---|---|
| Ankylosing spondylitis | n | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| | mean | 7.9 | 11.2 | 13.4 | 12.3 | 7.2 | 4.4 | 4.0 | 18.6 |
| | SEM | 1.4 | 2.0 | 1.7 | 2.2 | 1.4 | 1.0 | 1.3 | 1.9 |
| Controls | n | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| | mean | 9.0 | 12.2 | 12.6 | 11.1 | 5.9 | 3.6 | 3.9 | 15.9 |
| | SEM | 1.2 | 1.6 | 1.7 | 1.7 | 1.0 | 0.9 | 1.3 | 1.8 |
| Significance | | NS | NS | NS | NS | NS | NS | NS | NS |

Table 9 Percentage lymphocytotoxicity of antiserum to formalin treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis patients and normal controls.

The results for the lymphocytotoxicity by antibodies to formalin treated bacteria are shown in Figure XI. When comparing the highest result, from each serum titration, there was no significant difference in lymphocytotoxicity from AS patients and normal controls.

HLA-B27 antiserum was used as indicated by the accompanying instructions. Pre-immunisation rabbit serum lymphocytotoxicity in AS patients and normal controls was 10.2 $\pm$ 1.6% and 10.7 $\pm$ 3.1% respectively. The difference between the two groups was not significant.

### 2.3.3 Lymphocytotoxicity of antibodies to heat treated Klebsiella

Heat treatment of the bacterial cell will denature its surface proteins and antiserum prepared against such a preparation will include antibodies to the major polysaccharides and lipopolysaccharides such as the O somatic antigen. A difference in cytotoxicity of antiserum to formalin treated bacteria and heat treated cells may be able to determine whether or not the cross-reacting antibody is derived from a heat labile component such as a protein.

No significant difference was found between AS patients and controls for each separate serum dulution (Table 10).

The serum did show a slight prozone effect but not as pronounced as the antiserum to formalin treated cells (Figure XII).

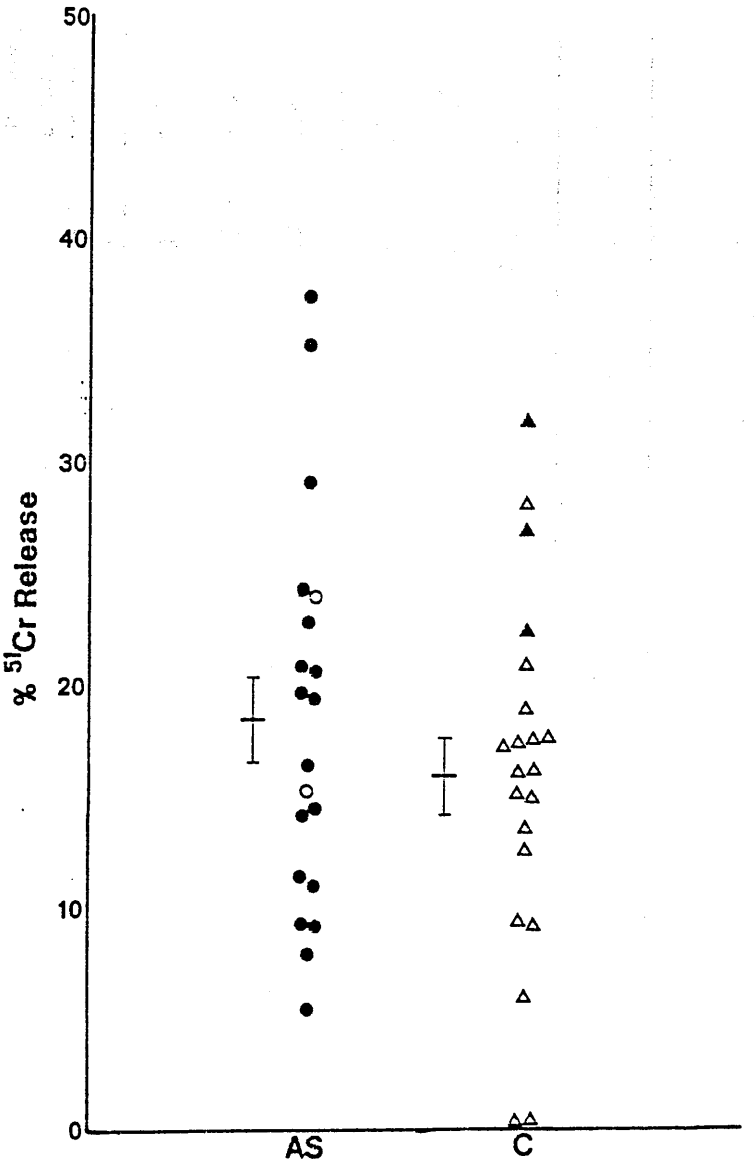



Fig XI Lymphocytotoxicity of antiserum to formalin treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis (AS) patients and normal controls (C).

Each point represents the highest result from each group of serum dilutions. Bars inidicate mean ± SEM.

● = $AB^{+}B27^{+}$ ▲ = $AS^{-}B27^{+}$
O = $AS^{+}B27^{-}$ △ = $AS^{-}B27^{+}$

| Serum dilution | | Undiluted | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | Highest single result |
|---|---|---|---|---|---|---|---|---|---|
| Ankylosing spondylitis | n | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| | mean | 3.8 | 5.2 | 5.6 | 4.5 | 4.1 | 3.1 | 4.1 | 8.9 |
| | SEM | 1.0 | 1.3 | 1.6 | 1.3 | 0.8 | 0.6 | 0.9 | 1.6 |
| Controls | n | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| | mean | 4.9 | 5.6 | 5.6 | 5.6 | 4.2 | 3.6 | 3.9 | 11.5 |
| | SEM | 1.4 | 1.8 | 1.4 | 1.3 | 0.9 | 0.8 | 1.1 | 1.8 |
| Significance | | NS | NS | NS | NS | NS | NS | NS | NS |

Table 10 Percentage lymphocytotoxicity of antiserum to heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis patients and normal controls.

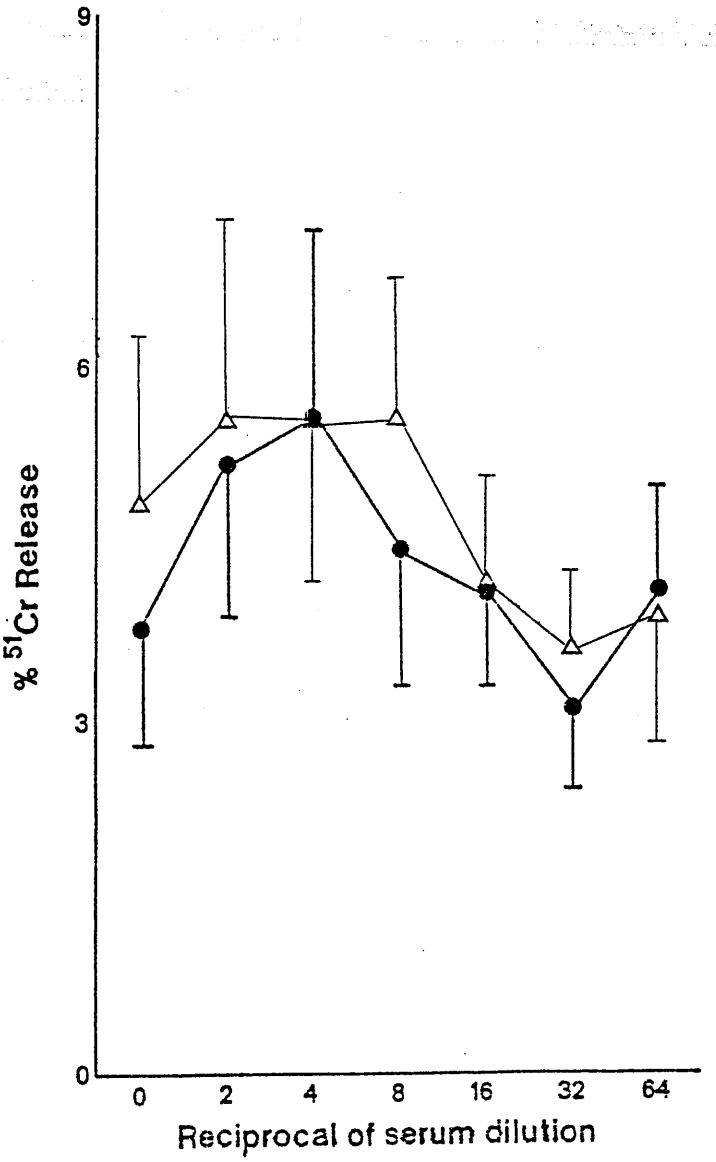



Fig XII Mean (± SEM) lymphocytotoxicity of dilutions of antiserum to heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes of ankylosing spondylitis (AS) patients and normal controls (C).

● = AS Δ = C

Taking the highest result from each titration, again no difference was seen between AS patients and controls (Figure XIII).

### 2.3.4 Lymphocytotoxicity of antibodies to the supernatant of heat treated bacteria

Supernatant fluids from heated suspensions of bacteria serve as a crude preparation of enterobacterial common antigen (ECA) (Ramia et al 1983). Klebsiella possesses ECA (Arburhnott, Owen & Russell 1984) although along with this antigen in the supernatant one will also find the O somatic antigen. Antiserum to the supernatant used in the assay will show if either of these two antigens produce cross-reacting antibodies.

There was no significant difference in percentage $^{51}Cr$ release between AS patients and controls (Table 11) nor was there any prozone effect (Figure XIV). There was also no differences between patients and controls when comparing the highest result from each titration (Figure XV).

### 2.3.5 Comparison of lymphocytotoxicity of B27 positive and B27 negative cells

AS patients and controls were divided into two groups depending on whether their PBL were B27 positive of B27 negative.

The lymphocytoxicity of HLA-B27 antiserum is shown in Figure XVI. The mean $^{51}Cr$ release from the B27 positive and negative groups is 47.8 $\pm$

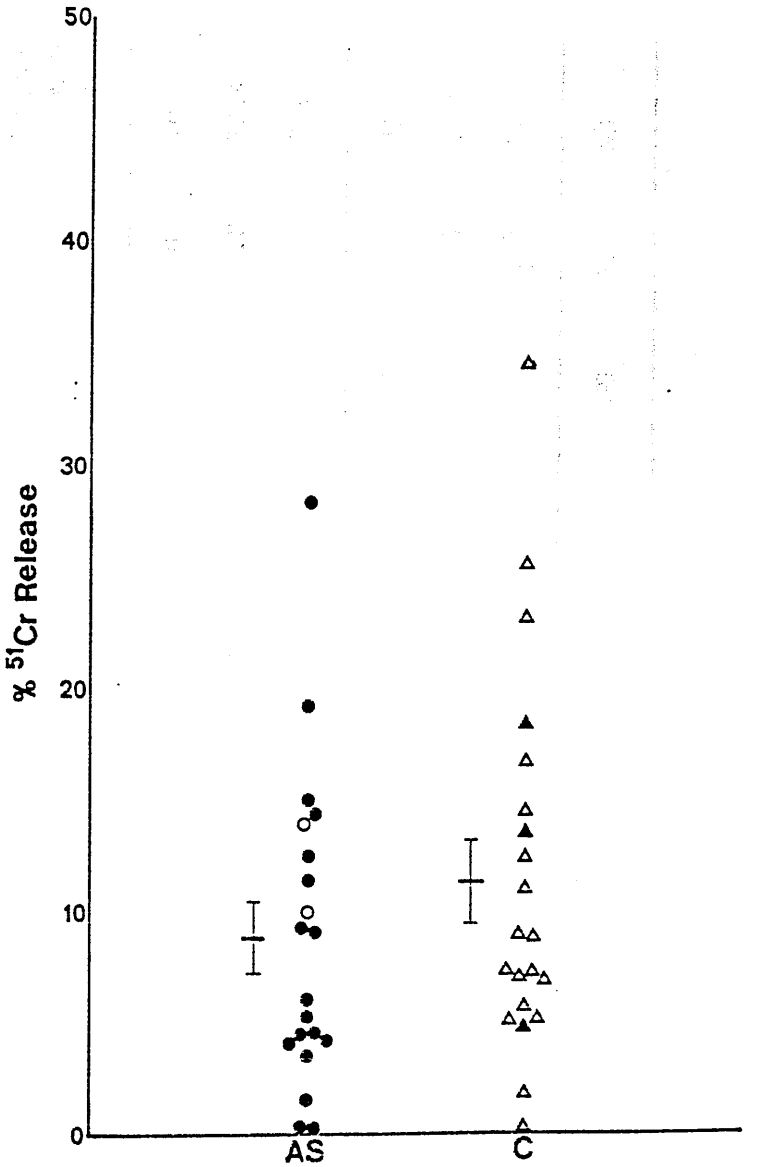



Fig XIII Lymphocytotoxicity of antiserum to heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis (AS) patients and normal controls (C).

Each point represents the highest result from each group of serum dilutions. Bars indicate mean ± SEM.

● = $AS^{+}B27^{+}$ ▲ = $AS^{-}B27^{+}$
O = $AS^{+}B27^{-}$ △ = $AS^{-}B27^{-}$

raised sensitivity to cytotoxic damage in B27 positive lymphocytes when compared to B27 negative lymphocytes. This study showed no recognition of the HLA-B27 antigen by Klebsiella antibodies.

It may be argued that these negative results imply lack of sensitivity in the experimental model to be able to repeat the findings of Geczy (1980). However, my assay was sensitive enough to differentiate between B27 positive and B27 negative lymphocytes using HLA-B27 antiserum, although the mean percentage $^{51}Cr$ release was only 47% with a range of 37 - 68%. One would have expected a much higher degree of cytotoxicity as shown by Cavendar and Ziff (1986).

Several problems that may be encountered in the process of this assay include the condition of the PBL, the medium and complement and the production of antiserum. Dilution of the complement solution or the use of similar volumes to Seager et al (1979) failed to work in my assay whereas others have reported that the concentration of complement is not critical (Prendergast et al 1983). Pre-immunisation serum should also be checked as Mackintosh & Pease (1982) have found that normal rabbit serum can produce higher cytotoxicity than that of specific Klebsiella serum. Studying antiserum to only one strain of Klebsiella may not give conclusive results but others have used many different sera and still have not been able to demonstrate lymphocytotoxicity using this assay (Beaulieu et al 1983; Singh et al 1986).

An explanation for the lack of corroboration was suggested by McGuigan et al (1986a) who thought a reduced carriage of HLA-B27 associated

cross-reactive marker on $B27^{+}AS^{+}$ PBL may occasionally escape detection by some antisera. For this reason they used neat serum in their assay. However, the present study has shown that the highest levels of $^{51}Cr$ release do not necessarily occur with undiluted serum.

Ogasawara et al (1986) have recently shown that formalin killed Klebsiella or the sonicated cell envelopes did not cross-react with HLA-B27 antibodies indicating that there are no reactive antigens on the surface of bacterial envelopes. By solubilising the bacterial envelopes, and therefore dissociating some of the individual components of the cell envelope from one another, they reasoned that additional antigenic determinants might be exposed. Such a preparation of Klebsiella pneumoniae K43 was found to cross-react with the anti-HLA-B27 antibody M2.

In conclusion, this study (confirming work by Cameron et al 1987) has failed to find evidence that antisera raised against Klebsiella pneumoniae K43 have any particular ability to react with B27 positive lymphocytes and the suggestion that this organism may have a key role in the pathogenesis of AS may not be tenable until the original observations are independently confirmed. If anti-lymphocytic antibodies are important in AS such antibodies may be difficult to identify using the lymphocyte cytotoxicity assay. Even if this assay could be repeated it is not certain whether this relationship between enteric bacteria and AS is of pathologic significance to the disease process.

| Serum dilution | | Undiluted | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | Highest single result |
|---|---|---|---|---|---|---|---|---|---|
| Ankylosing spondylitis | n | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| | mean | 10.3 | 5.8 | 6.1 | 5.5 | 4.8 | 5.1 | 3.8 | 13.4 |
| | SEM | 1,7 | 1.3 | 1.6 | 1.6 | 1.5 | 1.4 | 1.0 | 2.1 |
| Controls | n | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |
| | mean | 8.0 | 6.8 | 4.1 | 5.3 | 4.7 | 4.9 | 5.3 | 11.5 |
| | SEM | 1.3 | 1.1 | 0.7 | 1.4 | 1.0 | 1.4 | 1.7 | 1.6 |
| Significance | | NS | NS | NS | NS | NS | NS | NS | NS |

Table 11 Percentage lymphocytotoxicity of antiserum to the supernatant of heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis patients and normal controls.

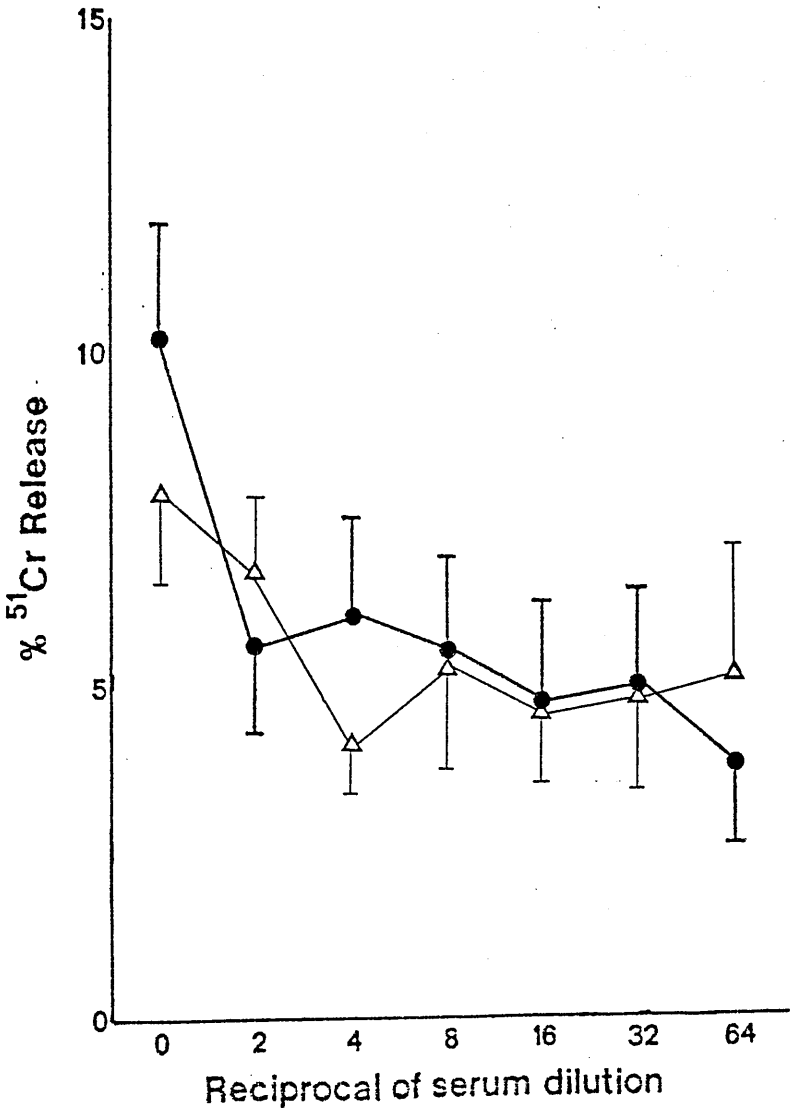



Fig XIV Mean (± SEM) lymphocytotoxicity of dilutions of antiserum to the supernatant of heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes of ankylosing spondylitis (AS) patients and normal controls (C).

● = AS Δ = C

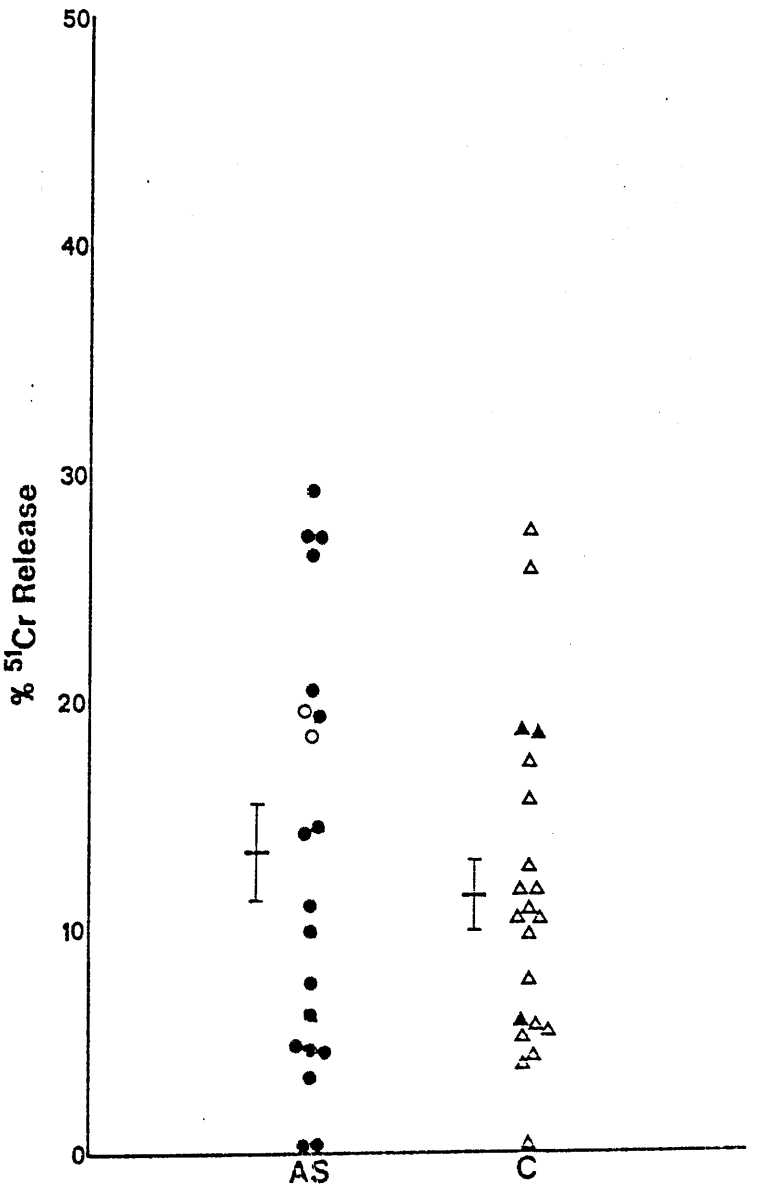



Fig XV Lymphocytotoxicity of antiserum to the supernatant of heat treated Klebsiella pneumoniae K43 against peripheral blood lymphocytes from ankylosing spondylitis (AS) patients and normal controls (C).

Each point represents the highest result from each group of serum dilutions. Bars indicate mean ± SEM.

● = $AS^+B27^+$ ▲ = $AS^-B27^+$
○ = $AS^+B27^-$ △ = $AS^-B27^-$

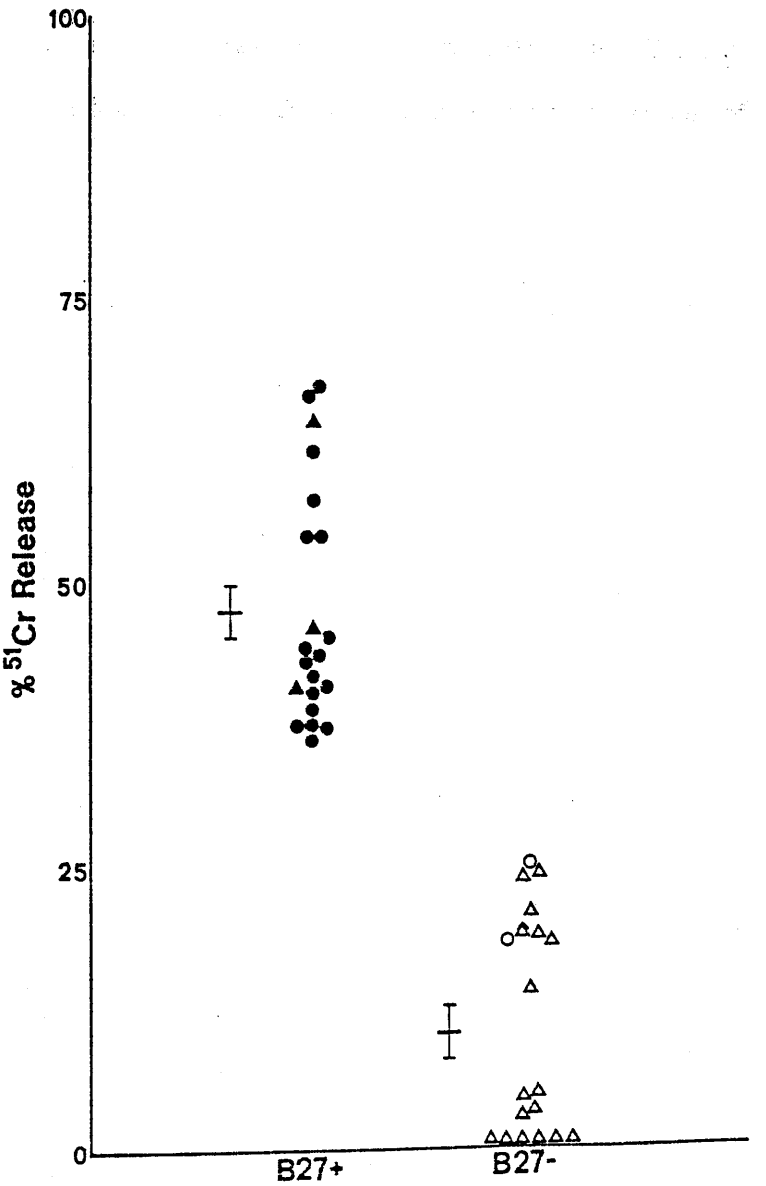



Fig XVI Lymphocytotoxicity of HLA-B27 antiserum.

Bars indicate mean ± SEM

● = $AS^{+}B27^{+}$ ▲ = $AS^{-}B27^{+}$

○ = $AS^{+}B27^{-}$ △ = $AS^{-}B27^{-}$

2.3% and 10.5 $\pm$ 2.3% respectively. The difference between the two groups is highly significant ($p < 0.001$).

The lymphocytotoxicity of antibodies to FT, HT and HTS Klebsiella were compared in those with B27 positive and negative PBL (Table 12). No significant difference was found between the two groups of PBL for any of the sera.

## 2.4 Discussion

One of the techniques used to study the cross-reactivity between lymphocytes from patients with ankylosing spondylitis and enteric bacteria is the $^{51}Cr$ release assay. Seager et al (1979) found that an antiserum to Klebsiella was cytotoxic for B27 positive PBL from AS patients but not for B27 negative patients or normal individuals. The Klebsiella species was later identified as Klebsiella pneumoniae K43. Culture filtrates of these bacteria were also capable of modifiying B27 positive PBL from normal individuals to become susceptible to lysis by antiserum to the Klebsiella species.

The present research has sought to confirm B27 cell specificity for anti-Klebsiella serum. Antiserum was raised to three different preparations of Klebsiella pneumoniae K43. Formalin treated bacterial cells were prepared as used by Seager et al (1979). Antiserum raised against this preparation should produce antibodies to all the major cell surface antigens. Heat-treated bacterial cells were compared with the formalin treated bacteria in an attempt to identify any heat labile cross-reactive antigen. Thirdly, using the supernatant of a heat treated suspension would detect the presence of cross-reactive ECA or O antigen.

Using the $^{51}Cr$ release lymphocytotoxicity assay the study failed to find any association between antibodies to Klebsiella and B27 PBL. This suggests that Klebsiella pneumoniae K43 does not possess antigens similar to those found on blood lymphocytes of AS patients. If there was an association between HLA-B27 and Klebsiella one would expect

Chapter 3

# Non-specific activation of peripheral blood lymphocytes

## 3.1 Introduction

Activation of T and B lymphocytes by non-specific mitogens or specific antigen provides one of the most readily available *in vitro* assays of immune function. The most commonly used method for detecting lymphocyte activation is incorporation of $^{3}$H-thymidine into the chromosomal DNA which correlates well with the number of lymphocytes in the S-phase of the cell cycle.

T cells recognise foreign antigens in association with cell-surface antigens coded for by the Major Histocompatibility Complex (MHC). The MHC, known as the HLA system in humans, has been of great interest in recent years due to the strong association between individual HLA antigens and disease. Such an association may reflect the ability or inability of T cells to respond to a particular combination of HLA antigen and virus or bacterium.

Mitogens are polyclonal activators that will stimulate a large proportion of the lymphocytes. A number of such mitogens are lectins - proteins or glycoproteins of non-immune origin that agglutinate and/or precipitate complex carbohydrates. They are isolated from a wide variety of natural sources such as plants, seeds and seaweed. Phytohaemagglutinin (PHA) simulates T helper ($T_H$) cells and concanavalin A (Con A) stimulates both $T_H$ and T suppressor ($T_S$) cells.

Pokeweed mitogen (PWM) will activate B cells only in the presence of T cells and is therefore a T cell dependent mitogen. Activation by PWM is a complex process requiring $T_H$ cells to induce B cells to differentiate. In the normal individual this is 'down regulated' by $T_S$ cells. Thus the B cell response is the net result of helper and suppressor regulatory influences. The pathogenesis of an auto-immune disease process may involve an excess of $T_H$ activity or deficiency of $T_S$ activity. A T cell dependent *in vitro* system of B cell activation will therefore allow the investigator to explore these potential immunoregulatory abnormalities.

The *in vitro* PBL transformation by mitogens has been studied with some controversy in AS. The responses to PWM and Con A have tended to be quite normal (Froebel et al 1975; Kinsella et al 1984) while decreased T lymphocyte responsiveness to PHA has been reported (Escanilla, Alepa and Reefe 1970; Sturrock, Froebel and MacSween 1975; Froebel et al 1975). Others have reported normal responses to this mitogen (Nikbin et al 1975; Fan et al 1977; Christiansen, Hawkins and Dawkins 1978; Kinsella et al 1984).

As there seems to be some evidence for abnormalities in cell mediated immunity (CMI) in AS patients, attempts have been made to ascertain whether these could be attributed to disturbances in the numbers of T and B cells. Several studies have found the numbers and proportions of T/B cells to be within the normal range (Nikbin et al 1975; Christiansen et al 1978; Hickling et al 1982; Veys et al 1983) while others have noted a significant T cell lymphopaenia (Fan et al 1977; Byrom et al 1979). It was suggested that this T cell lymphopaenia was

caused by C-reactive protein (CRP) coating the T cells and impairing the recognition of T cell markers resulting in a reduction in the number of T cells (Sotnik 1979). However, the finding of normal T cell numbers in AS patients with raised CRP levels casts doubts on this theory (Hickling et al 1982).

Byrom et al (1979) found an overall depletion of T cells in patients with anterior uveitis and AS that remained low for many months after the condition returned to normal. Patients' household contacts also showed a significant T cell lymphopaenia. Lymphocyte depletion in the contacts of uveitis patients as well as in the patients themselves suggests that there may have been a lateral transmission of an infective agent during or before the uveitis attack.

T cell subpopulations have also been demonstrated using monoclonal antibodies against T cell surface markers. Results are again conflicting. Veys et al (1983) found an increase in the percentage of helper-inducer cells compared to controls, but no differences in the ratio of $T_H$ to $T_S$ cells while Nilsson and Biberfield (1980) reported increased numbers of $T_S$ cells. In contrast Vinje et al (1982) found no differences in the percentage of $T_S$ and $T_H$ cells between AS patients and controls. CMI in AS therefore presents as a conflicting picture needing greater clarification of T cell function in the pathogenesis of the disease.

## 3.2 Materials and methods

### 3.2.1 Patient groups

PBL transformation by mitogens was studied in patients with AS, CD and RA. Healthy laboratory and hospital staff were used as controls. Clinical details of the groups are given in Table 13. AS and CD patients were attending the rheumatology and gastroenterology out-patient clinic respectively. RA patients were in-patients at the Centre for Rheumatic Diseases, Royal Infirmary.

### 3.2.2 Lymphocyte transformation assay

PBL were separated, under sterile conditions, as previously described (Section 2.2.3). The cells were resuspended to give a final concentration of $1 \times 10^6$/ml in RPMI medium containing 20% human pooled serum.

Phytohaemagglutinin (PHA, type V-S, Sigma) and concanavalin A (Con A) (type IV, Sigma) were reconstituted in sterile distilled water and diluted to the required concentration in RPMI medium. Pokeweed mitogen (PWM) (Sigma) was reconstituted and diluted in RPMI medium.

100µl of PBL and 100µl of mitogen solution were distributed, in triplicate, into round bottomed microtitre plates (Gibco) and incubated for 72 hours at 37°C in 5% $CO_2$ and humidified air. 4 hours before harvesting 20µl of $^3$H-thymidine (50µlCi/ml:49Ci/mmol, Amersham) were added to each well. The cultures were harvested onto filter

| | AS | CD | RA | C |
|---|---|---|---|---|
| Number of patients | 20 | 20 | 20 | 20 |
| Mean age (range) | 42 (23-69) | 36 (21-60) | 63 (41-75) | 31 (21-45) |
| Sex | M = 16<br>F = 4 | M = 7<br>F = 13 | M = 15<br>F = 5 | M = 13<br>F = 7 |
| Drug therapy | All patients on NSAID | Salazopyrin 6<br>Mesalazine 3<br>NSAID 1<br>No drug treatment 10 | NSAID 13<br>Steroids 4<br>Gold/ Penicillamine 3 | Not applicable |

Table 13 Clinical details of patients and controls involved in the lymphocyte transformation study.

discs with a multiple cell-culture harvester (Skatron, Norway). The discs were washed twice with 5% trichloro-acetic acid and methanol, placed in scintillation vials and allowed to dry. 5ml scintillation fluid (BDH) was then added to each vial and the incorporation of thymidine into the DNA measured on an LKB gamma counter.

The optimal reponse for each mitogen was obtained by varying the mitogen concentration. PHA was used at 5, 10 and 25µg/ml, Con A at 25, 100 and 250µg/ml and PWM at 0.1, 0.2 and 0.5µg/ml.

A stimulation index (SI) was calculated for each suspension as

$$SI = \frac{\text{cpm stimulated cells}}{\text{cpm unstimulated cells}}$$

## 3.3 Results

### 3.3.1 Optimal dose response

The mean lymphocyte responses to PHA, PWM and Con A, in normal controls, are given in Table 14. The optimal concentration for each mitogen was 10, 0.2 and 100µg/ml for PHA, PWM and Con A respectively. These concentrations were therefore used in subsequent experiments.

### 3.3.2 Unstimulated cultures

The mean cpm ± SEM of unstimulated PBL were 60 ± 34, 59 ± 35, 65 ± 22 and 55 ± 21 for AS, CD and RA patients and normal controls respectively. The responses with unstimulated cultures were not significantly different between any of the groups thereby permitting the application of the SI to compare group responses.

### 3.3.3 In vitro peripheral blood lymphocyte stimulation by phytohaemagglutinin

The responses to PHA are illustrated in Figure XVII. In response to PHA, the mean SI for AS, CD, RA patients and normal controls was 483 ± 80, 454 ± 63, 194 ± 28 and 529 ± 60 respectively. When compared by the Wilcoxon rank sum test the results for AS were not significantly different from the CD group or normal controls. The CD group was also not significantly different from the controls. The results for the RA patients were significantly lower than all the other three groups (AS $p < 0.001$, CD $p < 0.02$, C $p < 0.001$).

| | | | | |
|---|---|---|---|---|
| PHA | Concentration (μg/ml) | 5 | 10 | 25 |
| | SI | 480 ± 62 | 684 ± 132 | 533 ± 148 |
| PWM | Concentration (μg/ml) | 0.1 | 0.2 | 0.5 |
| | SI | 124 ± 16 | 160 ± 34 | 114 ± 28 |
| Con A | Concentration (μg/ml) | 25 | 100 | 250 |
| | SI | 234 ± 30 | 866 ± 253 | 440 ± 93 |

Table 14 Mean lymphocyte responses to varying doses of PHA, PWM and Con A.

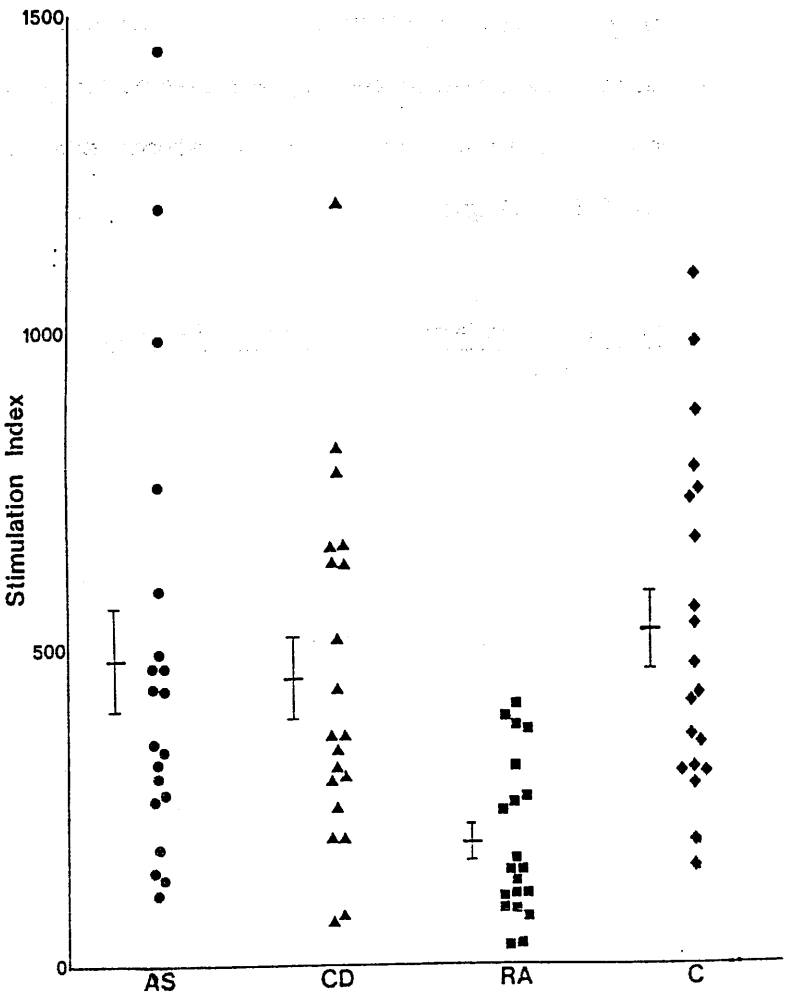



Fig XVII Lymphocyte transformation responses to PHA in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

Bars indicate mean ± SEM.

### 3.3.4 In vitro peripheral blood lymphocyte stimulation by pokeweed mitogen

The responses to PWM are illustrated in Figure XVIII. The mean SI for AS, CD and RA patients and normal controls was 216 ± 46, 249 ± 53, 140 ± 20 and 248 ± 31 respectively. No significant difference was found between any of the groups except for the RA patients whose mean SI was significantly lower than the control group ($p < 0.01$).

### 3.3.5 In vitro peripheral blood lymphocyte stimulation by concanavalin A

The responses to Con A are illustrated in Figure XIX. The mean SI was 429 ± 58, 397 ± 46, 175 ± 24 and 630 ± 73 for AS, CD and RA patients and normal controls respectively. All three patient groups had significantly lower SI than normal controls (AS $p < 0.05$, CD $p < 0.01$, RA $p < 0.001$). The SI for RA patients was also lower than that of the AS and CD patients ($p < 0.001$).

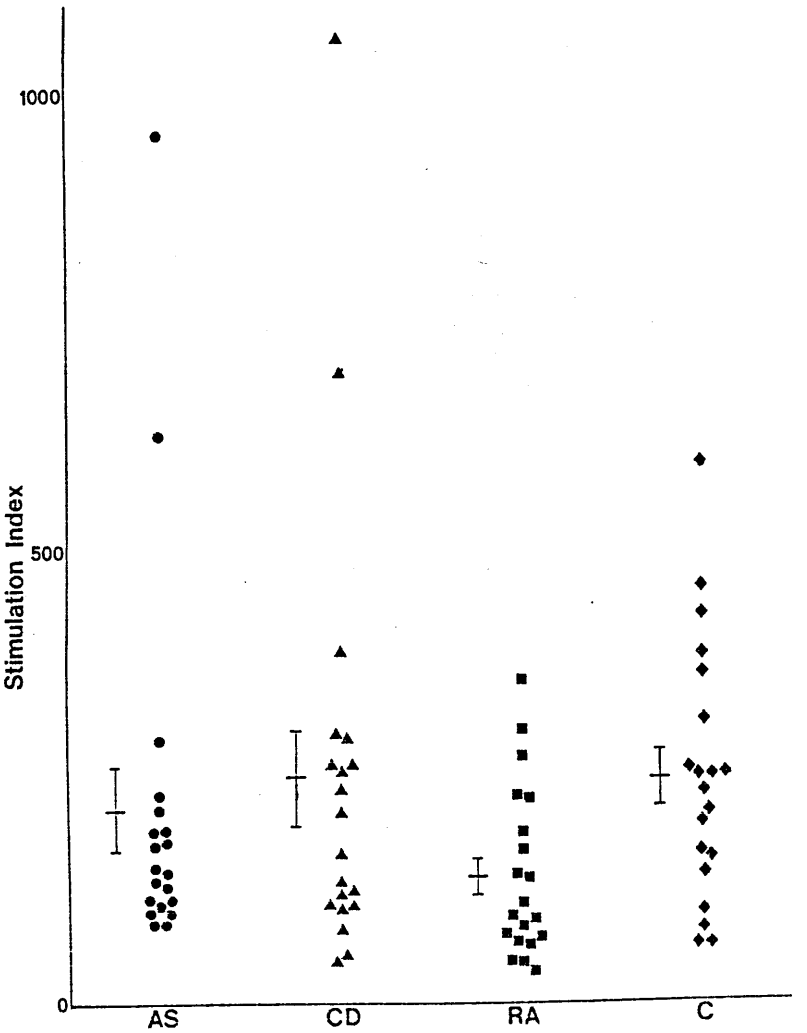



Fig XVIII Lymphocyte transformation responses to PWM in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

Bars indicate mean ± SEM.

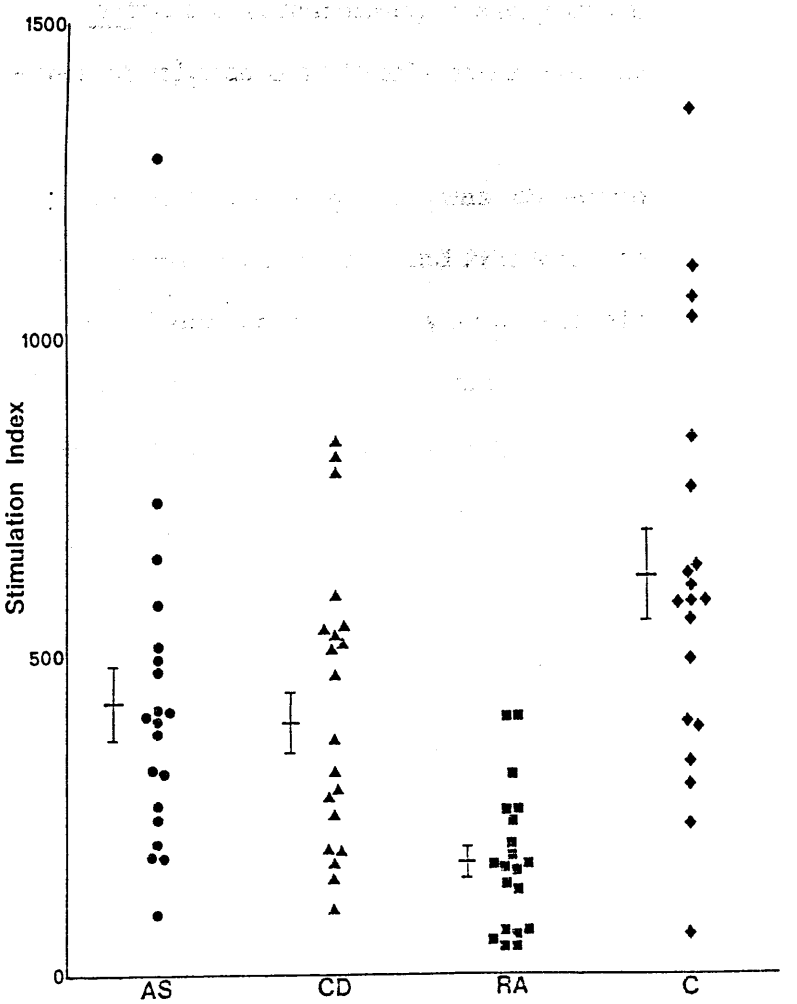



Fig XIX Lymphocyte transformation responses to Con A in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

Bars indicate mean ± SEM.

## 3.4 Discussion

The aim of this study was to examine the cell mediated immunity (CMI) in patients with ankylosing spondylitis and to compare them with two other inflammatory disorders, Crohn's disease and rheumatoid arthritis. An *in vitro* PBL transformation assay was used to determine whether there was an imbalance in T-cell immunoregulation.

A reduction in Con A responsiveness was detected in the PBL of patients with AS. Responses to PHA and PWM were not significantly different from normal controls. PHA is a $T_H$ cell mitogen and Con A a $T_H$ and $T_S$ cell mitogen. As the response to PHA was normal the reduced transformation by Con A could be explained by an abnormality in the $T_S$ cell population. This could be due to a shortage of these cells or a diminished $T_S$ cell activity. As there is conflicting data on $T_S$ cell numbers in AS we assume that these results stem from an abnormality in the cells' activity. Confirming this assumption, Vinje et al (1982), showed reduced Con A-induced $T_S$ cell activity as detected in a Con A/mixed lymphocyte culture assay. They also demonstrated T lymphocyte subpopulations of $T_S$ and $T_H$ cell markers were within the normal range and suggested that this lack of suppressor cell activity corresponded with the moderately elevated humoral responses that were detected.

The *in vitro* proliferative response to Con A was also diminished in patients with Crohn's disease. Selby and Jewell (1983) have examined the proportion and numbers of T lymphocytes and their subsets in peripheral blood of patients with inflammatory bowel disease (IBD).

The proportion of T cells was normal but the actual number of T lymphocytes was reduced in active disease. The ratio of $T_H/T_S$ cells was not significantly altered. Although T cell numbers seem to be normal there is evidence of impairment of some aspects of CMI in IBD in particular a defective suppressor cell function (Selby et al 1983). Functional analysis of peripheral blood T cells in patients with IBD has demonstrated an impairment of Con A induced and spontaneous suppressor cell activity (Hodgson, Wands and Isselbacher 1978; Shorter 1981; Victorino and Hodgson 1981).

Many patients with autoimmune diseases have an increased ratio of $T_H/T_S$ cells reflecting a relative or absolute deficiency of suppressor/cytotoxic T cells (Raeman et al 1981). The normal ratio seen in AS and IBD may be evidence against these disorders being primarily auto-immune. However, the defective suppressor cell function may cause an imbalance of T helper cell function leading to an abnormal increase in B cell activity which may lead to the production of antibodies that are auto-immune.

It may be more pertinent to study the numbers of T lymphocyte subpopulations in the intestinal mucosa of patients with IBD as this is where the pathological symptoms occur. Abnormal numbers or a redistribution or an alteration in the mucosal micro-environment may be important in the pathogenesis of IBD. James et al (1986) have analysed lymphocytes from the lamina propria of patients with Crohn's disease. They found an increase in the number of $T_H$ cells and a decrease in $T_S$ cells when compared to similar cell types in peripheral blood.

Since AS may result from a gut initiated immunological response a study of intestinal lymphocytes may be more appropriate in AS. An increase in IgA antibody levels has been reported in patients with AS (Chapter 1). Production of this antibody occurs predominantly in mucosal surfaces such as those in the intestine. The possibility exists that there may also be increased levels of $T_H$ cells (or abnormalities in $T_S$ cell function) leading to such raised antibody responses.

Mitogen transformation of rheumatoid arthritis PBL in the present study has shown a diminished response to all three mitogens (PHA, PWM and Con A). This suggests that there is both a $T_H$ and $T_S$ cell abnormality in these patients. Other studies have also reported decreased PBL transformation to these mitogens (Lance and Knight 1974; Lockshin et al 1975). The reduced responsiveness may be explained by the absence or inactivation of responder cells or by a lack of processing of antigen by macrophages. The proportion of T cells in peripheral blood has been well documented with most investigators finding an increased ratio of $T_H/T_S$ cells. This is caused by both raised levels of $T_H$ cells and reduced levels of $T_S$ cells in RA patients when compared to normal levels (MacKenzie and Williamson 1983). The decreased response to Con A can be explained by a lack of suppressor cells whereas the responses to PHA and PWM may be due to an abnormality in helper function or lack of processing by macrophages.

Drug therapy may also explain some of the results from this study. The mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is thought to be secondary to the inhibition of prostaglandin

production by inhibiting the action of cyclo-oxygenase. It is thought that by blocking the effects of prostaglandin E (PGE) the final stages of the inflammatory process may be affected. However, PGE also plays an important role in modulating the immune response (Goodwin and Webb 1980).

Goodwin, Ceuppens and Rodriguez (1983) have examined the effect of the NSAID, piroxicam, on PHA reactivity and T cell populations. It had previously been reported that PHA responses were depressed in RA patients and that this could be reversed by inhibiting endogenous PG production. They also found that the PHA response in patients, after starting piroxicam therapy, gradually increased until there was no significant difference from controls. Therefore the in vitro administration of NSAIDs had the effect of reversing the lowered response to PHA towards normality. The lower proportions of $T_S$ cells were also seen to rise after piroxicam treatment presumably due to the inhibition of prostaglandin action.

The effect of NSAIDs in AS patients could therefore explain the normal PBL response to PHA. However, there was a diminished response in RA patients which could not be attributed to drug treatment. If NSAIDs depress $T_S$ cells one would expect that the response to Con A should also be normal but this was not seen in either AS or RA patients. Probably PG were without effect on T cells in the first place as PG are local hormones whose site of action remains at the site of production (Goodwin et al 1980) which presumably would be the synovial joints. Circulating PG have no systemic activities and therefore NSAIDs are unlikely to be responsible for the observed results.

Steroids may have a greater effect on T cell function. The administration of corticosteroid in vivo results in a rapid lymphopaenia due to redistribution of circulating lymphocytes to other lymphoid compartments. They are able to bind to a specific intracytoplasmic receptor therefore washing cells would not release the drug (Cupps and Fauci 1982). Monocytes (MN) appear to be particularly sensitive to glucocorticoid (GC) induced changes (Cupps et al 1982). Since MN are important accessory cells and function in many lymphocyte responses GC induced effects on MN may indirectly or directly alter the functions of other cell subsets such as T cells. In fact blastogenesis by PHA, Con A and PWM has been reported to be suppressed (Cupps et al 1982). Gold also interferes with the ability of MN to serve as accessory cells in the initiation of a mitogen response (Lipsky and Ziff 1977). As only 7 RA patients, in this study, were on steroid or gold therapy yet every patients' mitogenic responsiveness was lowered, it seems that this is not alone in causing diminished mitogenic responsiveness in RA patients.

The lymphocyte transformation response in CD patients does not seem to be affected by the main drug used in the treatment of this disease - sulphasalazine. Sachar et al (1973) found no differences in the mitogenic response between patients who were or were not taking medication and the addition of salicylazosulfapyridine to the culture medium had no effect on transformation. If there is an effect it would be to increase the already diminished response as shown by Rubinstein et al (1978). The proportion of peripheral blood T cells is also not altered (Selby et al 1983). Therefore the impairment of the lymphocyte response to Con A reflects an intrinsic defect in the T

cell regulatory system in Crohn's disease.

In summary this study has shown that patients with AS and CD have lowered responses to Con A indicating that there may be abnormal suppressor cell activity. RA patients seem to have abnormalities in both suppressor and helper T cell function due to the decreased responses to PHA, Con A and PWM. This imbalance of immunoregulatory T cells may therefore be important in the pathogenesis of these three inflammatory disorders.

Chapter 4

## Lymphocyte proliferative responses to bacterial antigens

### 4.1.1 Introduction

In contrast to the non-specific proliferative responses discussed in Chapter 3, where up to 30% of cells may be triggered by the binding of the mitogen to specific sugars on the lymphocyte membrane, antigen stimulation will only transform those cells that have specific receptors for that antigen. A positive response requires that the individual has had prior exposure to the antigen and therefore transformation assays may be used as an indication of such exposure.

A specific response requires the help of accessory cells (eg macrophages) which capture and break down micro-organisms and soluble antigens into smaller molecular fragments (known as epitopes) that are of an appropriate size to react with receptors on the surface of lymphocytes. These accessory cells shuttle epitopes to their surface where they are readily accessible to the lymphocytes. The T-cell receptor, for antigen, is derived in a manner similar to that of B cell and antibody development (Nossal 1987). When a T cell encounters an antigen, specific for that T cell, multiplication and differentiation of a T cell clone occurs. The T cell does not recognise the antigen itself, but antigen in molecular association with MHC antigens. Therefore a T-cell receptor is directed towards 'foreign' epitopes held in loose association with 'self' molecules.

Banck and Forsgren (1978) have studied the transformation of lymphocytes to various formalin treated bacteria and found that most of them caused an increase in DNA synthesis. However, most of this was not due to a specific reaction but a mitogenic effect. Some bacteria, such as Mycobacterium tuberculosis, produced both a mitogenic and specific antigen response.

The lymphocyte response to bacterial antigens has been investigated with varying degrees of success in AS. Nikbin et al (1975) previously reported reduced lymphocyte transformation to a crude mixed Yersinia enterocolitica and Yersinia pseudotuberculosis cell antigen but later were able to find no differences between AS patients and controls to Yersinia, Salmonella and E.coli antigens (Mawle et al 1978). With the apparent association between AS and Klebsiella the transformation response to Klebsiella in AS patients was investigated and a diminished in vitro cell response to Klebsiella but not Yersinia or Shigella was found (Seager et al 1979; Geczy et al 1980b). They suggested that this may reflect impaired processing of antigen by macrophages or the production of suppressor cells and/or suppressor factors. Several other groups have attempted to confirm the decrease in in vitro proliferative responses to Klebsiella and other enteric bacteria but have failed to do so (Enlow et al 1982; Kinsella et al 1984; Sheldon and Pell 1985).

Two other rheumatic disorders, that may help to elucidate the disease pathogenesis of AS, are reactive arthritis and Reiter's syndrome. They are clinically distinct from AS but share certain characteristics, such as, a tendency to affect young males, with a

similar location and character of articular and extra-articular affected lesions. They are also linked genetically by the presence of HLA-B27. In these two disorders the aetiological agent is known and may be involved in the initiation of both diseases. A lymphocyte proliferative response whether augmented or diminished in patients with AS or RS compared with normal controls might suggest prior antigenic exposure. Enlow et al (1982) and Sheldon et al (1985), however, have not been able to demonstrate any abnormalities in CMI to various bacterial antigens in RS. The response in patients with yersiniosis and Yersinia arthritis seems to be more promising. Vuento (1983) studied lymphocyte transformation in patients who had recently recovered from yersiniosis and found significantly higher responses to Yersinia species and E.coli antigens when compared to healthy controls. Patients who had developed arthritis after a Yersinia infection had weaker lymphocyte responses to Yersinia enterocolitica and E.coli than those without arthritis (Leino et al 1983). This response was persistent even years after the Yersinia infection (Vuento et al 1983). Their results are similar to the findings that the lymphocyte transformation response to Salmonella typhi persists in those who had once had typhoid fever (Mogensen 1979). This lowered response in those who had once had Yersinia arthritis cannot be due to a suppression of lymphocyte activity generated during the arthritis as the reactivity persisted for many years. It may reflect an impaired lymphocyte function where the cell does not recognise the foreign antigen thus making these patients more susceptible to infection and arthritic complications.

The results of lowered or increased CMI in ReA and AS to different

enterobacteriaceae suggest that there is a common antigenic determinant triggering or inhibiting the lymphocyte transformation response. A candidate for such a structure could be the enterobacterial common antigen (ECA). In the study by Vuento et al (1984) they tried to demonstrate this possibility by stimulating lymphocytes from patients with Yersinia and Salmonella infection, with or without arthritis, using several different bacterial antigens. To specifically test the importance of ECA for the response a pair of ECA positive and negative Salmonella typhimurium strains was included. When whole bacteria were used in the assay the presence of ECA made no difference to the response - maybe due to the masking of ECA by other antigens. When the supernatant (soluble antigens) of heat treated cells was used as an antigen a higher response was observed with the ECA positive strain. ECA may have stimulated the lymphocytes and if ECA positive bacterial strains are involved in pathogenesis of ReA ECA may be important in this disease and perhaps AS.

Granfors (Granfors 1979; Granfors et al 1980) has shown that anti-Yersinia IgA and IgG levels in serum are higher and persist for longer in patients with Yersinia arthritis than non-arthritic patients. With the previous findings of depressed lymphocyte transformation responses to gram-negative bacteria in arthritic patients a reverse relationship exists between the humoral and cell mediated immune responses to Yersinia in these patients. An explanation could be that CMI is impaired leading to the persistence of Yersinia in the cells of the intestine resulting in a prolonged antibody response. How this disturbed immune response results in ReA is still unknown.

### 4.1.2 Aims of the study

If an organism is involved in the pathogenesis of AS it may imply that there is also a disturbed immune respone. This study set out to investigate the cellular immune responses to various enterobacterial antigens in an attempt to find any similarities or differences that may emerge.

## 4.2 Materials and methods

### 4.2.1 Patient groups

The same groups of patients were used in this study as in Section 3.2.1.

### 4.2.2 Bacterial antigens

The bacterial strains used included Klebsiella pneumoniae K43 and K25 (NCTC 9163 and NCTC 9145); Yersinia enterocolitica serotype 3 (NCTC 11176); known ECA positive strains Escherichia coli 2387 (014 : K7 : $H^-$, kindly supplied by Dr H Mayer, Max-Plank Institute for Immunobiology, West Germany) and Salmonella typhimurium SH892 (kindly supplied by Dr P H Mäkelä, Central Public Health Laboratory, Helsinki, Finland) and Pseudomonas aeruginosa, isolated from an AS patient.

The bacteria were grown O/N at 37°C in Mueller-Hinton broth (Oxoid). The cultures were then washed in saline and killed with formalin and heat as previously described in Section 2.2.1. A soluble antigen was also prepared by centrifuging 10ml of cell suspension and resuspending in 10ml of saline. The cells were then heat killed (100°C, Koch steamer, 2 hrs), centrifuged and the supernatant retained after filtering through a 0.45µm cellulose acetate filter (Millipore). All antigen preparations were stored at -20°C before use. Before killing the bacteria, for standardisation of antigen, a correlation between absorbance at 620nm (Cecil, spectrophotometer) and the concentration of bacteria was determined (Appendix B).

### 4.2.3 Lymphocyte transformation assay

The assay has been previously described in Section 3.2.3. 100µl of antigen was added to the microtitre plates instead of mitogen and the cells were pulsed with $^{3}$H-thymidine 18 hours before harvesting.

### 4.2.4 The effect of bacterial antigen preparations on the viability of peripheral blood lymphocytes

The viability of PBL after 4 days incubation with each bacterial antigen preparation was assessed using trypan blue (0.1%) exclusion.

## 4.3 Results

### 4.3.1 Viability of peripheral blood lymphocytes

The viability of PBL after 4 days incubation with bacterial antigens was found to exceed 90% in all the patient groups and normal controls.

### 4.3.2 Optimum dose of antigen and kinetics of the response

In order to determine the optimal dose of antigen, concentrations of $10^6$, $10^7$, $10^8$ and $10^9$ bacterial cfu/ml and supernatants at dilutions of 1/100, 1/10 and undiluted were used to stimulate normal control PBL for 3, 4, 5 and 6 days (Tables 15 - 21). The experiments were repeated three times and the results are expressed as SI $\pm$ SEM.

The maximum response was, in most cases, observed after 4 days therefore a 4-day culture period was used in subsequent experiments. The optimal response with E.coli, Y.enterocolitica and Ps.aeruginosa was observed with a concentration of $10^6$ cfu/ml. The optimal concentration for K.pneumoniae K43 FT and HT antigens was $10^7$ and $10^9$ cfu/ml respectively and that for S.typhimurium HT and FT antigens was $10^8$ and $10^6$ cfu/ml. Optimal concentrations for K.pneumoniae K25 was $10^7$ cfu/ml. A dilution of 1/10 of the heat treated supernatant produced, in most cases, the highest result. These afore mentioned concentrations were subsequently used in further experiments.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| | FT | 3 | 2.73 ± 0.47 | 7.60 ± 0.64 | 0.85 ± 0.22 | 0.46 ± 0.02 |
| | | 4 | 3.13 ± 0.46 | 5.37 ± 0.09 | 0.80 ± 0.05 | 2.74 ± 1.98 |
| | | 5 | 4.67 ± 0.80 | 1.71 ± 0.10 | 0.46 ± 0.02 | 0.14 ± 0.01 |
| Klebsiella | | 6 | 6.14 ± 1.78 | 2.85 ± 0.62 | 0.62 ± 0.04 | 0.16 ± 0.01 |
| pneumoniae | | 3 | 1.70 ± 0.30 | 0.38 ± 0.02 | 0.50 ± 0.04 | 16.18 ± 0.30 |
| K43 | HT | 4 | 2.56 ± 0.95 | 0.27 ± 0.03 | 0.32 ± 0.05 | 10.07 ± 0.20 |
| | | 5 | 1.27 ± 0.38 | 0.13 ± 0.01 | 0.14 ± 0.01 | 3.81 ± 0.20 |
| | | 6 | 3.22 ± 0.76 | 0.12 ± 0.01 | 0.12 ± 0.01 | 4.51 ± 1.00 |

Table 15 Time course and dose response of lymphocyte transformation by Klebsiella pneumoniae K43 antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| | FT | 3 | 12.18+ 3.23 | 11.44 ± 3.63 | 0.50 ± 0.02 | 0.59 ± 0.02 |
| | | 4 | 13.31 ± 0.96 | 14.80 ± 3.51 | 0.29 ± 0.02 | 0.23 ± 0.01 |
| | | 5 | 12.54 ± 1.94 | 9.66 ± 3.29 | 0.10 ± 0.01 | 0.11 ± 0.01 |
| Klebsiella | | 6 | 11.02 ± 1.38 | 8.13 ± 4.75 | 0.04 ± 0.01 | 0.04 ± 0.01 |
| pneumoniae | | 3 | 13.98 ± 2.28 | 12.56 ± 1.67 | 0.79 ± 0.05 | 0.34 ± 0.01 |
| K25 | HT | 4 | 18.75 ± 1.13 | 21.58 ± 2.07 | 1.06 ± 0.26 | 0.14 ± 0.01 |
| | | 5 | 17.52 ± 2.08 | 15.29 ± 0.46 | 0.46 ± 0.20 | 0.80 ± 0.01 |
| | | 6 | 16.31 ± 0.65 | 11.82 ± 0.93 | 0.41 ± 0.32 | 0.10 ± 0.01 |

Table 16 Time course and dose response of lymphocyte transformation by Klebsiella pneumoniae K25 antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| | FT | 3 | 0.92 ± 0.08 | 0.70 ± 0.01 | 0.74 ± 0.03 | 0.61 ± 0.03 |
| | | 4 | 1.82 ± 0.09 | 1.79 ± 0.15 | 0.84 ± 0.03 | 0.96 ± 0.09 |
| | | 5 | 1.22 ± 0.08 | 0.86 ± 0.02 | 0.77 ± 0.16 | 0.74 ± 0.01 |
| Escherichia | | 6 | 1.40 ± 0.44 | 1.00 ± 0.04 | 0.96 ± 0.08 | 1.08 ± 0.04 |
| coli | | 3 | 0.98 ± 0.02 | 0.78 ± 0.01 | 0.72 ± 0.06 | 0.58 ± 0.01 |
| 2387 | HT | 4 | 1.30 ± 0.05 | 1.18 ± 0.06 | 0.94 ± 0.03 | 0.96 ± 0.05 |
| | | 5 | 1.17 ± 0.10 | 0.83 ± 0.02 | 0.82 ± 0.03 | 0.81 ± 0.04 |
| | | 6 | 0.96 ± 0.08 | 0.98 ± 0.06 | 0.92 ± 0.01 | 1.02 ± 0.06 |

Table 17 Time course and dose response of lymphocyte transformation by Eschericia coli 2387 antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| | FT | 3 | 1.37 ± 0.14 | 0.90 ± 0.06 | 0.83 ± 0.05 | 0.80 ± 0.04 |
| | | 4 | 2.42 ± 0.09 | 1.64 ± 0.12 | 1.36 ± 0.04 | 1.53 ± 0.05 |
| | | 5 | 1.40 ± 0.16 | 0.68 ± 0.02 | 0.69 ± 0.03 | 0.74 ± 0.01 |
| Yersinia | | 6 | 1.07 ± 0.07 | 0.82 ± 0.04 | 0.75 ± 0.04 | 0.86 ± 0.07 |
| enterocolitica | | 3 | 1.24 ± 0.04 | 0.83 ± 0.01 | 0.84 ± 0.01 | 0.72 ± 0.01 |
| 3 | HT | 4 | 1.81 ± 0.36 | 1.16 ± 0.04 | 1.15 ± 0.06 | 1.15 ± 0.04 |
| | | 5 | 1.10 ± 0.16 | 0.64 ± 0.02 | 0.59 ± 0.02 | 0.58 ± 0.04 |
| | | 6 | 0.85 ± 0.15 | 0.78 ± 0.01 | 0.74 ± 0.01 | 0.75 ± 0.01 |

Table 18 Time course and dose response of lymphocyte transformation by Yersinia enterocolitica 3 antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| Salmonella typhimurium SH4892 | FT | 3 | 1.26 ± 0.06 | 1.20 ± 0.13 | 0.23 ± 0.01 | 0.24 ± 0.01 |
| | | 4 | 1.62 ± 0.05 | 1.55 ± 0.02 | 0.51 ± 0.01 | 0.52 ± 0.03 |
| | | 5 | 1.94 ± 0.11 | 2.32 ± 0.39 | 0.63 ± 0.02 | 0.62 ± 0.05 |
| | | 6 | 1.95 ± 0.22 | 2.14 ± 0.29 | 0.87 ± 0.05 | 0.93 ± 0.04 |
| | HT | 3 | 0.97 ± 0.05 | 0.56 ± 0.04 | 2.55 ± 0.24 | 0.25 ± 0.02 |
| | | 4 | 1.07 ± 0.02 | 0.84 ± 0.06 | 3.12 ± 0.06 | 0.49 ± 0.02 |
| | | 5 | 1.27 ± 0.06 | 0.74 ± 0.06 | 2.19 ± 0.09 | 0.57 ± 0.01 |
| | | 6 | 1.00 ± 0.02 | 0.68 ± 0.02 | 1.88 ± 0.10 | 0.60 ± 0.01 |

Table 19 Time course and dose response of lymphocyte transformation by Salmonella typhimurium SH4892 antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | | Incubation time (days) | Antigen concentration (cfu/ml) | | | |
|---|---|---|---|---|---|---|
| | | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| | FT | 3 | 1.60 ± 0.09 | 1.15 ± 0.05 | 1.11 ± 0.06 | 0.92 ± 0.07 |
| | | 4 | 2.03 ± 0.13 | 1.06 ± 0.07 | 1.07 ± 0.04 | 0.67 ± 0.01 |
| | | 5 | 1.45 ± 0.18 | 1.20 ± 0.06 | 1.31 ± 0.14 | 1.12 ± 0.06 |
| Pseudomonas | | 6 | 1.05 ± 0.02 | 0.68 ± 0.02 | 0.94 ± 0.04 | 0.80 ± 0.04 |
| aeruginosa | HT | 3 | 1.38 ± 0.16 | 1.04 ± 0.09 | 1.13 ± 0.14 | 1.14 ± 0.03 |
| | | 4 | 1.22 ± 0.14 | 0.72 ± 0.06 | 1.09 ± 0.20 | 0.68 ± 0.04 |
| | | 5 | 1.37 ± 0.10 | 1.05 ± 0.06 | 1.05 ± 0.02 | 1.17 ± 0.09 |
| | | 6 | 1.06 ± 0.02 | 0.84 ± 0.05 | 0.89 ± 0.01 | 0.78 ± 0.03 |

Table 20 Time course and dose response of lymphocyte transformation by Pseudomonas aeruginosa antigens.

Optimum SI underlined.
FT - formalin treated; HT - heat treated.

| Bacterial antigen | Antigen dilution | Incubation Time (days) | | | |
|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 |
| Klebsiella pneumoniae K43 | Un | 4.69±0.46 | 5.74±0.73 | 2.77±0.54 | 2.92±0.57 |
| | 1/10 | 5.60±0.12 | 5.75±0.44 | 4.70±1.50 | **16.18±4.02** |
| | 1/100 | 2.16±0.31 | 1.19±0.15 | 0.92±0.12 | 2.70±0.94 |
| Klebsiella pneumoniae K25 | Un | 1.70±0.52 | 1.94±0.45 | 1.12±0.20 | 0.56±0.14 |
| | 1/10 | 6.34±0.97 | 5.03±0.54 | **8.48±2.08** | 5.76±1.34 |
| | 1/100 | 2.35±0.47 | 2.03±0.31 | 3.00±0.20 | 2.94±0.63 |
| Escherichia coli 2387 | Un | 0.56±0.03 | 0.84±0.01 | 0.68±0.02 | 1.04±0.04 |
| | 1/10 | 0.66±0.01 | 0.91±0.04 | 0.78±0.03 | 0.96±0.01 |
| | 1/100 | 0.79±0.04 | **1.13±0.03** | 0.93±0.07 | 0.98±0.06 |
| Yersinia enterocolitica 3 | Un | 0.69±0.03 | 0.95±0.03 | 0.43±0.02 | 0.69±0.01 |
| | 1/10 | 0.83±0.04 | 0.86±0.04 | 0.52±0.03 | 0.65±0.01 |
| | 1/100 | 0.80±0.01 | **0.99±0.02** | 0.47±0.01 | 0.62±0.03 |
| Salmonella typhimurium SH4892 | Un | 0.84±0.03 | 1.07±0.04 | 0.97±0.08 | 0.86±0.04 |
| | 1/10 | 1.92±0.06 | 2.70±0.58 | **3.81±0.72** | 1.70±0.30 |
| | 1/100 | 0.79±0.08 | 1.57±0.08 | 1.30±0.13 | 0.93±0.03 |
| Pseudomonas aeruginosa | Un | 1.78±0.04 | 1.68±0.12 | 1.64±0.25 | 0.77±0.08 |
| | 1/10 | **2.73±0.14** | 1.38±0.13 | 1.4-±0.04 | 1.03±0.09 |
| | 1/100 | 1.37±0.06 | 1.21±0.06 | 1.15±0.09 | 0.96±0.02 |

Table 21 Time course and dose response of lymphocyte transformation by soluble antigen(s) in the supernatant of heat treated bacteria.

Optimum SI underlined.
Un - Undiluted.

### 4.3.3 Unstimulated cultures

The mean cpm ± SEM of unstimulated PBL from AS, CD, RA patients and normal controls was 147 ± 21, 116 ± 17, 101 ± 11 and 112 ± respectively. The responses for the 4 day cultures were not significantly different thereby permitting the application of the stimulation index to compare group results.

### 4.3.4 Lymphocyte transformation responses to formalin treated bacteria

The mean stimulation indices for each of the patient groups with respect to each of the bacterial antigens are shown in Table 22. The highest proliferative response occurred against K.pneumoniae K43. Lymphocytes for RA patients had a significantly lower in vitro proliferative response to both K.pneumoniae K43 and K25 ($p < 0.05$). Lymphocyte responses from CD patients were also significantly lower than normal controls to K.pneumoniae K25 ($p < 0.05$). No other differences were apparent for the response of patients' lymphocytes to these antigens when compared to controls.

Banck and Forsgren (1978) divided the response of lymphocytes to bacterial antigens into groups depending on their stimulation index. A SI > 20 indicated a strong effect, SI of 5 - 20 a moderate effect, SI 3 - 5 a weak effect and an SI < 3 essentially no effect. From this the results to formalin treated bacteria show K.pneumoniae K43 to have a moderate effect on lymphocyte transformation whereas the other bacteria studied produced essentially no response.

| | AS | CD | RA | C |
|---|---|---|---|---|
| Klebsiella pneumoniae K43 | 6.15+1.41 (0.1-18.19) | 6.18+1.42 (0.61-24.74) | *3.92+0.93 (0.43-17.6) | 10.57+2.65 (0.1-41.95 |
| Klebsiella pneumoniae K25 | 2.75+0.49 (0.32-8.16) | *1.97+0.23 (0.53-4.09) | *1.84+0.23 (0.46-3.69) | 3.08+0.39 (0.61-6.55) |
| Esherichia coli 2387 | 1.78+0.24 (0.28-4.75) | 1.46+0.18 (0.43-4.18) | 1.26+0.13 (0.39-2.35) | 1.59+0.16 (0.71-2.9) |
| Yersinia enterocolitica 3 | 3.54+0.99 (0.05-16.59) | 2.19+0.38 (0.56-7.21) | 1.48+0.17 (0.44-3.3) | 2.11+0.32 (0.44-6.87) |
| Salmonella typhimurium SH4392 | 4.73+1.12 (0.26-19.31) | 2.70+0.59 (0.71-11.57) | +1.95+0.33 (0.16-5.37) | 4.19+0.82 (0.13-10.92) |
| Pseudomonas aeruginosa | 1.86+0.21 (0.83-3.99) | 1.59+0.17 (0.65-3.65) | 3.18+1.13 (0.56-16.69) | 2.1+0.25 (0.89-4.74) |

Table 22 Lymphocyte transformation by formalin treated gram-negative bacteria in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

* significant difference from controls $p < 0.05$.
\+ AS > RA $p < 0.05$.

### 4.3.5 Lymphocyte transformation responses to heat treated bacteria

The SI for the *in vitro* proliferative response of lymphocytes to heat treated bacteria are shown in Table 23. The highest response in all the four groups occurred with *K.pneumoniae* K43. Lymphocytes from AS patients did not respond any differently from normal controls and CD patients' lymphocytes only had a lower response to *Ps.aeruginosa* ($p < 0.05$). However, a significantly decreased transformation by *K.pneumoniae* K43 and K25 ($p < 0.05$), *Y.enterocolitica* ($p < 0.005$) and *S.typhimurium* ($p < 0.02$) was seen for RA lymphocytes. *K.pneumoniae*, again, produced a moderate effect. The responses to the other bacteria was virtually nil.

### 4.3.6 Lymphocyte transformation responses to soluble antigen(s) released into the supernatant of heat treated bacteria

The mean stimulation indices for each patient group with respect to each of the soluble antigen preparations are shown in Table 24.

Lymphocytes from RA patients had a significantly lower *in vitro* proliferative response to all of the bacterial antigens (*K.pneumoniae* K43 and K25, $p < 0.02$; *Y.enterocolitica*, $p < 0.005$; *E.coli* ($p < 0.005$) and *S.typhimurium* ($p < 0.002$).

Lymphocytes from AS patients had comparable results to controls except to *Ps.aeruginosa* where the response was lower ($p < 0.05$).

| | AS | CD | RA | C |
|---|---|---|---|---|
| Klebsiella pneumoniae K43 | 8.91+1.76 (0.87-25.06) | 12.63+3.29 (0.12-47.74) | ‡*6.22+1.74 (0.21-21.74) | 19.33+4.14 (0.14-59.83) |
| Klebsiella pneumoniae K25 | 2.69+0.59 (0.23-9.29) | 1.79+0.25 (0.5-5.36) | *1.57+0.27 (0.15-3.95) | 3.13+0.52 (0.1-7.73) |
| Esherichia coli 2387 | 2.27+0.37 (0.28-6.09) | 1.43+0.14 (0.5-3.48) | +1.3+0.18 (0.3-3.31) | 1.79+0.27 (0.14-4.84) |
| Yersinia enterocolitica 3 | 3.76+0.88 (0.8-15.1) | ●1.86+0.28 (0.58-5.05) | ***1.31+0.19 (0.26-3.3) | 2.29+0.43 (1.01-6.6) |
| Salmonella typhimurium SH4892 | 4.7+0.88 (0.06-12.78) | 4.65+0.99 (0.07-16.14) | ***3.78+1.46 (0.1-30.52) | 10.11+1.95 (0.19-27.62) |
| Pseudomonas aeruginosa | 2.32+0.28 (0.88-7.33) | *1.34+0.14 (0.11-2.53) | 2.56+0.83 (0.48-17.71) | 2.21+0.31 (0.26-6.93) |

Table 23 Lymphocyte transformation by heat treated gram-negative bacteria in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

* significant difference from controls $p < 0.05$.
** $p < 0.02$. *** $p < 0.05$.
‡ AS > RA $p < 0.001$.
\+ CD > RA $p < 0.05$.
● AS > CD $p < 0.05$.

| | AS | CD | RA | C |
|---|---|---|---|---|
| Klebsiella pneumoniae K43 | 3.16±0.6 (0.81-11.86) | 2.96±0.83 (0.1-15.41) | **2.18±0.46 (0.33-8.86) | 3.86±0.55 (0.35-8.67) |
| Klebsiella pneumoniae K25 | 1.61±0.19 (0.37-3.72) | 1.37±0.17 (0.63-3.4) | †**1.22±0.18 (0.24-3.43) | 2.01±0.31 (0.33-5.69) |
| Escherichia coli 2387 | 2.15±0.33 (0.9-6.8) | ***1.63±0.27 (0.63-6.39) | ****1.47±0.15 (0.58-2.92) | 2.90±0.35 (1.09-6.15) |
| Yersinia enterocolitica 3 | 6.4±2.31 (0.25-37.8) | 3.08±0.95 (0.91-19.35) | ***‡1.4±0.21 (0.21-3.48) | 4.96±1.25 (0.14-23.28) |
| Salmonella typhimurium SH4892 | 6.47±2.26 (0.46-34.27) | ****2.11±0.38 (0.39-6.7) | *****†1.64±0.32 (0.23-5.94) | 7.06±1.97 (1.21-37.69) |
| Pseudomonas aeruginosa | *3.03±0.46 (0.84-7.45) | *3.7±1.31 (0.97-27.43) | ****2.61±0.83 (0.95-17.8) | 4.92±0.77 (1.08-13.44) |

Table 24 Lymphocyte transformation by soluble antigen(s) released into the supernatant of heat treated gram-negative bacteria in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

* significant difference from controls $p < 0.05$.
** $p < 0.02$, *** $p < 0.005$, **** $p < 0.002$,
***** $p < 0.001$.
AS > RA † $p < 0.05$ ‡ $p < 0.02$ ‡ $p < 0.005$.

## 4.4 Discussion

This study found no differences in the responses of AS PBL to various bacterial antigens when compared to controls except for the response to antigen(s) released into the supernatant of heat treated Pseudomonas aeruginosa, which was lower. PBL from CD patients showed a decreased transformation to formalin treated K.pneumoniae K25, heat treated Ps.aeruginosa and to the supernatants from heat treated E.coli, S.typhimurium and Ps.aeruginosa. The greatest differences in responses was shown by PBL from RA patients especially to the soluble antigens released into the supernatants of all the heat treated bacteria. These diminished responses may be due to a lowered responsiveness of T cells or to an abnormal processing of antigen by macrophages. It could also indicate a lack of B cell function to bacterial antigens as bacteria are said to be mitogenic for B lymphocytes (Banck and Forsgren 1978). As the lowered responses were seen mainly with the supernatants of heat treated bacteria this could mean that the antigens are normally hidden by other antigens (eg the capsular (K) antigen) when the whole cell cell is being used as a stimulant. Only with heat are they released from the bacterial cells. Such antigens could be the 'O' somatic antigen, ECA or some other component of the cell. However, the response is not likely to be due to ECA alone as Ps.aeruginosa does not possess ECA. Ps.aeruginosa heat treated supernatant was the only bacterial antigen that failed to stimulate a response in all the three patient groups which suggests that such an antigen(s) is either not being recognised by the lymphocytes or is similar to some self antigen in which case it will not be recognised as foreign.

Banck and Forsgren (1978) have studied the mitogenic capacity of more than 30 bacterial species for human B and T lymphocytes. They found the majority of formalin killed bacteria produced a polyclonal B cell response. This response was mitogenic and not specific as it occurred after 3-4 days. There was virtually no activation of T cells and even the B cell response was poor with such bacteria as E.coli and Klebsiella sp. Räsänen et al (1980), however, have found that some bacterial strains induce a proliferative response in both human B and T lymphocytes after 4 days incubation although the bacterium-induced transformation seemed to be rather weak. They suggested that in vitro transformation may be an artifact with no in vivo relevance. However, animal experiments with bacterial products have shown an induction of auto-antibody production (Fournie, Lambert and Miescher 1974; Hammerström et al 1976) and in several infectious diseases in man a polyclonal antibody response has been detected (Fudenberg et al 1976). The significance of the polyclonal lymphocyte response in microbial, infections is unclear. Petit and Unanue (1974) suggested non-specific activation represents an early mechanism of protection against micro-organisms. Yet polyclonal proliferation of lymphocytes together with subsequent antibody production would seem harmful if auto-antibodies are formed. In infectious diseases, depending on the host's immune reactivity, specific antibody or a variety of antibodies are detected. A specific antibody response will often dominate even if the stimulant is capable of polyclonal activation. The mechanism by which cells are able to escape from polyclonal antibody production, if non-specifically activated, are not clear. Perhaps in diseases where there is thought to be some auto-immunity (such as AS) the non-specific polyclonal antibody production is not able to be switched to

a specific antibody response and auto-antibodies may then result.

The in vitro proliferative responses of PBL from all the groups in this study were all poor even though some differences were detected from normal controls. The time course experiments on normal controls resulted in a peak response after 4 days therefore this incubation period was used for the other patient groups. Perhaps a longer incubation time is required to produce a specific T cell response, even though it has been shown that there is virtually no T cell response to bacteria in normals (Banck and Forsgren 1978). The low results may also mean that the patients' cells had not been sensitised to these particular antigens. However, it is almost certain that the patients would have met these bacteria before. Perhaps, peripheral blood lymphocytes are not the right cells to be studying. Ford et al have studied the responses of synovial lymphocytes from patients with sexually transmitted and enteric reactive arthritis (Ford, da Roza and Shah 1981; Ford, da Roza and Schulzer 1982; Ford 1983). Lymphocytes from patients with sexually transmitted ReA responded to Chlamydia and Ureaplasma but not to bacteria associated with enteric ReA. Lymphocytes from patients with enteric ReA responded to enteric antigens only. Synovial lymphocytes from RA patients did not respond to Chlamydia or Ureaplasma and there was only a slight response to Salmonella. Of the lymphocytes from synovial fluid from AS patients none responded to Klebsiella. Comparing synovial fluid and peripheral blood lymphocytes greater responses were seen with lymphocytes from the synovial fluid. They concluded that peripheral blood lymphocytes were poor and unreliable indicators of the cause of sexually transmitted and enteric ReA. The synovial fluid lymphocytes, however,

can recognise the precipitating microbiological cause. These findings may be of diagnostic value when clinical and microbiological evidence is lacking especially in patients with arthritis restricted to the knee joints and without intestinal or gastrointestinal symptoms. This may also be the case in AS where the main symptoms are in the spine and sacro-iliac joint. Lymphocytes around these sites may be more reactive to specific antigens than those from peripheral blood.

Chapter 5

# Humoral Immunity to Klebsiella pneumoniae

## 5.1 Introduction

It has been shown that in patients with Yersinia-induced reactive arthritis high titres of serum IgA antibodies to Yersinia persisted much longer than in those who did not have arthritis and that the titres correlated with disease severity in the presence of HLA-B27 (Granfors 1979; Granfors et al 1980). In view of the seemingly elevated serum IgA levels in AS and the presence of Klebsiella in the gastrointestinal tract Trull et al (1983a) decided to investigate the possibility that part of this increase might be due to an IgA immune response to a micro-organism. They found that AS patients with active disease (ESR > 15mm/hr) had elevated serum IgA anti-Klebsiella antibody levels when compared to patients with inactive disease or healthy controls. The antibody could be absorbed out from the serum by Klebsiella pneumoniae cells but not by Pseudomonas aeruginosa cells. They suggested that patients with AS were reacting in a similar immunological manner to some micro-organism present in their gastrointestinal tract as patients with ReA do following infection by known precipitating antigens. Further studies showed changes in the immune response to Klebsiella in the saliva of AS patients. The secretory IgA immune response to Klebsiella was elevated in 39% of patients with AS although this was not associated with an increase in CRP or disease activity (Trull and Panayi 1983b).

The present study set out to investigate the humoral immune response to Klebsiella in AS and other inflammatory disorders.

## 5.2 Materials and methods

### 5.2.1 Patient groups

Antibodies to Klebsiella pneumoniae K43 were measured in the serum of patients with ankylosing spondylitis, Crohn's disease and rheumatoid arthritis. Healthy laboratory and hospital staff were used as controls. Clinical details of the groups are given in Table 25.

### 5.2.2 Bacterial antigens

Klebsiella pneumoniae K43 (NCTC 9163) was grown overnight (O/N) in minimal salts medium supplemented with 2g/l glucose. The bacteria were centrifuged (1540g, 20 mins), the supernatant decanted and filtered through a cellulose acetate filter (0.45μm, Millipore) and concentrated to a final concentration of 1mg/ml using a total protein kit from Sigma. This was used as the culture filtrate antigen preparation (CF). The bacterial pellet was washed in 0.9% saline and inactivated with 1% buffered formalin O/N. The bacterial suspension was then washed and resuspended in saline at $10^8$ cfu/ml (FT antigen). Pellets were also sonicated (12 microns, 10 minutes), centrifuged, the supernatant decanted and adjusted to a concentration of 1mg/ml protein (SON antigen). All antigen preparations were stored at -20°C until required.

| | AS | CD | RA | C |
|---|---|---|---|---|
| No of patients | 65 | 32 | 25 | 35 |
| Mean age (yrs) | 41.9 | 39.6 | 60.6 | 26.1 |
| (range) | (22-68) | (14-68) | (26-82) | (19-50) |
| Sex | M = 54 | M = 14 | M = 5 | M = 18 |
| | F = 11 | F = 18 | F = 20 | F = 17 |
| Drug therapy | NSAID 53<br>None/occasional NSAID 12 | None 15<br>Sulphasalazine 11<br>NSAID 13<br>Steroids 1 | NSAID 18<br>Steroids 5<br>Gold 2 | Not applicable |
| Joint disease | Peripheral joint involvement 39 | AS 3<br>Peripheral arthropathy 2<br>Peripheral arthritis 2<br>Sacroiliitis 1 | All patients | Not applicable |

Table 25 Clinical details of patients and controls in the study of the humoral immune response to Klebsiella pneumoniae.

### 5.2.3 Enzyme linked immunosorbent assay

Before use, the formalin treated (FT) bacteria were centrifuged and resuspended to the original concentration in 0.2M sodium bicarbonate-carbonate buffer (pH 8.6). The CF and sonicated (SON) antigen preparations were diluted in the above buffer to 0.05mg/ml protein. 200µl of each antigen was absorbed onto flat bottomed microtitre plates (Sterilin) O/N at 37°C. The suspensions were decanted and the wells washed four times in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20, 0.5% BSA and 0.05% sodium azide. Serum samples were diluted in PBS (with 0.5% BSA and 0.05% sodium azide) and 100µl samples added in triplicate to the wells. Control wells containing serum alone were included for each sample. A control serum was also added to each plate in every assay and if this result varied by more than 15% the test samples' results were discarded and the assay repeated.

After 2 hours incubation at 37°C the plates were washed and 100µl of goat anti-human IgA, IgG or IgM alkaline phosphatase conjugate (Sigma), diluted 1/1000 in PBS was added to each well and incubated for a further 2 hours at 37°C. The plates were then washed again and 100µl of p-nitrophenol (PNP) substrate (Sigma) added and the colour developed for 30 minutes at 37°C. The reaction was stopped with the addition of 100µl 3M NaOH. The absorbance was measured spectrophotometrically on a Titertek Multiskan at 405nm.

Results were obtained by substracting the absorbance of serum in untreated wells from the absorbance of serum in the treated wells.

Comparison of results between the different groups was analysed using the Wilcoxon rank sum test.

### 5.2.4 Asborption studies

The specificity of the ELISA was assessed by absorption studies. 1ml of $10^9$ cfu/ml formalin treated bacteria was centrifuged and to the pellet 1ml of serum, diluted 1/10 in PBS, added. A control serum was prepared without any bacteria. The serum was then mixed and incubated O/N at $4^{\circ}C$ on a Rolamix (Luckham Ltd). The bacteria were removed by centrifugation and the sera retested. Results are expressed as % absorption -

$$\% \text{ Absorption} = \frac{\text{A405nm after absorption}}{\text{A405nm pre-absorption}} \times 100\%$$

## 5.3 Results

### 5.3.1 Optimal sera concentrations

Using formalin treated bacteria at $10^8$ cfu/ml normal serum concentrations were varied to obtain the dilution that gave an absorbance at 405nm of approximately 1.0. An absorption of 1.0 was chosen as this is half the maximum absorption able to be measured by the spectrophotometer and also because any differences in patients' serum from normal levels would be detected more easily. Table 26 expresses the results from 3 normal controls.

The serum dilutions that gave the most appropriate results are 1/10, 1/500 and 1/100 for IgA, IgG and IgM respectively. These serum dilutions were therefore used in subsequent experiments.

### 5.3.2 Optimal antigen concentrations

Using the above serum dilutions the optimal response to each antigen was obtained by varying the antigen concentration. The responses from three normal controls are given in Table 27. There was little variation in immunoglobulin levels to various concentrations of CF and SON antigens. A concentration of 0.05mg/ml protein was therefore chosen to be used in subsequent experiments. Similar results were also found with the higher concentrations of FT antigen. The lower of the two, ie $10^8$ cfu/ml was therefore used in further experiments.

| | Absorbance (A405nm) | | | |
|---|---|---|---|---|
| Serum dilution | 1/10 | 1/100 | 1/500 | 1/1000 |
| IgA | 1.236 ± 0.050 | 0.574 ± 0.068 | 0.242 ± 0.035 | 0.126 ± 0.025 |
| IgG | 1.785 ± 0.025 | 1.461 ± 0.038 | 1.064 ± 0.081 | 0.820 ± 0.076 |
| IgM | 1.660 ± 0.053 | 0.954 ± 0.084 | 0.419 ± 0.034 | 0.254 ± 0.049 |

Table 26 Antibody levels to formalin treated Klebsiella in dilutions of normal control serum (mean ± SEM).

| | Antigen concentration | | | |
|---|---|---|---|---|
| | Culture filtrate (mg/ml protein) | | | |
| | 0.25 | 0.1 | 0.05 | 0.025 |
| IgA | 0.148±0.032 | 0.16±0.042 | 0.152±0.031 | 0.146±0.023 |
| IgG | 0.172±0.054 | 0.199±0.058 | 0.207±0.07 | 0.188±0.062 |
| IgM | 0.244±0.026 | 0.337±0.039 | 0.458±0.06 | 0.453±0.01 |
| | Sonicate (mg/ml protein) | | | |
| | 0.25 | 0.1 | 0.05 | 0.025 |
| IgA | 0.342±0.038 | 0.312 0.041 | 0.334±0.054 | 0.385±0.061 |
| IgG | 0.739±0.089 | 0.651±0.088 | 0.659±0.125 | 0.707±0.137 |
| IgM | 0.64±0.098 | 0.945±0.028 | 1.028±0.077 | 1.012±0.222 |
| | Formalin treated (cfu/ml) | | | |
| | $10^9$ | $10^8$ | $10^7$ | $10^6$ |
| IgA | 0.448±0.26 | 0.398±0.175 | 0.328±0.099 | 0.182±0.035 |
| IgG | 0.906±0.128 | 0.826±0.028 | 0.754±0.198 | 0.379±0.17 |
| IgM | 1.257±0.031 | 1.258±0.036 | 1.152±0.141 | 0.79±0.072 |

Table 27 The effect of antigen concentrations on levels of antibody to Klebsiella.

### 5.3.3 IgA antibodies to Klebsiella pneumoniae

The IgA responses to Klebsiella CF are shown in Figure XX. The mean ± SEM values were 0.565 ± 0.045, 0.617 ± 0.102, 0.425 ± 0.076 and 0.428 ± 0.045 for AS, CD and RA patients and normal controls respectively. There was no significant difference between any of the patient groups and the controls.

The IgA responses to SON antigen are shown in Figure XXI. The mean ± SEM values for AS, CD and RA patients and normal controls were 0.651 ± 0.044, 0.896 ± 0.095, 0.661 ± 0.082 and 0.473 ± 0.057 respectively. The differences between the three patients groups and the controls were significantly raised (AS, $p < 0.002$; CD, $p < 0.001$; RA, $p < 0.05$). Anti-Klebsiella IgA was also raised in CD patients when compared to AS patients ($p < 0.05$).

The IgA responses to FT antigen are shown in Figure XXII. The mean ± SEM values were 0.764 ± 0.031, 0.915 ± 0.096, 0.746 ± 0.076 and 0.492 ± 0.05 for AS, CD and RA patients and normal controls respectively. All three patient groups had significantly raised IgA antibody levels compared to normal controls (AS, $p < 0.001$; CD, $p < 0.002$; RA, $p < 0.02$).

### 5.3.4 IgG antibodies to Klebsiella pneumoniae

Figure XXIII shows the IgG antibody levels to the CF antigen. The mean ± SEM for each patient group and normal controls was AS, 0.22± 0.026; CD, 0.272 ± 0.047; RA, 0.15 ± 0.03 and C, 0.206 ± 0.033. There

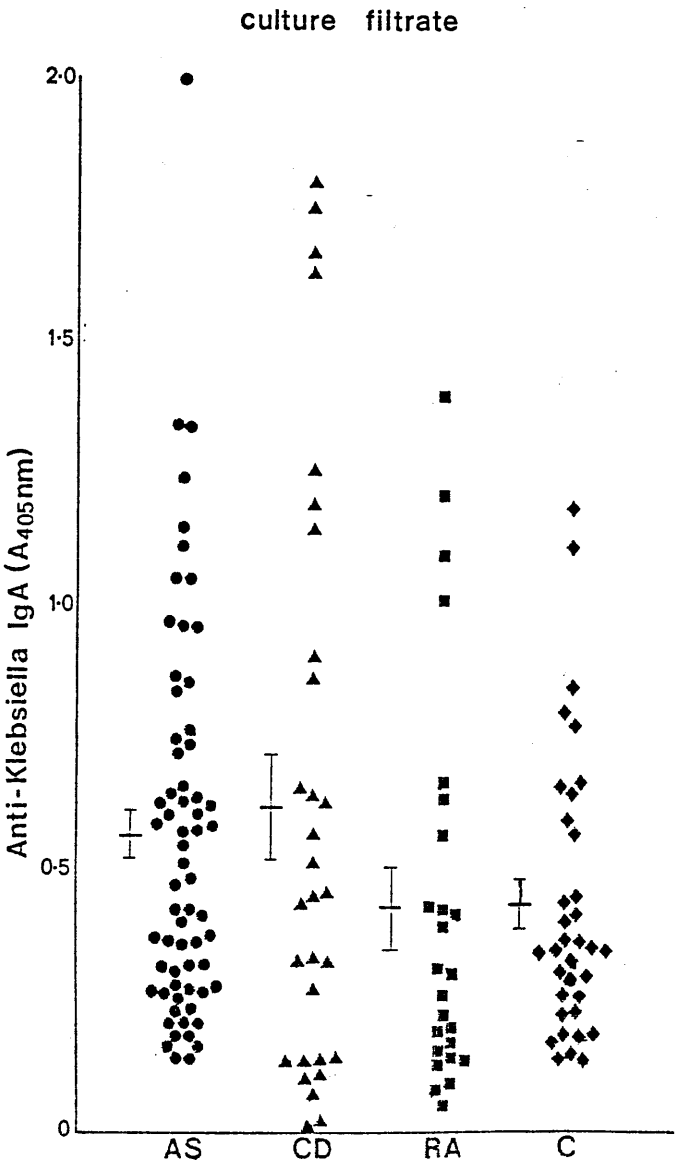



Fig XX Serum IgA antibody levels (mean ± SEM) to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spodylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal control (C).

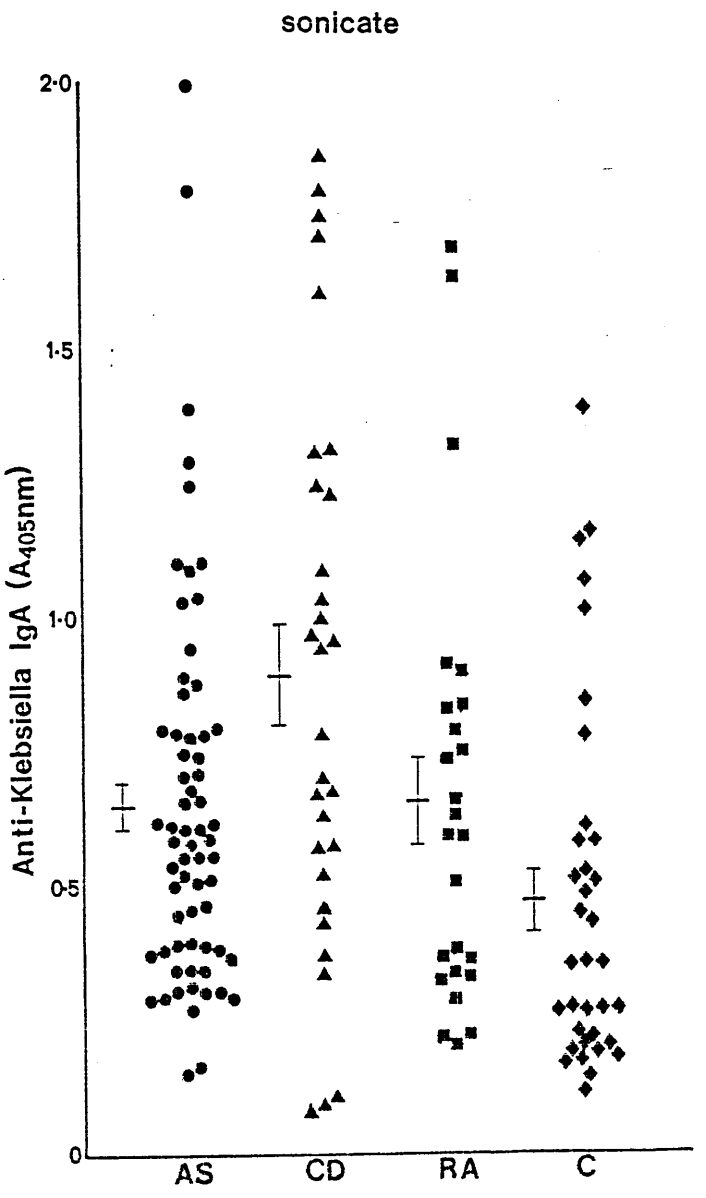



Fig XXI Serum IgA antibody levels (mean ± SEM) to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

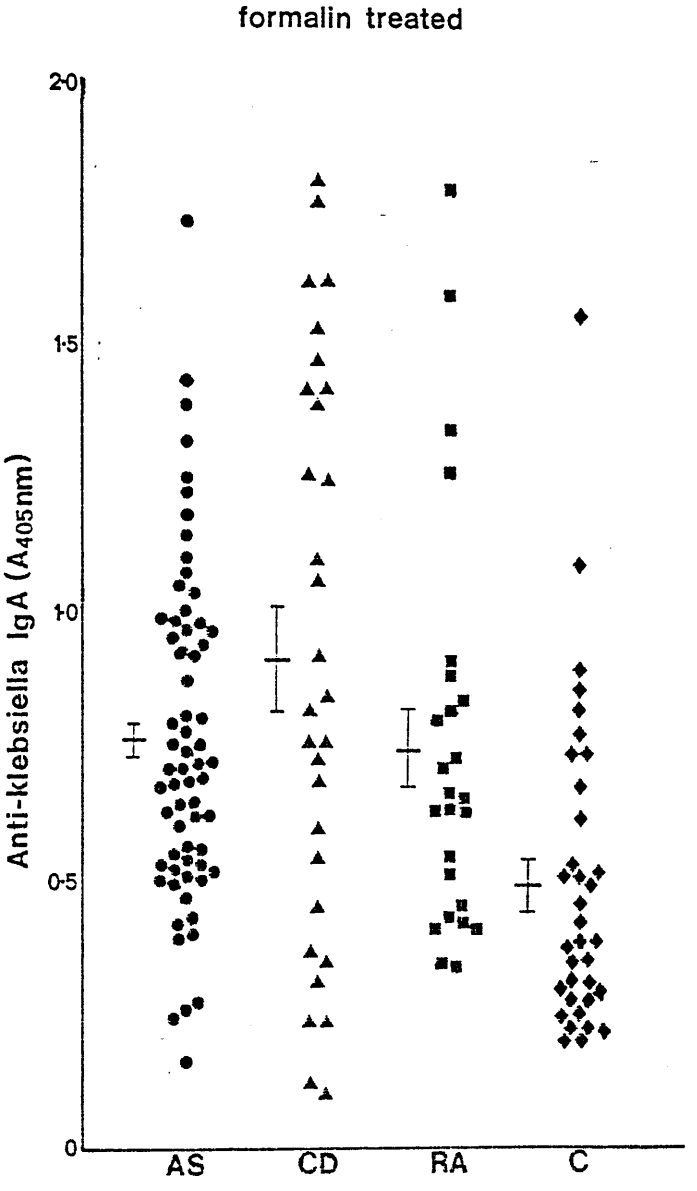



Fig XXII Serum IgA antibody levels (mean ± SEM) to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

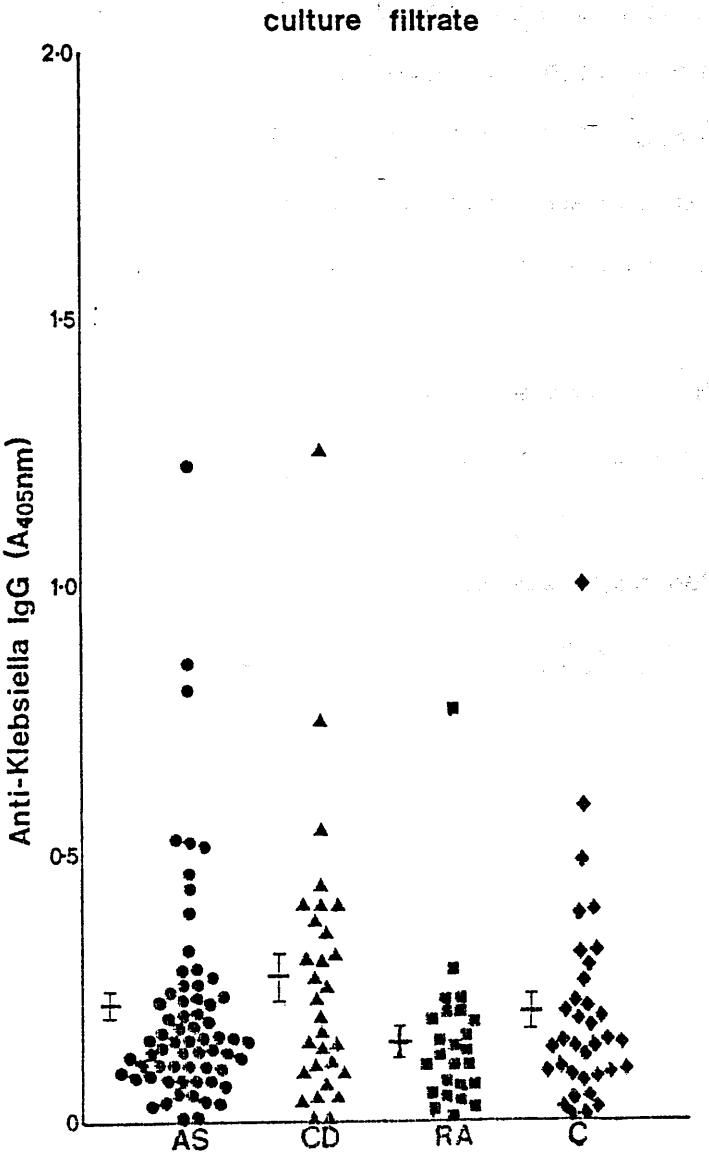



Fig XXIII Serum IgG antibody levels (mean ± SEM) to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

was no statistically significant difference between any of the patient groups and the control group.

The IgG responses to the SON antigen are shown in Figure XXIV. The mean ± SEM values for each group was AS, 0.947 ± 0.058; CD, 0.929 ± 0.105; RA, 0.808 ± 0.047 and C, 0.727 ± 0.067. None of the patient groups were significantly different from the normal controls except the AS patients who had slightly raised levels ($p < 0.05$).

The IgG antibody levels to FT antigen are shown in Figure XXV. The mean ± SEM values for the AS, CD, RA and normal control groups were 0.764 ± 0.039, 0.82 ± 0.086, 0.746 ± 0.09 and 0.648 ± 0.052 respectively. No significant difference was found between any of the groups.

### 5.3.5 IgM antibodies to Klebsiella pneumoniae

Figure XXVI shows the IgM antibody responses to CF antigen. The mean ± SEM antibody level for AS, CD and RA patients and normal controls was 0.076 ± 0.013, 0.208 ± 0.047, 0.111 ± 0.27 and 0.174 ± 0.028 respectively. The only difference between the groups was with the AS patients who had significantly lower IgM levels when compared to normal controls ($p < 0.005$).

The IgM antibody responses to SON antigen for patients with AS, CD and RA and normal controls were 0.449± 0.038, 0.809 ± 0.084, 0.636 ± 0.088 and 0.777 ± 0.075 respectively (Figure XXVII). The AS patients had a significantly lower IgM response compared to normal controls and

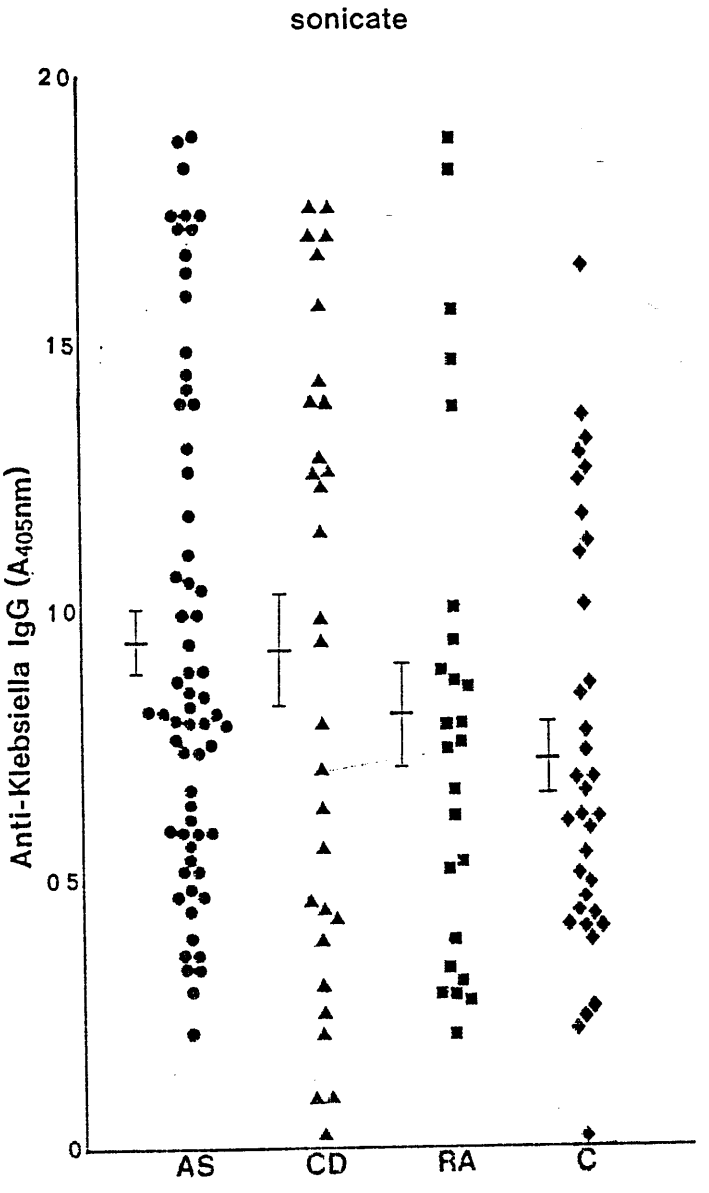



Fig XXIV Serum IgG antibody levels (mean ± SEM) to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

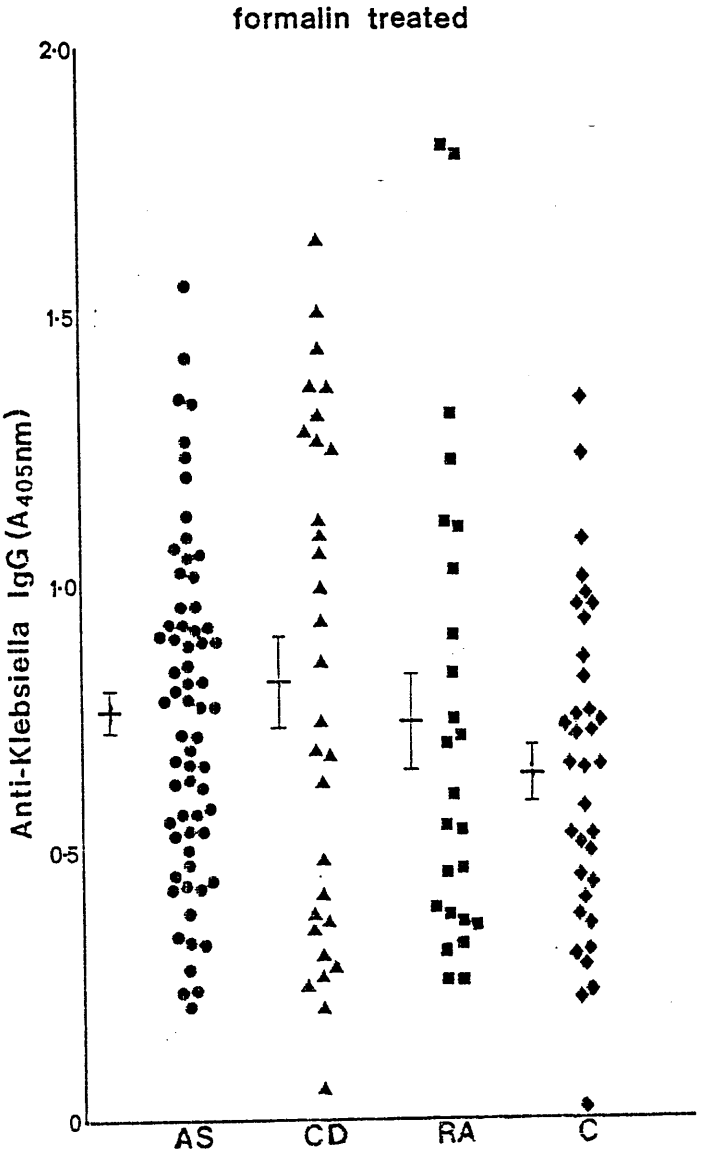



Fig XXV Serum IgG antibody levels (mean ± SEM) to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD, rheumatoid arthritis (RA) and normal controls (C).

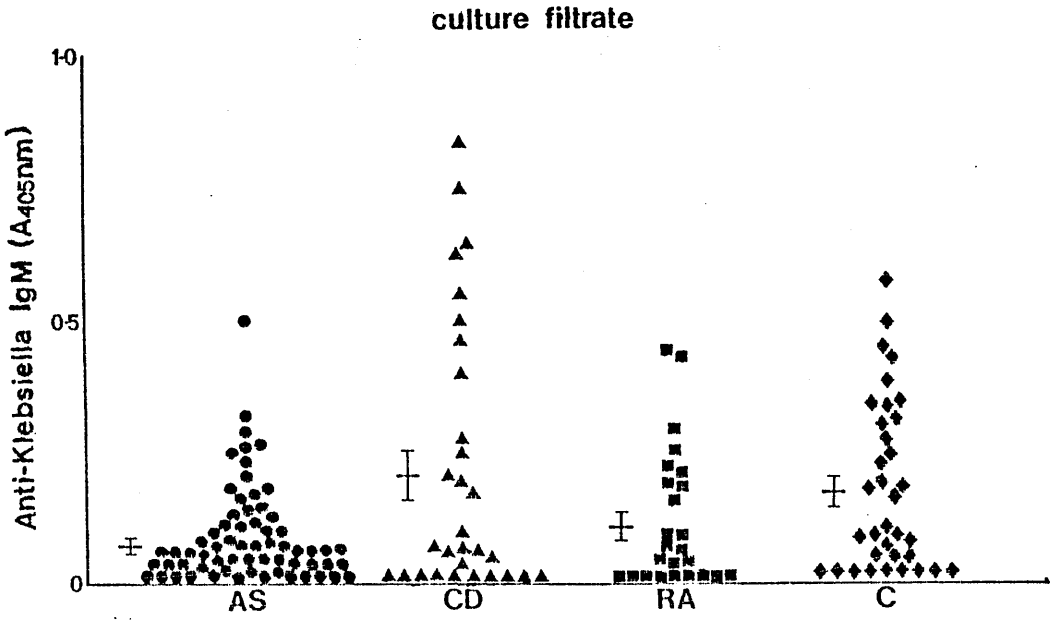



Fig XXVI Serum IgM antibody levels (mean + SEM) to a Klebsiella pneumoniae K43 culture filtrate in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

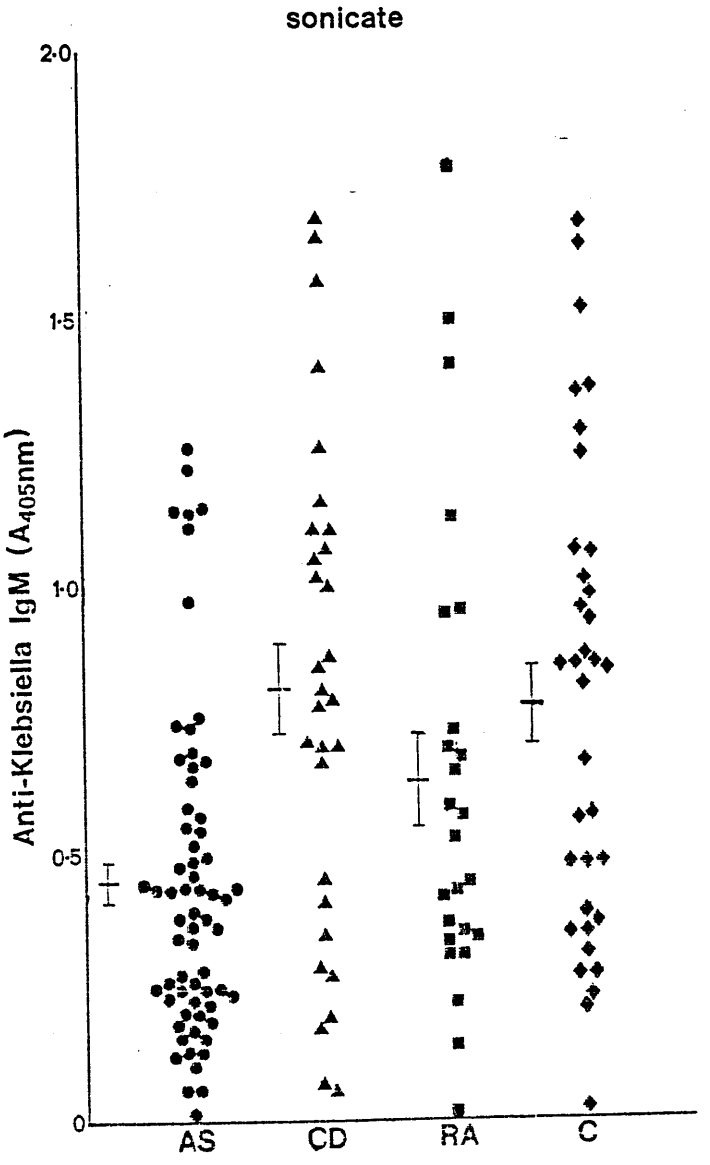



Fig XXVII Serum IgM antibody levels (mean ± SEM) to sonicated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

patients with CD (both $p < 0.001$).

Figure XXVIII shows the IgM antibody levels to FT antigen. The mean $\pm$ SEM values were 0.425 $\pm$ 0.034, 0.922 $\pm$ 0.098, 0.553 $\pm$ 0.085 and 0.72 $\pm$ 0.072 for AS, CD and RA patients and normal controls respectively. AS patients had significantly lowered IgM antibody levels compared to normal controls ($p < 0.001$) and RA patients' IgM antibody levels were lower than those of CD patients ($p < 0.02$).

### 5.3.6 Absorption studies

The absorption of 3 sera from each patient group with Klebsiella FT antigen reduced the level of IgA antibodies whereas absorption with Escherichia coli, Yersinia enterocolitica and Campylobacter jejuni was considerably less (Table 28).

### 5.3.7 Correlation of anti-Klebsiella antibodies with disease activity and laboratory parameters in ankylosing spondylitis patients

AS patients were divided into groups depending on their disease activity. As IgA antibodies to SON and FT Klebsiella antigens in AS patients, were raised it was investigated as to whether this was due to disease activity or peripheral joint involvement (PJI). No significant difference was found in IgA antibody levels to SON and FT antigens between any of the three disease activity groups (Table 29). IgA antibodies to SON antigen were raised in the active ($p < 0.05$) and inactive ($p < 0.005$) groups when compared to normal controls. IgA antibodies to FT Klebsiella were increased in all three AS groups

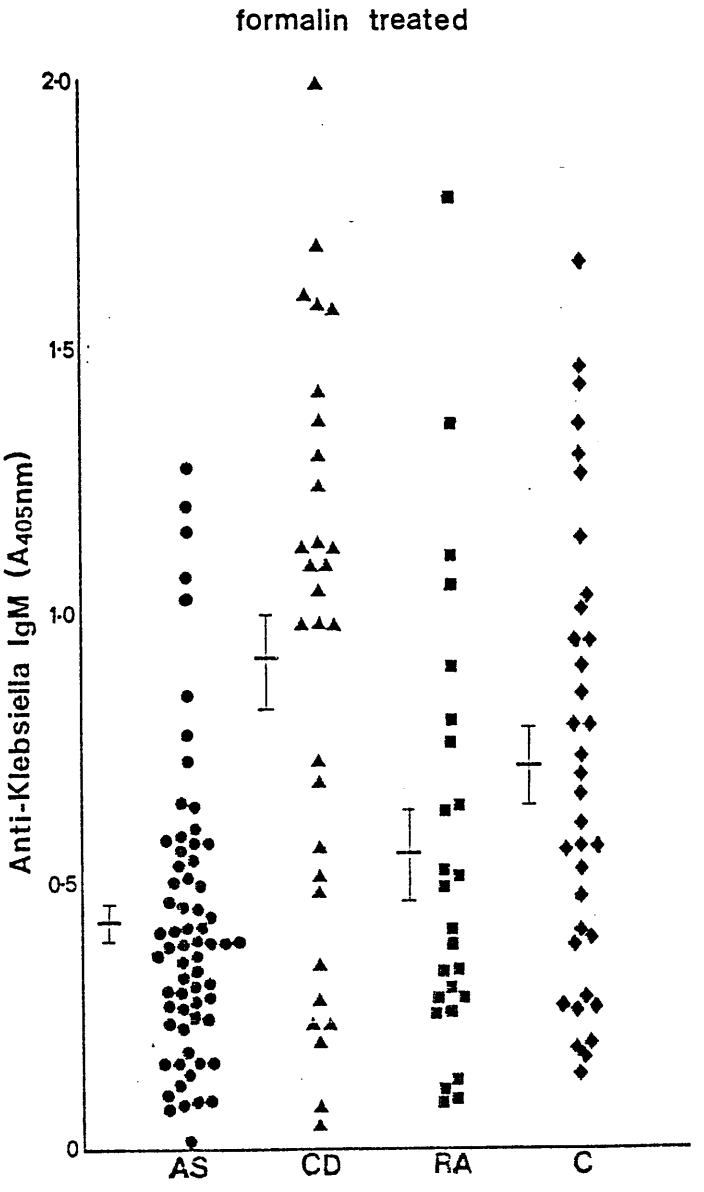



Fig XXVIII Serum antibody levels (mean ± SEM) to formalin treated Klebsiella pneumoniae K43 in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls (C).

| Serum | Absorption (%) with | | | |
|---|---|---|---|---|
| | Klebsiella | E.coli | Yersinia | Campylobacter |
| AS | 80.4 ± 4.0 | 23.3 ± 12.4 | 28.9 ± 6.8 | 22.9 ± 11.9 |
| CD | 60.2 ± 10.2 | 14.4 ± 1.8 | 30.0 ± 9.2 | 23.9 ± 7.1 |
| RA | 73.4 ± 10.4 | 28.9 ± 9.6 | 26.4 ± 8.5 | 21.7 ± 5.4 |

Table 28 Percentage absorption of anti-Klebsiella IgA antibodies by whole formalin treated bacteria.

| | Anti-Klebsiella IgA (A405nm) | | | |
|---|---|---|---|---|
| Antigen | A (n = 20) | PA (n = 20) | IN (n = 19) | C (n = 35) |
| SON | *0.649 ± 0.094 | 0.586 ± 0.064 | *0.698 ± 0.058 | 0.473 ± 0.057 |
| FT | *0.848 ± 0.084 | *0.67 ± 0.063 | *0.744 ± 0.059 | 0.492 ± 0.05 |

Table 29 Comparison of anti-Klebsiella IgA antibody levels and disease activity in ankylosing spondylitis (mean ± SEM).

* significantly higher than controls.

compared to controls (A, $p < 0.001$; PA, $p < 0.02$; IN, $p < 0.001$).

Patients with PJI did not have significantly different anti-Klebsiella IgA antibody levels from those without PJI (Table 30).

No correlation was found between IgA antibody levels to SON and FT Klebsiella and ESR, CRP or total IgA levels (Table 31).

The lowered anti-IgM antibody levels were also investigated to find if they were due to disease activity (Table 32). There was no differences in anti-Klebsiella IgM levels between the three AS groups. All the AS groups had lowered anti-Klebsiella IgM levels when compared to controls except for antibodies to the Klebsiella culture filtrate in the active AS patients.

No correlation was found between anti-Klebsiella IgM, CRP and ESR although a positive correlation was found between anti-SON/FT Klebsiella IgM and total IgM levels (Table 33).

Having found total serum IgM levels to be raised in AS patients with PJI (Section 1.3.4) a comparison of anti-Klebsiella IgM antibodies was made between patients with and without PJI (Table 34). No difference in anti-Klebsiella IgM antibody levels was found between the two groups of patients.

| | Anti-Klebsiella IgA (A405nm) | |
|---|---|---|
| Antigen | PJI (n = 39) | No PJI (n = 26) |
| SON | 0.611 ± 0.045 | 0.661 ± 0.075 |
| FT | 0.766 ± 0.048 | 0.747 ± 0.066 |

Table 30 Comparison of anti-Klebsiella IgA antibody levels in ankylosing spondylitis patients with and without peripheral joint involvement.

| | ESR | CRP | Total IgA |
|---|---|---|---|
| Anti-SON IgA | -0.086 | -0.288 | -0.204 |
| Anti-FT IgA | -0.01 | 0.064 | 0.082 |

Table 31 Correlation of anti-Klebsiella IgA with laboratory parameters (expressed as r values).

| | Anti-Klebsiella IgM (A405nm) | | | |
|---|---|---|---|---|
| Antigen | A (n = 22) | PA (n = 20) | IN (n = 19) | C (n = 35) |
| CF | 0.091±0.025 | *0.081±0.023 | **0.055±0.019 | 0.186±0.033 |
| SON | **0.455±0.063 | **0.466±0.066 | ***0.434±0.074 | 0.777±0.075 |
| FT | **0.404±0.058 | **0.418±0.061 | *0.438±0.055 | 0.72±0.072 |

Table 32 Comparison of anti-Klebsiella IgM antibody levels and disease activity in ankylosing spondylitis (mean ± SEM).

* significantly lower compared to controls.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

| | ESR | CRP | Total IgM |
|---|---|---|---|
| Anti-CF IgM | 0.06 | 0.233 | 0.213 |
| Anti-SON IgM | 0.095 | 0.141 | 0.467 ($p < 0.001$) |
| Anti-FT IgM | -0.026 | 0.134 | 0.398 ($p < 0.01$) |

Table 33 Correlation of anti-Klebsiella IgM with laboratory parameters, expressed as r values (significance level).

| | Anti-Klebsiella IgM (A405nm) | |
|---|---|---|
| Antigen | PJI (n = 39) | No PJI (n = 26) |
| CF | 0.08 ± 0.014 | 0.064 ± 0.024 |
| SON | 0.482 ± 0.051 | 0.396 ± 0.058 |
| FT | 0.444 ± 0.042 | 0.396 ± 0.061 |

Table 34 Comparison of anti-Klebsiella IgM antibody levels in ankylosing spondylitis patients with and without peripheral joint involvement (mean ± SEM).

## 5.4 Discussion

The present study investigated the immune response to Klebsiella pneumoniae K43 in the serum of patients with various inflammatory disorders. The results showed that IgA antibodies to sonicated and formalin treated bacteria are raised in the serum of patients with ankylosing spondylitis, Crohn's disease and rheumatoid arthritis when compared to normal healthy controls. Anti-Klebsiella IgA antibodies to a culture filtrate preparation and anti-Klebsiella IgG antibodies were not increased in these patients. As there was no increase in IgA antibodies to the culture filtrate it may be presumed that Klebsiella does not release antigen(s) from its cell that are able, by itself, to cause a specific immune response in the patients studied. The presence of normal levels of IgG but raised IgA antibodies suggests that the response is occurring in the gastrointestinal tract of these patients. The increase in these IgA levels may be due to an increased permeability of the gut to bacterial antigens leading to an increased gut mucosal IgA response allowing greater release of IgA into the circulation. As the increased antibody levels were seen in Crohn's disease and rheumatoid arthritis patients as well as AS patients there may be a non-specific response occurring whenever there is a gut disorder. In Crohn's disease there is chronic intestinal inflammation and the use of non-steroidal anti-inflammatory drugs (NSAIDs) in rheumatoid arthritis may cause increased bowel permeability. The increase of serum IgA in AS patients may be due to the effects of NSAIDs and/or low grade inflammatory bowel disease.

Trull et al (1983) found anti-Klebsiella IgA to be raised in patients with active disease as measured by ESR. He also showed a correlation between anti-Klebsiella IgA antibody and CRP levels. This study showed that the increase in anti-Klebsiella IgA antibodies was found in all AS patients irrespective of disease activity or peripheral joint involvement. Absorption studies also confirmed a specific antibody response. There was also no correlation between anti-Klebsiella IgA levels and ESR, CRP or total serum IgA levels.

It was interesting to find that anti-Klebsiella IgM levels were decreased in AS patients. The reason for the lower IgM antibody levels is unclear but could perhaps be due to the formation of immune complexes. Such a possibility could be investigated by measuring Klebsiella specific immune complexes in AS patients as Lahesmaa-Rantala et al (1987) have done with Yersinia specific immune complexes in patients with reactive arthritis.

Anti-Klebsiella IgM antibodies to sonicated and formalin treated bacteria also correlated positively with total serum IgM levels. Earlier I found that total serum IgM levels were raised in patients with peripheral joint involvement. One would therefore expect an increase in anti-Klebsiella IgM antibodies in patients with peripheral arthritis. However, no difference was seen in specific IgM levels between patients with and without peripheral joint involvement. Therefore if the increase in total IgM in patients with peripheral arthritis is due to a recent or ongoing infection it is probably not caused by Klebsiella.

If Klebsiella is involved in ankylosing spondylitis one would expect the raised anti-Klebsiella IgA antibodies to be found specifically in these patients. The fact that raised levels of these antibodies are found in Crohn's disease and rheumatoid arthritis suggests a non-specific immunological response in the intestinal mucosa of patients with some gut abnormality. The elevated antibodies to Klebsiella in ankylosing spondylitis have also been shown not to reflect events associated with active inflammatory disease.

Chapter 6

Antibodies to enterobacterial antigens

## 6.1 Introduction

If there is a non-specific antibody response occurring as a result of increased gut permeability one would expect an increased antibody response to other enteric bacteria. Trull et al (1984) found elevated titres of antibodies to Klebsiella pneumoniae compared to Salmonella, Yersinia and Pseudonomas in AS patients. Ebringer et al (1985c) also reported a selective antibody response to Proteus in rheumatoid arthritis patients which was not observed in patients with AS.

Van Bohemen et al (1983) found that serum from patients with yersiniosis and Yersinia arthritis displayed an IgA response directed towards two cell envelope proteins, both the so called major out membrane proteins (MOMPs), of Yersinia enterocolitica. 2 of 25 AS patients also gave a positive reaction and 5 showed an IgG or IgM antibody response directed towards the major proteins of the same organism. It therefore seems that the major cell envelope proteins of Yersinia may have immunological significance in yersiniosis, Yersinia arthritis and ankylosing spondylitis.

After their initial observations van Bohemen et al studied the antibody reactivities with separated cell envelope antigens of seven incriminated bacteria (van Bohemen et al 1985 and 1986a). Using a gel-immuno-radioassay there was no qualitative differences in

reactivities between separated bacterial cell envelope antigens and serum from patients with AS, AS and acute anterior uveitis, healthy first degree relatives of AS patients or normal healthy controls, ie, no antigens were noted for their exceptional reactivity or lack of reactivity. They also investigated serum antibodies to B27 M1+ bacterial antigens in AS. M1 and M2 are epitopes of the B27 antigen and have been identified on the cell surface of several enterobacteria (van Bohemen et al 1984). The M1 epitope has been identified on three enteric bacterial species - E.coli, K.pneumoniae and Y.enterocolitica. Initial studies of reactivity of the three M1 epitopes with AS serum failed to detect any antibody that reacted with all three antigens. Therefore no antibody actually reacted with the M1+ epitope. However, at higher serum dilutions 5 of the 35 AS patients retained a clear response to the MOMPs of the bacteria thus confirming their earlier results. As MOMPs are known to cross-react with several enterobacteriaceae (Hofstra and Dankert 1980) it may be that the aetiology of AS entails infection by a group of enteric bacteria rather than a few individual species.

Another antigen that is common to several species of enterobacteriaceae is the enterobacterial common antigen (ECA). Raised serum levels of antibodies to ECA have been found in patients with ulcerative colitis and Crohn's disease (Bull and Ignaczak 1973; Bartnick and Kaluzewski 1979), alcoholic cirrhosis (Turunen et al 1981) and severe pancreatitis (Kivilaakso et al 1984).

The measurement of antibodies to ECA may be helpful in studying the escape, into the circulation, of other bacterial cell wall components of which the highly reactive endotoxin (LPS) is a major one. The increased levels of antibodies to ECA in patients with the above mentioned diseases indicates an immune response to gram-negative bacteria which may in turn suggest the participation of ECA in the pathogenesis of non-specific inflammatory bowel diseases and other diseases.

## 6.2 Materials and methods

### 6.2.1 Patient groups

Antibodies to Escherichia coli (a routine laboratory strain, Yersinia enterocolitica 3 and Campylobacter jejuni were measured in the serum of patients with ankylosing spondylitis, Crohn's disease, ulcerative colitis and rheumatoid arthritis. Healthy laboratory and hospital staff were used as controls. Clinical details of the groups are given in Table 35.

### 6.2.2 Bacterial antigens

Formalin treated bacterial antigens were prepared as previously described in Section 5.2.2.

Purified ECA was a gift from Dr H Mayer (Max-Planck Institute for Immunobiology, West Germany). The purification procedure is described elsewhere (Männel and Mayer 1978).

### 6.2.3 Enzyme linked immunosorbent assay

The ELISA has been previously described (Section 5.2.3). ECA was initially used at 10, 5 and 1µg/ml for coating the plates to obtain the optimal concentration to use in the assay. Each concentration was tested with rabbit antiserum raised to known ECA positive and negative strains of E.coli 014 - 2387 and F1327 respectively.

| | AS | CD | UC | RA | C |
|---|---|---|---|---|---|
| No of patients | 20 | 20 | 20 | 20 | 20 |
| Mean age (yrs) | 41 | 41 | 45 | 60 | 25 |
| (range) | (25-64) | (19-68) | (21-68) | (26-82) | (19-44) |
| Sex | M = 18 | M = 9 | M = 10 | M = 5 | M = 10 |
| | F = 2 | F = 11 | F = 10 | F = 15 | F = 10 |
| Drug therapy | NSAID 18<br>None 2 | Steroids 1<br>NSAID 1<br>Sulphasalazine 6<br>NSAID/ Sulphasalazine 1<br>None 11 | Sulphasalazine 19<br>Mesalazine 1 | Gold 2<br>Steroids 5<br>NSAID 13 | Not applicable |
| Joint disease | Peripheral joint involvement 9 | AS 3<br>Peripheral arthropathy 1 | Peripheral arthropathy 1 | All patients | Not applicable |

Table 35 Clinical details of patients and controls in the study of the humoral immune response to enteric bacterial antigens.

A 1/10 serum dilution was used for all immunoglobulin classes. Every plate included the known ECA+ and ECA- sera as controls.

### 6.2.4 Absorption studies

The specificity of the ELISA was assessed as previously described (Section 5.2.4). Three sera from each patient group were absorbed with E.coli, Y. enterocolitica, C. Jejuni and K. pneumoniae.

## 6.3 Results

### 6.3.1 Antibodies to Escherichia coli

The IgA responses to E.coli are shown in Figure XXIX. The mean values (± SEM) for AS, CD, UC and RA patients and normal controls were 0.77 ± 0.079, 0.79 ± 0.095, 0.771 ± 0.104, 0.671 ± 0.1 and 0.536 ± 0.075 respectively. The AS and CD patients had significantly raised anti-E.coli IgA levels when compared to controls ($p < 0.02$ and $p < 0.05$ respectively).

Figure XXX shows the IgG response. The mean values (± SEM) were 0.355 ± 0.042, 0.554 ± 0.087, 0.613 ± 0.065, 0.448 ± 0.082 and 0.338 ± 0.047 for AS, CD, UC and RA patients and normal controls respectively. Anti-E.coli IgG levels in CD and UC patients were significantly increased when compared to normal controls ($p < 0.05$ and $p < 0.002$). AS and RA patients had significantly lower levels when compared to UC patients ($p < 0.005$ and $p < 0.05$).

The IgM responses are shown in Figure XXI. The mean values (± SEM) for AS, CD, UC and RA patients and normal controls were 0.43 ± 0.079, 0.572 ± 0.104, 0.421 ± 0.055, 0.396 ± 0.112 and 0.396 ± 0.052 respectively. No significant difference was found between any of the groups.

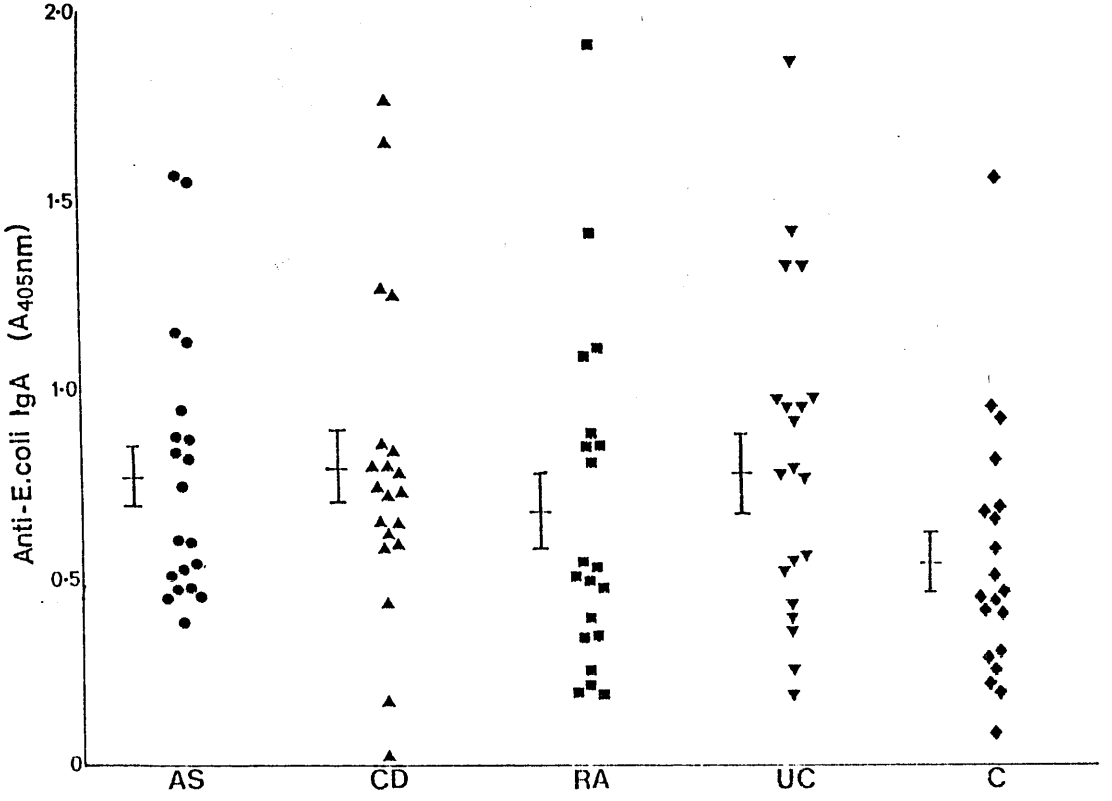



Fig XXIX Anti-E.coli IgA antibody levels (mean ± SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

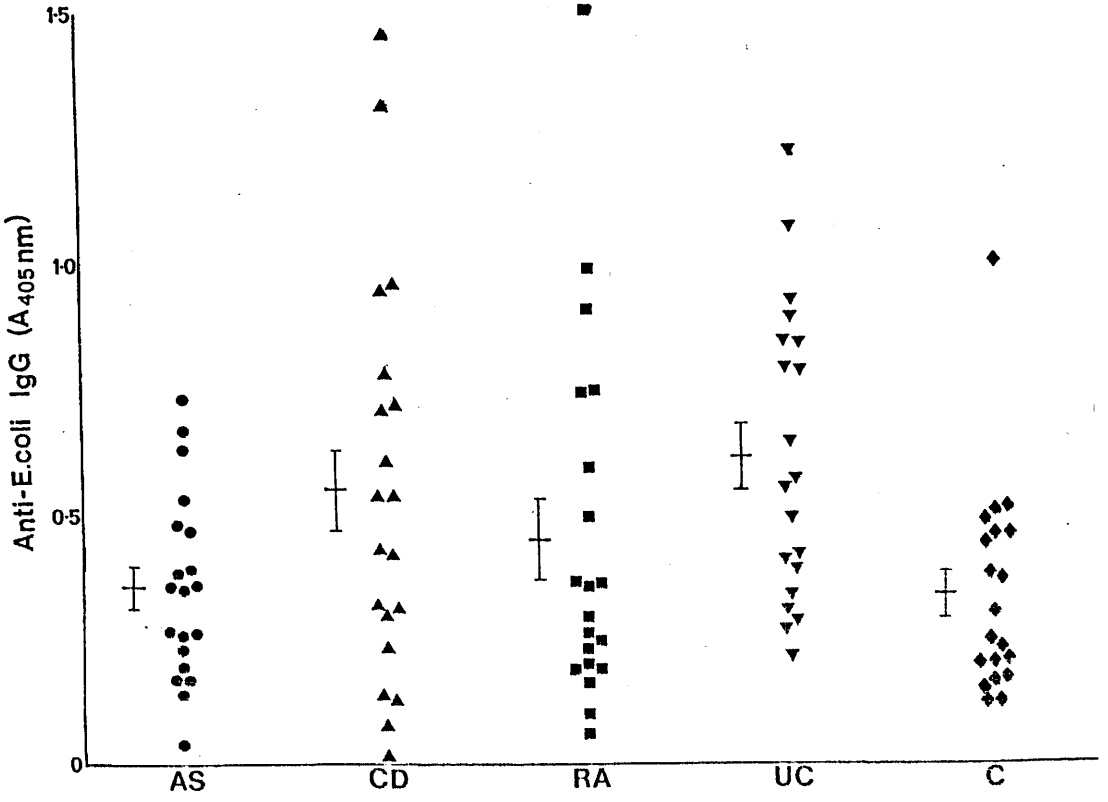



Fig XXX Anti-E.coli IgG antibody levels (mean + SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

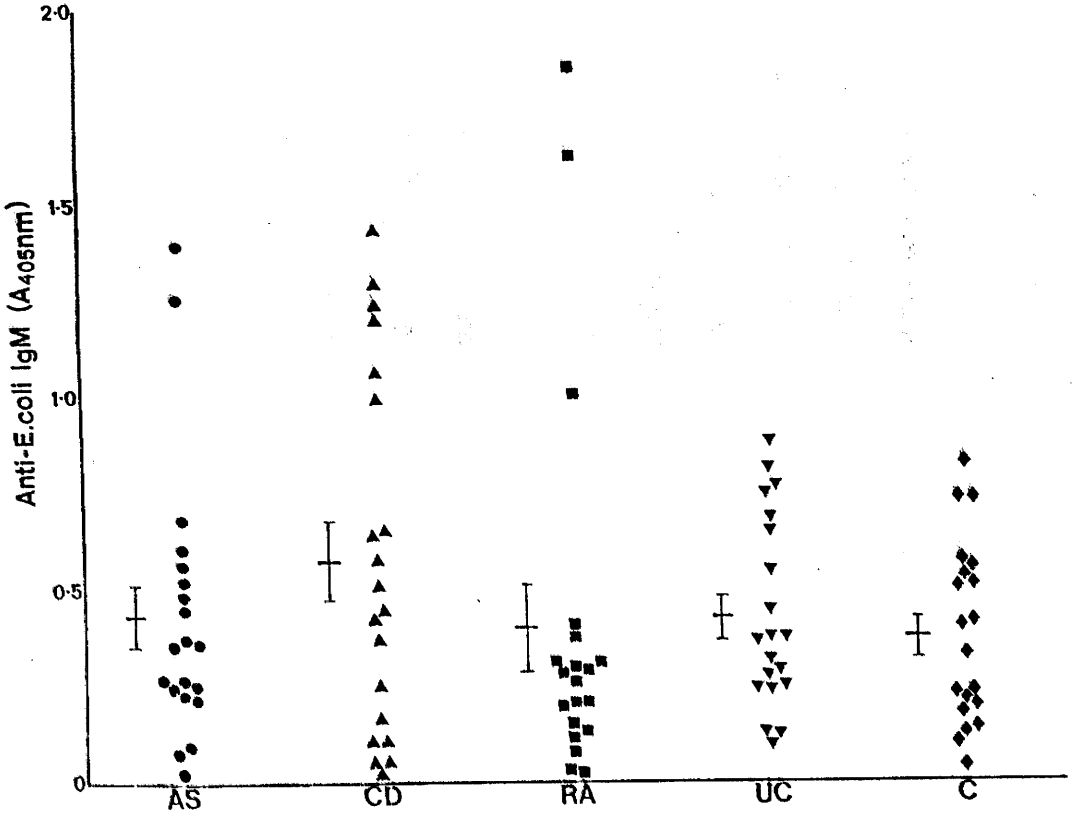



Fig XXXI Anti-E.coli IgM antibody levels (mean ± SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

### 6.3.2 Antibodies to Yersinia enterocolitica

The mean (± SEM) anti-Yersinia IgA levels in AS, CD, UC and RA patients and normal controls were 0.392 ± 0.055, 0.418 ± 0.079, 0.305 ± 0.061, 0.369 ± 0.061 and 0.161 ± 0.018 respectively (Figure XXXII). All the patient groups had significantly raised levels of anti-Yersinia IgA when compared to normal controls (AS, $p < 0.001$; CD, $p < 0.005$; UC, $p < 0.05$; RA, $p < 0.01$).

The IgG responses are shown in Figure XXXIII. The mean (± SEM) anti-Yersinia IgG levels in AS, CD, UC and RA patients and normal controls were 0.303 ± 0.05, 0.371 ± 0.067, 0.417 ± 0.076, 0.252 ± 0.037 and 0.199 ± 0.026 respectively. Patients with UC had slightly raised anti-Yersinia IgG when compared to normal controls ($p < 0.05$). No other differences were found between the groups.

The mean (± SEM) anti-Yersinia IgM responses were 0.435 ± 0.059, 0.502 ± 0.097, 0.578 ± 0.106, 0.156 ± 0.026 and 0.295 ± 0.05 for patients with AS, CD, UC and RA and normal controls respectively (Figure XXXIV). Patients with RA had significantly lowered anti-Yersinia IgM levels than all the other groups (AS, $p < 0.001$; CD, $p < 0.005$; UC, $p < 0.01$; C, $p < 0.05$). UC patients had raised levels when compared to controls ($p < 0.02$).

### 6.3.3 Antibodies to Campylobacter jejuni

The anti-Campylobacter IgA responses are shown in Figure XXXV. Mean (± SEM) values for AS, CD, UC and RA patients and normal controls were

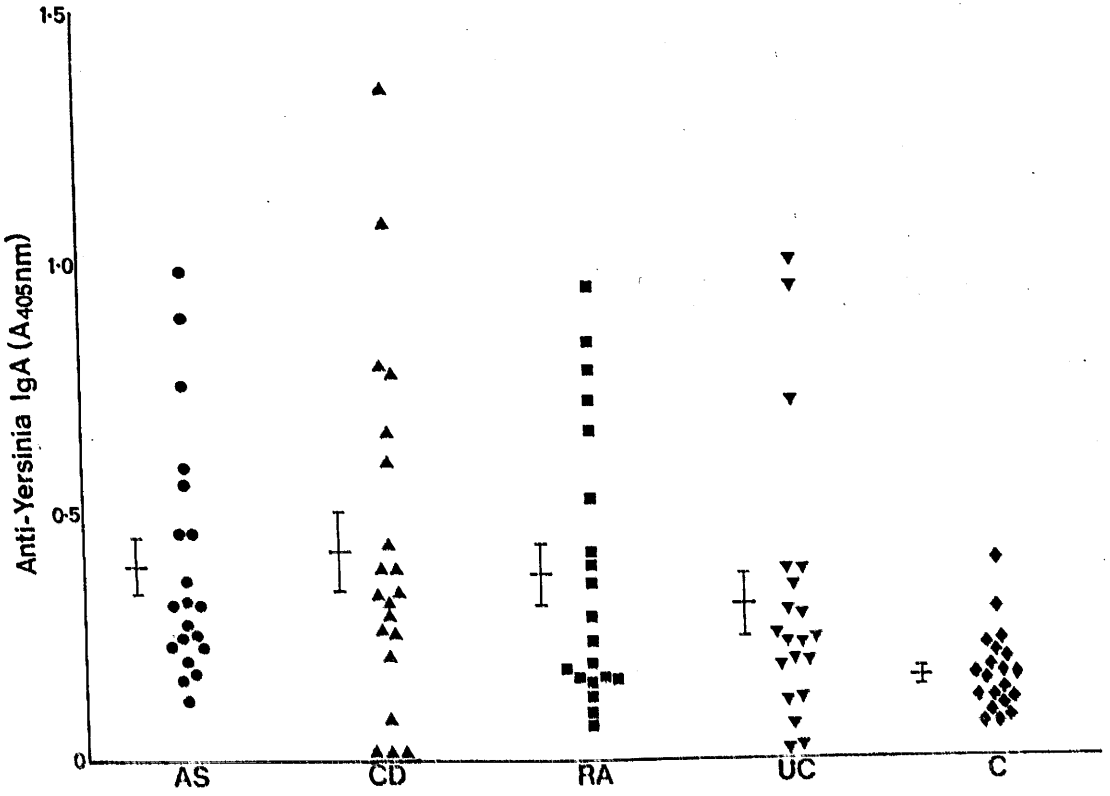



Fig XXXII Anti-Yersinia IgA antibody levels (mean + SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

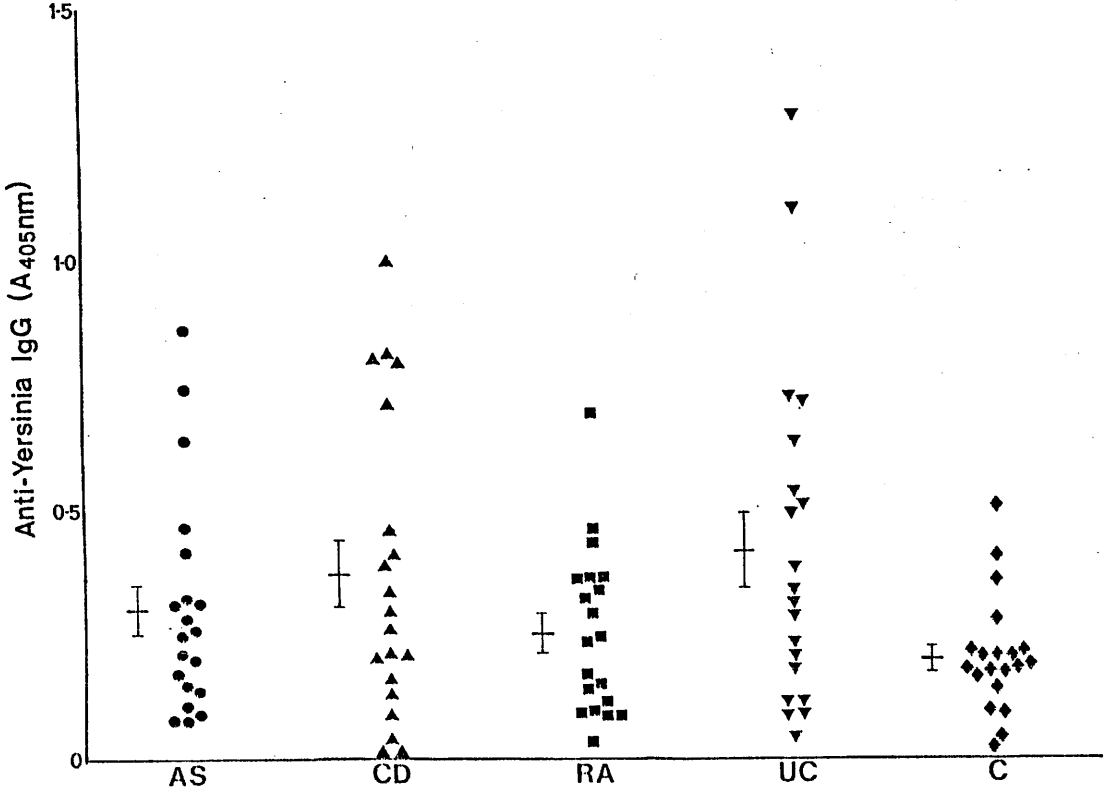



Fig XXXIII Anti-Yersinia IgG antibody levels (mean + SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

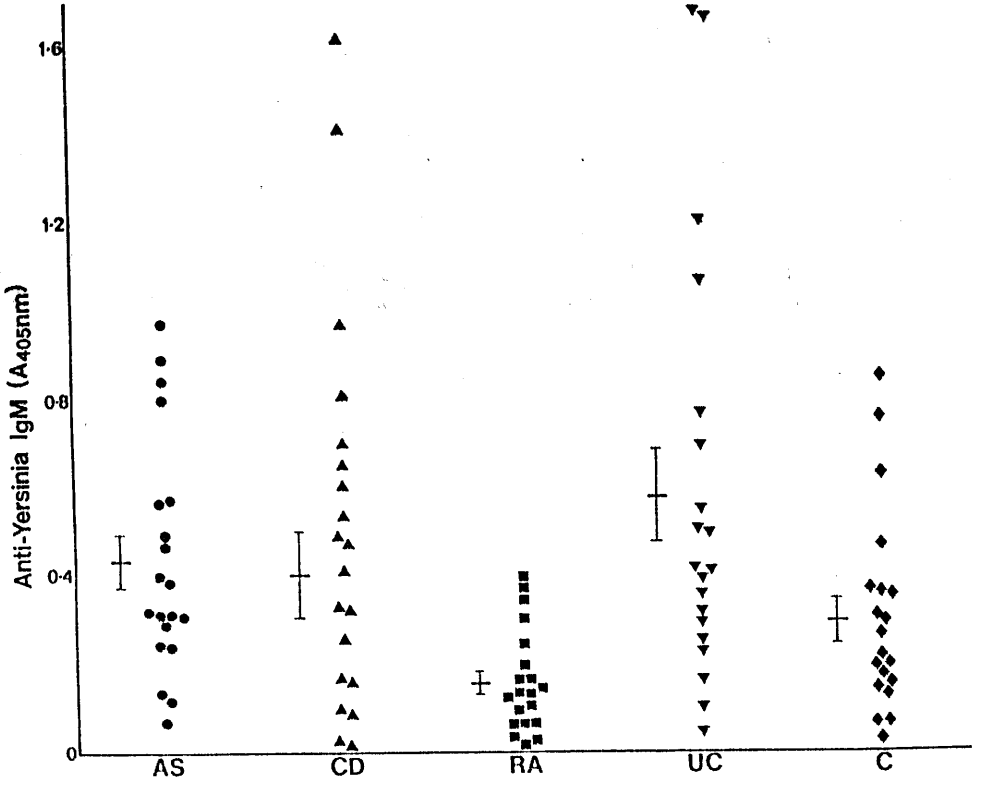



Fig XXXIV Anti-Yersinia IgM antibody levels (mean + SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

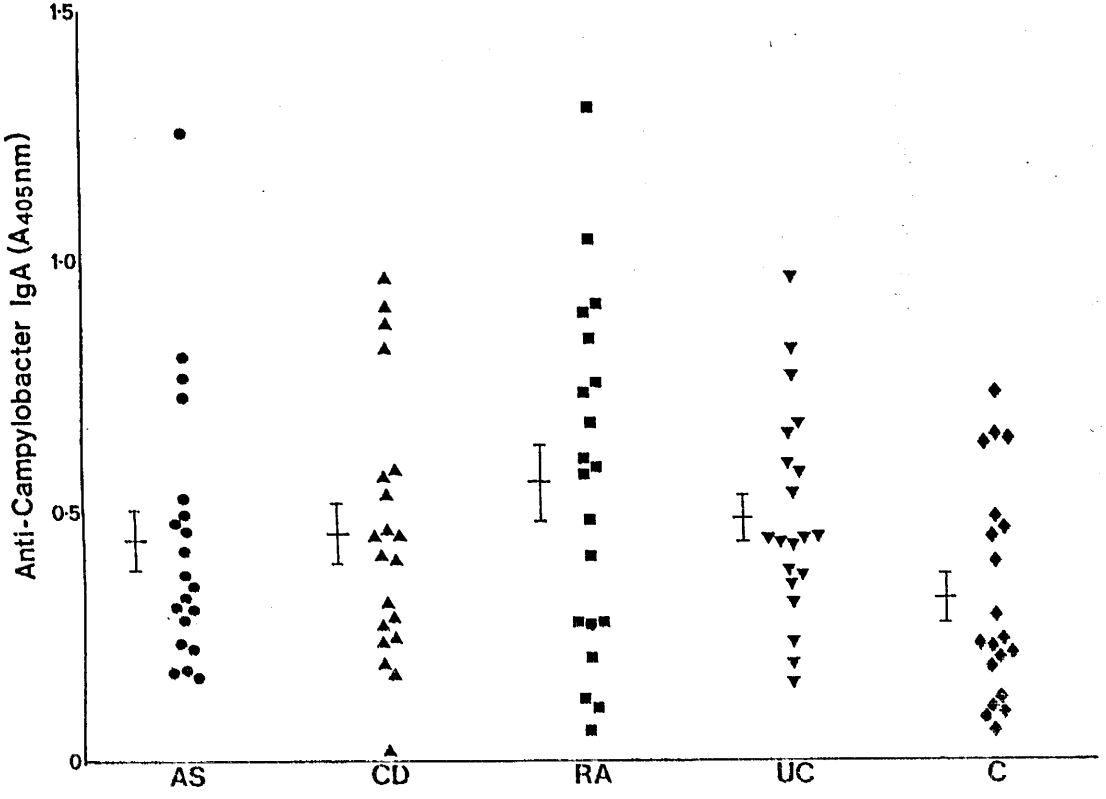



Fig XXXV Anti-Campylobacter IgA antibody levels (mean ± SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

0.443 ± 0.06, 0.455 ± 0.059, 0.484 ± 0.047, 0.554 ± 0.077 and 0.322 ± 0.048 respectively. Patients with UC and RA had raised levels of anti-Campylobacter IgA compared to normal controls ($p < 0.05$).

For AS, CD, UC and RA patients and normal controls the mean (± SEM) anti-Campylobacter IgG values were 0.498 ± 0.065, 0.475 ± 0.088, 0.622 ± 0.104, 0.462 ± 0.077 and 0.537 ± 0.069 respectively (Figure XXXVI). These values were not significantly different from one another.

The anti-Campylobacter IgM results are shown in Figure XXXVII. The mean (± SEM) levels were 0.215 ± 0.026, 0.306 ± 0.059, 0.372 ± 0.047, 0.293 ± 0.049 and 0.31 ± 0.036 for AS, CD, UC and RA patients and normal controls respectively. The only significant difference between the groups was a lowered response in AS patients compared to patients with UC ($p < 0.01$).

### 6.3.4 Anti-Klebsiella antibodies in ulcerative colitis patients

Anti-Klebsiella antibodies were also measured in the serum of UC patients. The mean (± SEM) IgA, G and M values were 0.764 ± 0.094, 0.857 ± 0.092 and 0.488 ± 0.073 respectively. The anti-Klebsiella IgA level was raised when compared to normal controls ($p < 0.02$) and the IgM response was significantly lower ($p < 0.05$). No significant difference was found in levels of anti-Klebsiella IgG when compared to normal controls.

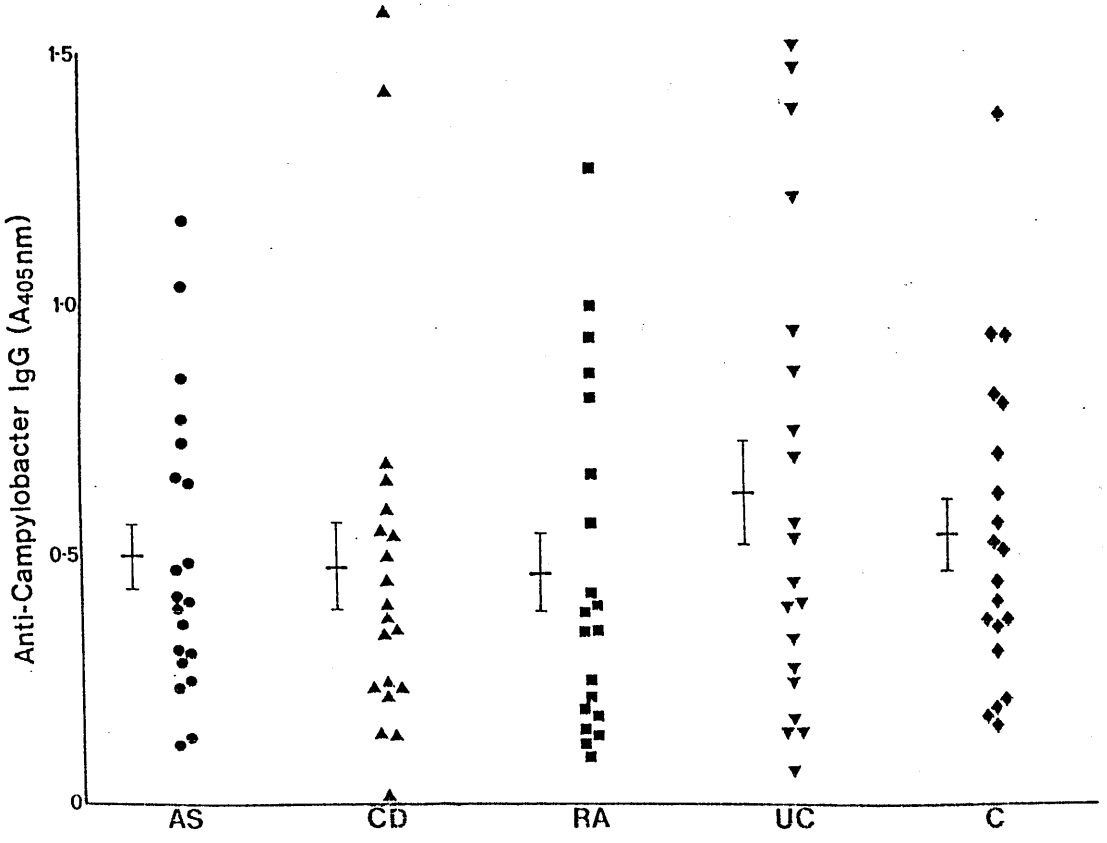



Fig XXXVI Anti-Campylobacter IgG antibody levels (mean ± SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

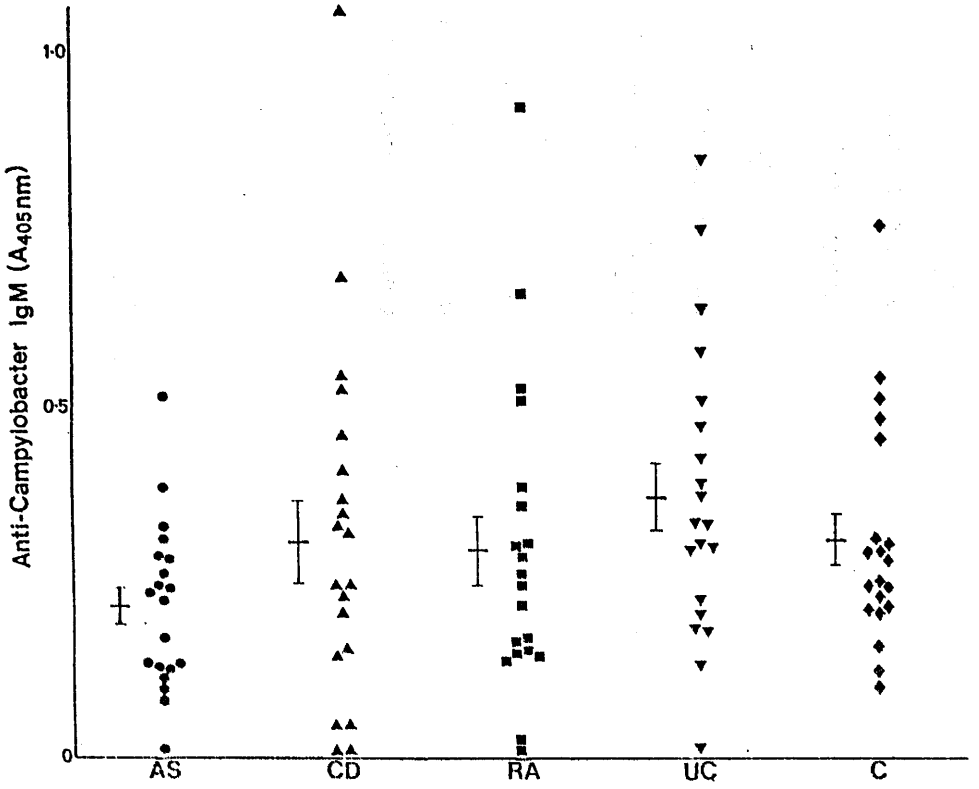



Fig XXXVII Anti-Campylobacter IgM antibody levels (mean ± SEM) in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA), ulcerative colitis (UC) and normal controls (C).

### 6.3.5 Absorption studies

The specificity of the raised IgA antibody levels was examined using bacterial cell absorption. The percentage absorption of the IgA antibodies with whole formalin killed bacteria is shown in Table 36. Absorption by homologous bacteria removed approximately 70% or more of the antibody. This was much greater than that obtained with heterologous bacteria. However, in sera from patients with CD, UC and RA, absorption by heterologous bacterial cells, of anti-Yersinia IgA was higher than that of anti-E.coli or anti-Campylobacter IgA suggesting that anti-Yersinia IgA antibodies were cross-reacting with antigens on K.pneumoniae, E.coli and C.jejuni. However, the reverse was not the same, ie, anti-E.coli, anti-Campylobacter and anti-Klebsiella (Section 5.3.6) IgA antibodies were not absorbed by Yersinia antigens. This indicates that anti-E.coli, Klebsiella and Campylobacter IgA antibodies are specific for antigens on these bacteria, but not Yersinia, whereas anti-Yersinia IgA antibodies are specific for Yersinia but also cross-react with antigens present on the other three bacterial cells. This is a unique happening which has not been reported before.

### 6.3.6 Antibodies to enterobacterial common antigen

Using rabbit serum to ECA+ E.coli the absorbance at 405nm with 10, 5 and 1µg/ml ECA was > 2, > 2 and 1.176. The readings with serum to ECA- E.coli were 0.104, 0.095 and 0.111 for the same concentrations of ECA. 1µg/ml of ECA was therefore enough antigen for coating the plates and the ECA+ and ECA- antisera were suitable positive and

| Serum | IgA antibody absorbed | %Absorption by | | | |
|---|---|---|---|---|---|
| | | Klebsiella | E.coli | Yersinia | Campylobacter |
| AS | E.coli | 9.8±0.7 | 75.5±15.3 | 12.0±6.4 | 7.4±3.8 |
| | Yersinia | 20.0±3.9 | 28.8±3.8 | 64.8±9.0 | 10.0±2.7 |
| | Campylobacter | 18.0±4.6 | 25.3±12.6 | 28.8±8.6 | 70.0±4.4 |
| CD | E.coli | 9.6±7.9 | 68.6±7.8 | 20.0±5.7 | 10.4±4.6 |
| | Yersinia | 43.7±7.4 | 46.9±7.7 | 86.7±5.5 | 36.5±4.4 |
| | Campylobacter | 19.3±10.4 | 31.7±9.1 | 32.9±6.6 | 75.2±4.4 |
| UC | E.coli | 12.8±6.6 | 88.6±5.2 | 17.8±5.1 | 17.7±4.6 |
| | Yersinia | 42.7±6.9 | 49.9±6.6 | 95.4±3.0 | 38.7±3.3 |
| | Campylobacter | 20.0±3.1 | 26.2±3.0 | 19.2±9.7 | 71.4±8.4 |
| RA | E.coli | 28.4±8.0 | 71.8±16.7 | 21.8±5.8 | 9.5±1.4 |
| | Yersinia | 40.6±21.8 | 46.4±22.5 | 73.6±8.6 | 23.4±5.2 |
| | Campylobacter | 31.3±7.2 | 29.4±11.5 | 31.1±13.5 | 70.0±13.4 |

Table 36 Percentage absorption of anti-bacterial antibodies by whole formalin killed bacteria.

negative controls to use in the assay.

Antibodies to ECA were measured in 7 patients with AS, CD and RA and 7 normal controls. Only 7 patients in each group were studied as there was a limited amount of ECA available. The results are expressed in Table 37. No significant difference in antibody levels was found between the groups for anti-ECA IgA, G or M.

### 6.3.7 Comparison of IgA antibody levels and disease activity

Anti-E.coli, Yersinia and Campylobacter IgA antibody levels were compared in AS patients with active, probably active and inactive disease (Table 38). No difference was found in IgA levels between these three groups.

IgA levels were also compared in AS patients with and without PJI (Table 39). There was also no significant difference in antibody levels between these two groups.

Of the four CD patients who had joint disease only one patient had increased anti-E.coli and anti-Yersinia IgA antibody levels above that of the CD patients' mean. None of the four patients had a higher anti-Campylobacter IgA level than the mean value. The values were, for anti-E.coli IgA, 0.711, 0.799, 0.588 and 0.776; for anti-Yersinia IgA, 0.074, 0.206, 0.283 and 0.534 and for anti-Campylobacter IgA, 0.266, 0.172 and 0.242.

| Patients | Anti-ECA antibody (A405nm) | | |
|---|---|---|---|
| | IgA | IgG | IgM |
| AS (n = 7) | 0.396 ± 0.111 | 0.612 ± 0.19 | 1.112 ± 0.187 |
| CD (n = 7) | 0.495 ± 0.109 | 1.057 ± 0.113 | 0.777 ± 0.152 |
| RA (n = 7) | 0.433 ± 0.117 | 0.814 ± 0.123 | 0.664 ± 0.187 |
| C (n = 7) | 0.419 ± 0.109 | 0.636 ± 0.187 | 0.809 ± 0.14 |

Table 37 Anti-ECA antibodies in patients with ankylosing spondylitis (AS), Crohn's disease (CD), rheumatoid arthritis (RA) and normal controls.

| | IgA antibodies to | | |
|---|---|---|---|
| | E.coli | Yersinia | Campylobacter |
| A (n = 7) | 0.732 ± 0.09 | 0.326 ± 0.082 | 0.448 ± 0.091 |
| PA (n = 6) | 0.964 ± 0.152 | 0.471 ± 0.124 | 0.525 ± 0.155 |
| IN (n = 6) | 0.671 ± 0.179 | 0.412 ± 0.102 | 0.398 ± 0.077 |

Table 38 Comparison of IgA antibodies (mean ± SEM) and disease activity in ankylosing spondylitis patients.

| | IgA antibodies to | | |
|---|---|---|---|
| | E.coli | Yersinia | Campylobacter |
| PJI (n = 9) | 0.757 ± 0.121 | 0.368 ± 0.09 | 0.484 ± 0.111 |
| No PJI (n = 11) | 0.78 ± 0.109 | 0.413 ± 0.071 | 0.408 ± 0.064 |

Table 39 Comparison of IgA antibodies (mean ± SEM) in ankylosing spondylitis patients with and without peripheral joint involvement.

## 6.4 Discussion

Serum from patients with AS displayed an IgA antibody response to Escherichia coli and Yersinia enterocolitica but not Campylobacter jejuni. Patients with CD also displayed a similar response. Serum from patients with UC and RA also had raised anti-Yersinia antibody levels although the response to E.coli was similar to that of controls. The anti-Campylobacter IgA response was also raised in these two patient groups. There was not a substantial difference in IgG and IgM responses except in patients with UC who had increased anti-E.coli and anti-Yersinia IgG levels and in CD patients who also had raised anti-E.coli IgG levels. These results and those from the previous study suggest that there is a non-specific antibody response to bacterial antigens occurring across the intestinal mucosa. It also suggests that not one, but perhaps several members of the Enterobacteriaceae species are involved in these disorders. The fact that anti-IgA antibodies were raised in the serum of patients with UC as well as CD points to large bowel as well as small bowel inflammatory disease being associated with increased mucosal IgA antibody responses.

If several bacteria are involved in these diseases it is more likely to be a response to a common antigen(s). Such a candidate is ECA. This study investigated the antibody response to ECA in 7 patients with AS, CD and RA and compared them to 7 normal controls. Although only a small number of patients were studied no differences in antibody levels were found, suggesting ECA alone is not responsible for these raised IgA antibody levels.

The increased IgA levels were also compared with disease activity in AS patients. The activity of the disease or the presence of peripheral joint involvement had no effect on the antibody levels.

In absorption studies the homologous bacteria absorbed approximately 76% of the anti-IgA antibodies whereas the heterologous bacteria absorbed only 20%. However, the absorption of anti-Yersinia antibodies by K.pneumoniae, E.coli and C.jejuni in the serum of CD, UC and RA patients was approximately 41% which could indicate that anti-Yersinia antibodies were cross-reacting with antigens on these bacteria. Antibodies to Klebsiella, E.coli and Campylobacter, however, did not cross-react with Yersinia antigens. This could mean that the IgA antibody response to Yersinia consists of antibodies specific for Yersinia and also antibodies that are cross-reactive to several other members of the enterobacteriaceae family whereas the IgA response to these other bacteria consist of antibodies specific for the homologous bacteria. Considering this point it was also interesting to find that anti-Yersinia antibodies were raised in all four patient groups whereas anti-E.coli and anti-Campylobacter IgA were only raised in two of the four patient groups. The Yersinia strain used in this study is more commonly found in patients with reactive arthritis in Scandanavian countries. In Britain Yersinia Spp is isolated in about 3.5% patients and the pathogenic strain, used in this study, is rarely isolated (Greenwood and Hooper 1987). It could be that the raised antibody levels are not specific for Yersinia antigens but common to antigens found on other bacteria that are stereochemically similar as suggested by the absorption studies.

Whether the increased responses to these bacteria are a cause of the disease or are a result of the disease cannot be concluded from these results. However, these diseases seem to have one common denominator, that of an increased permeability of the gut to bacterial antigens and that the raised levels of anti-Klebsiella antibodies are found not only in AS serum but the serum of patients with other diseases. Also the suggestion that the increased IgA antibody levels in AS are due specifically to Klebsiella have not been substantiated in these experiments.

The permeability of a solute across the intestinal wall occurs in two ways. Molecules of low molecular weight are thought to be absorbed by diffusion across the lipid cell membrane through polar regions incorporated into the membrane structure, ie, intracellular diffusion. Larger hydrophilic molecules do not diffuse across cell membranes and yet they still diffuse across the intestinal mucosa presumably by a different intercellular route.

Three main techniques have been used to investigate intestinal permeability - differential sugar absorption, $^{51}$Cr-EDTA absorption and absorption of polyethylene glycol (PEG) polymers. They are all based on urinary recovery of an oral probe molecule. Bjarnason et al (1984a) have suggested that the increased gut permeability in AS and other disorders may reflect the ingestion of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs have been shown to cause ulceration in the intestine of man (Sturges et al 1973; Madhok et al 1986). Withdrawal of the drug has also been associated with the absence of ulceration (Silvoso et al 1979; Caruso et al 1980;

Bjarnason et al 1984a) and Jenkins et al (1987) have shown increases in intestinal permeability using, $^{51}$Cr-EDTA as the marker, of rheumatoid arthritis and osteoarthritis (OA) patients taking NSAIDs and suggested that prostaglandins may play a role in maintaining intestinal integrity. The increase in $^{51}$Cr-EDTA absorption may occur in the stomach due to gastric lesions, however, increased permeability in chronic alcoholism occurs independently of the presence of gastritis (Bjarnason, Ward and Peters 1984a) indicating that gastric lesions are not necessary for increased permeability. The increase in intestinal permeability may facilitate antigen absorption and thus indirectly contribute to persistence of the joint symptoms. One possible sequence of events is intestinal presentation of antigens, biological activity in the mucosa, of bacterial proteolytic enzymes or degradation products, followed by systemic absorption of the antigens through the damaged mucosa. Joint inflammation could then be caused by an increased immunological response, to these antigens, which concentrates in the joint tissues or by a process of synovial deposition of immune complexes.

Ileocolonoscopy has also been used to demonstrate intestinal abnormalities. Mielants and Veys (1985b) could not find any histological abnormalities in the distal ileum or colon of RA patients taking NSAIDs although they found that in AS patients inflammation was observed in B27 positive and negative patients with PJI (Mielants et al 1985a). None of the controls, who were all taking NSAIDs, showed signs of gut inflammation.

Smith, Gibson and Brooks (1985) have measured bowel permeability in AS and RA patients and compared them to OA patients administered with NSAIDs. They looked at PEG absorption by the gut and found that it was increased in active RA patients and all AS patients when compared to the OA group. Therefore the increased permeability was not due to NSAIDs. Struthers et al (1985) used differential sugar absorption to measure small bowel permeability in RA patients and found that it was normal in patients on NSAIDs and second line drugs. They did not detect any abnormality that could be due to NSAIDs and suggested that if one was to demonstrate that passage of antigenic material through the intestinal mucosa was abnormal in RA, molecules which resemble such material would have to be used in the tests. One would also have to determine by which route such molecules diffused through the intestinal wall. Simpson (1985) has, however, pointed out that unless the increase in urinary levels of the marker molecule correlated with an increase of marker in the blood the possibility exists that the greater excretion of the marker may indicate an increase in glomerular filtation rate rather than enhanced absorption. In response, Bjarnason et al (1986) has recently shown increased gut permeability in control subjects taking NSAIDs. Additionally indomethacin was shown to actually reduce the glomerular filtration rate. However, these studies would need to be repeated with AS and RA patients.

## Discussion

The aim of these studies was to investigate the possible association between enterobacteriaceae, especially Klebsiella, and ankylosing spondylitis and whether these bacteria were involved as immunological triggers. Patients with rheumatoid arthritis, Crohn's disease and ulcerative colitis were also studied to provide a disease control population.

One of the problems, when studying patients with AS, has been the division of the patients into groups depending on their disease activity. Active patients may have different immunological responses to those in an inactive state. The first investigation was therefore to determine what parameters could be used as measures of disease activity. The AS patients were initially divided, by a clinician, into three groups, active, probably active and inactive disease. Klebsiella carriage, ESR, CRP and immunoglobulin levels were then measured accordingly. Klebsiella carriage was not increased in patients with AS, no matter what their disease state was, when compared to normal community controls. Therefore Klebsiella carriage in the gut is not associated, specifically, with AS patients. ESR and CRP levels, two parameters frequently used as disease markers in various conditions, were found to be raised in AS patients with active disease. These levels were not altered in the patients with peripheral joint involvement. IgA, IgG and IgM levels were not different in any of the three patient groups suggesting that IgA, or either of the other two immunoglobulin classes, should not be used as indicators of disease activity. However, serum IgM levels were

increased in patients with PJI. These patients may have recently had or were currently undergoing some infectious episode. As there was no corresponding increase in IgA it may be concluded that this 'infection' was not gut-associated.

The cross-tolerance hypothesis to explain disease pathogenesis has been well documented in recent years but to test this theory antiserum to three antigen preparations of Klebsiella pneumoniae K43 were produced and reacted with lymphocytes from patients with AS. Antibodies to Klebsiella antigens would react with antigens on the cells of B27 positive AS patients and in the presence of complement the cells would be lysed. Of the sera tested, none produced greater lysis of AS lymphocytes when compared to normal control cells. Normal rabbit serum even produced similar results to that of anti-Klebsiella sera. Therefore, antibodies to Klebsiella antigens do not cross-react with antigens on the surface of AS peripheral blood lymphocytes thus rejecting the cross-tolerance theory of disease pathogenesis. It may be that there is cross-reactivity but with several enterobacteriaceae and not one specific bacterial strain. To investigate this further immunoblotting procedures could be used to determine whether there are common antigens present on several different enteric bacteria (especially bacteria isolated from AS patients) that could react with antibodies to B27 or antibodies in AS serum. If such an antigen existed it could be used to produce antiserum which could then be used to look for antigens in synovial fluid or membranes from AS patients.

However, finding cross-reactivity between a bacterial antigen and a cell surface antigen still does not explain how the disease is

initiated. The 'one gene' and 'two gene' theories although plausible seem far too simplistic. One could hypothesise a 'three gene' theory where the three genes involved are the B27 gene, a second gene which leads to tissue damage and a third or repressor gene that controls the amount of damage produced. In normal circumstances the repressor acts upon the B27 gene and a gene product that acts upon the second or disease gene. With infection by antigen in large amounts the repressor is totally switched off leading to the production of an acute disease such as Reiter's syndrome or reactive arthritis. With small amounts of antigen present continuously the repressor will still be working but the B27 gene will still be switched on and acting on the 'disease' gene. The constant expression of the 'disease' gene will lead to the production of a chronic disease such as ankylosing spondylitis. If the 'repressor' gene was sited on the X chromosome, in males only half as much repressor would be produced which would mean more of the B27 gene product would be expressed to act upon the 'disease' gene. This would then explain the male dominance of AS. For those who are B27 negative they may have the gene but only enough is expressed to activate the 'disease' gene to produce tissue damage. This theory therefore suggests that there is a common antigen, perhaps on bacteria, acting on an inhibitor or repressor and not the B27 gene itself.

From my initial observations Klebsiella did not seem to be associated with AS. The next step was to study the immune response to bacterial antigens in these patients. Firstly, cell mediated immunity was investigated and two other patient groups were included - those with rheumatoid arthritis and those with Crohn's disease. Non-specific

lymphocyte transformation by PHA, PWM and Con A was studied to look for any overall abnormality in cell function/activity. AS and CD lymphocytes responded poorly to Con A but the response to PHA and PWM was normal suggesting that suppressor cell activity was abnormal or that there was a depletion of suppressor cells in these patients. The responses to all three mitogens were lowered in RA patients confirming that these patients have abnormal T cell activity. Further studies investigated the lymphocyte responses to various enterobacterial antigens. Although the AS and CD lymphocyte responses to some of the antigens were lowered when compared to controls the overall response was normal. The RA patients, however, had decreased responses to many of the antigens indicating again a lymphocyte abnormality. These studies have therefore found decreased suppressor cell activity but normal lymphocyte responses to bacterial antigens in AS. One would need to follow up this study by investigating T cell numbers and ratios and suppressor cell function in AS patients in more detail. It would also be important to study lymphocytes from other sources in the body other than peripheral blood, for instance synovial and intestinal lymphocytes. In Reiter's syndrome and reactive arthritis synovial lymphocytes respond more to the causal infective organism than peripheral blood lymphocytes (Ford et al, 1981, 1982; Ford 1983). It could be that in AS peripheral blood lymphocytes are the wrong cells to be studying. T helper cell numbers are increased and T suppressor cell numbers lowered in the intestinal lamina compared to peripheral blood in patients with inflammatory bowel disease (James et al 1986). If AS is though to be gut-initiated then it would make more sense to study the responses occurring in that site.

The humoral immune response to bacterial antigens was also investigated. Serum IgA has been found to be raised in AS patients especially in those with active disease. Although this study found no correlation between disease activity and serum IgA levels it does not rule out the possibility that there may be a specific response to bacterial antigens occurring in these patients and an increased response may indicate a current or previous exposure of the patients to enterobacteriaceae.

Initial experiments looked at the humoral immune response to Klebsiella and raised levels of anti-Klebsiella antibodies were found in AS serum. This increase did not correlate with total serum IgA, disease activity or PJI. It was also found that anti-Klebsiella antibody levels were raised in patients with Crohn's disease, ulcerative colitis and rheumatoid arthritis. Therefore, the increase in antibody levels was not specific to AS patients. In AS serum anti-Klebsiella IgM was lowered and this was specific only for this patient group. It was lowered in all patients irrespective of disease activity or PJI. As total serum IgM was increased in patients with PJI it can be concluded that Klebsiella was not responsible. The lowered anti-Klebsiella IgM antibodies could have occurred because of an ongoing immunological response to Klebsiella in AS patients. IgM is the first antibody to be produced in response to an antigen and later this is switched to IgA or IgG. If, due to a continuous antigen stimulation IgA or IgG were to continue to be produced, IgM antibodies would be switched off and the levels would decrease. Alternatively, anti-Klebsiella IgM antibodies may be forming immune complexes which would not be detected in the system used. The measurement of immune

complexes and their specificity, if any, for bacterial antigens would be worthy of investigation.

The raised levels of anti-Klebsiella IgA antibodies in all four patient groups suggests that a non-specific immune response is occurring or that Klebsiella is involved in all these diseases. As the latter would seem unlikely it would be important to determine whether the reaction to other enteric bacterial antigens was similar. Anti-Yersinia IgA antibodies were, in fact, raised in all the patient groups, anti-E.coli IgA was raised in AS and CD serum and anti-Campylobacter jejuni IgA was raised in the UC and RA patients. No correlation was found between these IgA levels and disease activity or PJI in AS patients. The results, therefore, confirm those for Klebsiella suggesting that there is a non-specific response to enteric bacteria occurring in patients with AS, CD, UC and RA. This increase in IgA antibody levels may be due to an increased permeability of the gut to bacterial antigens leading to an increased mucosal production of IgA. Absorption studies showed that the IgA response to each bacterium was specific although in patients with CD, UC and RA approximately 40% of the anti-Yersinia antibodies were absorbed by Klebsiella, E.coli and Campylobacter antigens suggesting that anti-Yersinia antibodies are cross-reacting with antigens from these bacteria and that Yersinia possesses antigens similar to those on other enterobacteriaceae.

The important question that arises from these studies of humoral immunity is whether the increased gut permeability is caused as a result of the disease or is the disease caused by immunological

reactions due to the increased gut permeability? If the increased permeability is caused by the disease what effects do these immunological reactions have on the disease process? CD and UC patients have "leaky" bowels as the diseases are known to be due to abnormalities of the gut so why should there be an increase in gut permeability in AS and RA patients? About 10% of AS patients may have low grade colitis which could account for some of the increased IgA antibody responses. However, some studies have suggested that the increased gut permeability may be due to the administration of NSAIDs. The association of NSAID and gut permeability and the increased IgA antibody response would need to be studied very precisely. Also, the increased antibodies that are produced - are they reactive for bacterial antigens alone or do they react with body tissues in the affected joints?

Taking into account the observations from these studies there does seem to be some immunological reaction occurring in AS which may be involved in the pathogenesis of the disease. This may be the production and persistence of anti-bacterial antibodies in the gut due to T suppressor cell abnormalities and the absorbance of these antibodies into the circulation where they may react to form immune complexes that are deposited in sites such as the sacroiliac joint. If these antibodies also have anti-B27 activity reactions could occur with B27 positive cells in the body - even T suppressor cells. Antibody/lymphocyte complexes may circulate through the lymphatics where again they could be deposited in specific areas. The usual fate of complexes is to be removed by phagocytic cells. It is possible that such complexes are poorly handled by phagocytic cells in AS patients.

Where complexes persist inflammation and damage may be caused leading to the destruction of joints or tissues.

However, these theories presume that there may be some cross-reactivity between the antibodies produced and body tissues and/or cells (this study did not find any reaction with HLA-B27 or AS lymphocytes). Also, if immune complexes are formed why should they specifically localize in the spinal and sacroiliac joints? These points and questions such as, what is the relevance of the association between B27 and AS, still need to be clarified before the pathogenesis of AS is fully understood.

Further areas of research which could be pursued as a result of my own studies would be to investigate the possibility of cross-reactivity between enteric bacteria and antibodies found in AS serum by the immunoblotting of bacterial antigens with B27 and AS serum. Any common antigen found could be identified and isolated to produce antiserum which could be used in further cross-reactivity studies. The numbers and ratios of T cells, T suppressor cell activity and the response to mitogens and bacterial antigens could be compared for lymphocytes from peripheral blood, the intestine and synovial fluid. An assay could be set up to investigate if T suppressor cells are more susceptible to damage and/or destruction by bacterial antigens, antibodies or toxins and one would need to look for bacterial antigens or immune complexes in synovial fluid or membranes. Finally a controlled study would need to be set up to investigate the effect of NSAIDs on gut permeability, comparing AS patients taking NSAIDs to those on no medication and to controls taking and not taking NSAIDs.

Simultaneously the glomerular filtration rate should be measured and serum samples taken for the measurement of immunoglobulin levels.

Appendix A

## Statistical analysis

Non-parametric statistics were used throughout this study as these tests make relatively few assumptions about the nature of the population distribution, ie, they take into consideration that the data may not be normally distributed. The tests were performed using a Royal Infirmary Nuclear Medicine Statistics package on an Apple Computer.

### 1. The Wilcoxon rank sum test

The Wilcoxon rank sum test was used to determine whether the values from one sample are significantly different from the values from another sample. The observations in the two samples are combined into a single series and ranked in order. The lowest score starting with a rank of 1, the next getting a rank of 2 and so on. When $n_1$ and $n_2$ are both less than 26 the test statistic is

$$S = 2R - n_2 (N + 1)$$

where R = sum of ranks in smaller sample

N = total number of values

$n_1$ = sample size of larger group

$n_2$ = sample size of smaller group

The significance of S is determined from a table of critical values for S (Leach 1983).

When $n_1$ or $n_2$ is greater than 25 the probability associated with the observed value of S may be determined by computing the value of Z.

$$Z = \frac{Sc - mean}{SD}$$

where Sc = S-1 or S+1

c = continuity correction

mean = mean of S = 0

SD = standard deviation of S

$$= \sqrt{\text{variance of S}}$$

$$= \sqrt{\frac{n_1 n_2 (N + 1)}{3}}$$

The significance of Z may then be determined by referring to a table of probabilities asociated with values of Z in the normal distribution (Leach 1983). If the observed value of Z has an associated probability of equal of less than 0.05 then there is a significant difference between the two groups.

In the case of there being many ties the corrected formula for Z is

$$Z = \frac{\left(S - \frac{2n - U_1 - Uk}{2(k-1)}\right) - \text{mean}}{\sqrt{\frac{n_1 n_2 (N^3 - \sum Ui^3)}{3N(N-1)}}}$$

where k = the number of distinct values on the response variable

U = the column marginal totals from an ordered contigency table (Leach 1983)

2. Spearman rank correlation

This test investigates whether one set of observations vary correspondingly with those in another. The scores on each variable must first be rank ordered. If ties occur in either population each tied value is assigned the midrank or average rank that they would have received had they not been tied. The test statistic is

$$r = 1 - \frac{6D}{n(n^2 - 1)}$$

where $D = \sum (Xi - Yi)^2$

Xi = the rank of the ith value of X

Yi = the rank of the ith value of Y

n = number of paired variables

If there are a large number of ties then the corrected equation is

$$r = \frac{\sum x^2 + \sum y^2 - D}{2\sqrt{\sum x^2 \sum y^2}}$$

where $x^2 = \frac{n^3 - n}{12} - \sum Tx$

$$y^2 = \frac{n^3 - n}{n} - \sum Ty$$

$$Tx = \frac{Tx^3 - tx}{12}$$

$$Ty = \frac{ty^3 - ty}{t}$$

tx and ty are the number of x and y observations that are tied for a particular rank.

When the sample size is 30 or less, the probability that the two variables are linked together is obtained by comparing the calculated value of r, for n pairs of observations with tabulated critical values of r (Leach 1983). When the sample size is greater than 30, the significance of a value as large as the observed value of r is determined by calculating t,

$$t = r\sqrt{\frac{n-2}{1-r^2}}$$

and then determining the significance of that value of t by referring to a table of critical values of t (Swinscow 1983).

3. Chi-square test

This test determines whether the distribution of a discrete variable in a sample is different from the discrete variable in another sample.

When the results can be set out in a "fourfold table" or a "2 x 2" contingency table:

$$X^2 = \frac{(ad - bc)^2 (a + b + c + d)}{(a + b)(c + d)(b + d)(a + c)}$$

where a = number of observations on row 1 column 1

b = number of observations in row 1 column 2

c = number of observations in row 2 column 1

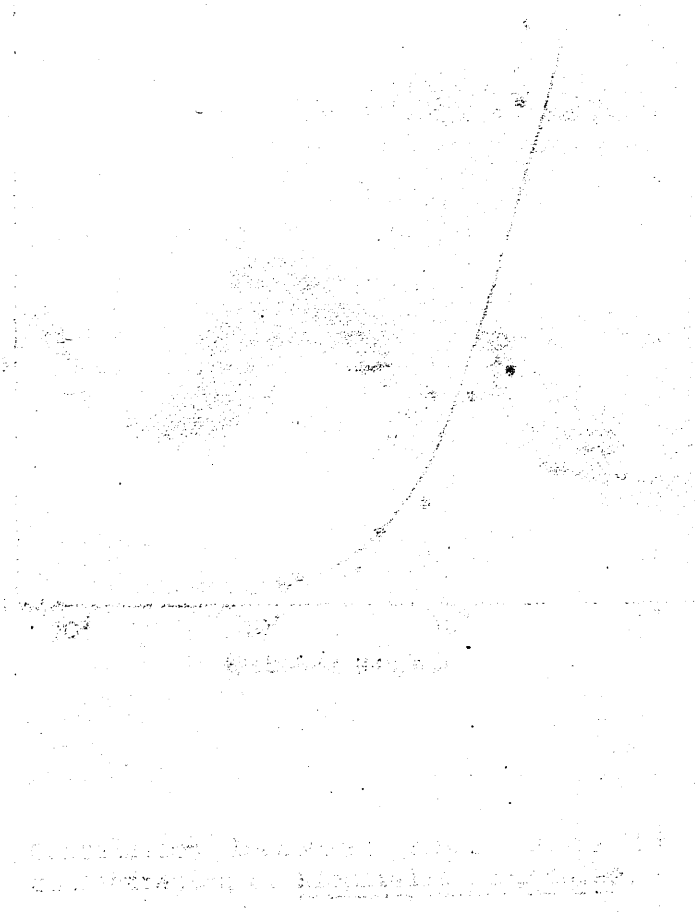
d = number of observations in row 2 column 2

For tables with a total of under 100 or with any number less than 10 Yates's correction should be used. The modified $X^2$ is then

$$X^2 = \frac{\left\{(|ad - bc|) - 1/2(a + b + c + d)\right\}^2 (a + b + c + d)}{(a + b)(c + d)(b + d)(a + c)}$$

where the vertical bars on either side of ad - bc mean the smaller of these two products is taken from the larger.

The significance of the value of $X^2$ is found by referring to a table of distribution of $X^2$ (Swinscow 1983) and the degrees of freedom is equal to (number of columns minus 1) x (number of rows minus 1).



Appendix B

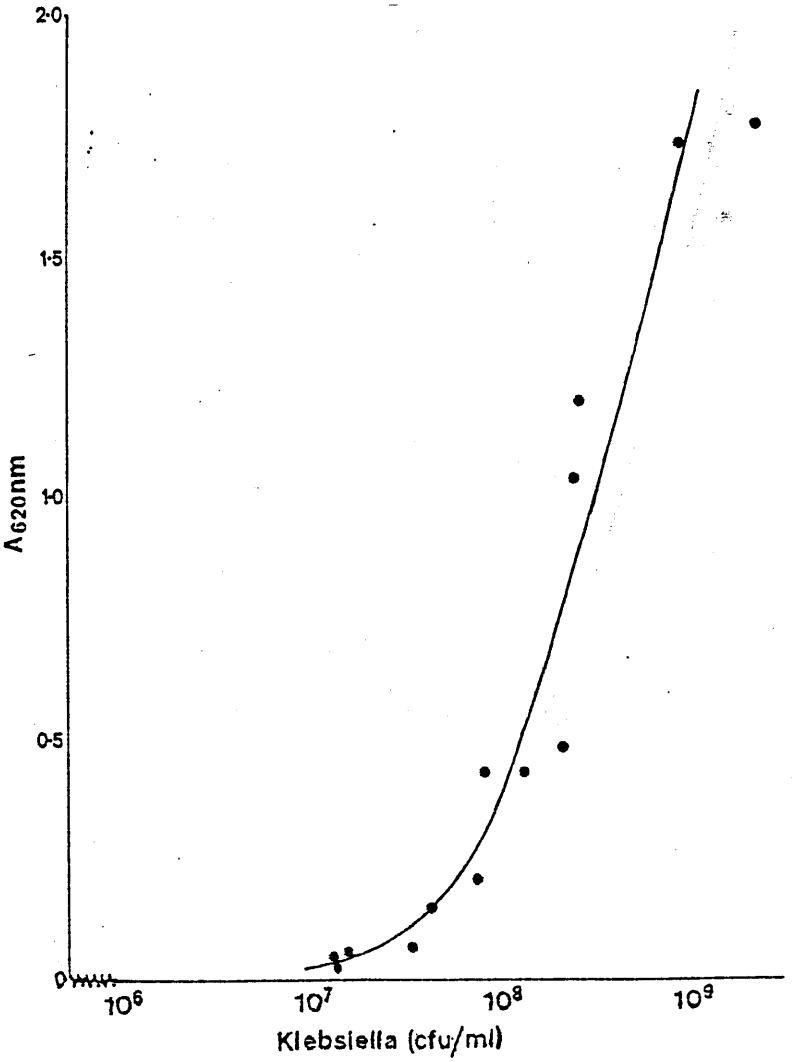



Fig XXXVIII Correlation between absorbance at 620nm and concentration of Klebsiella pneumoniae.

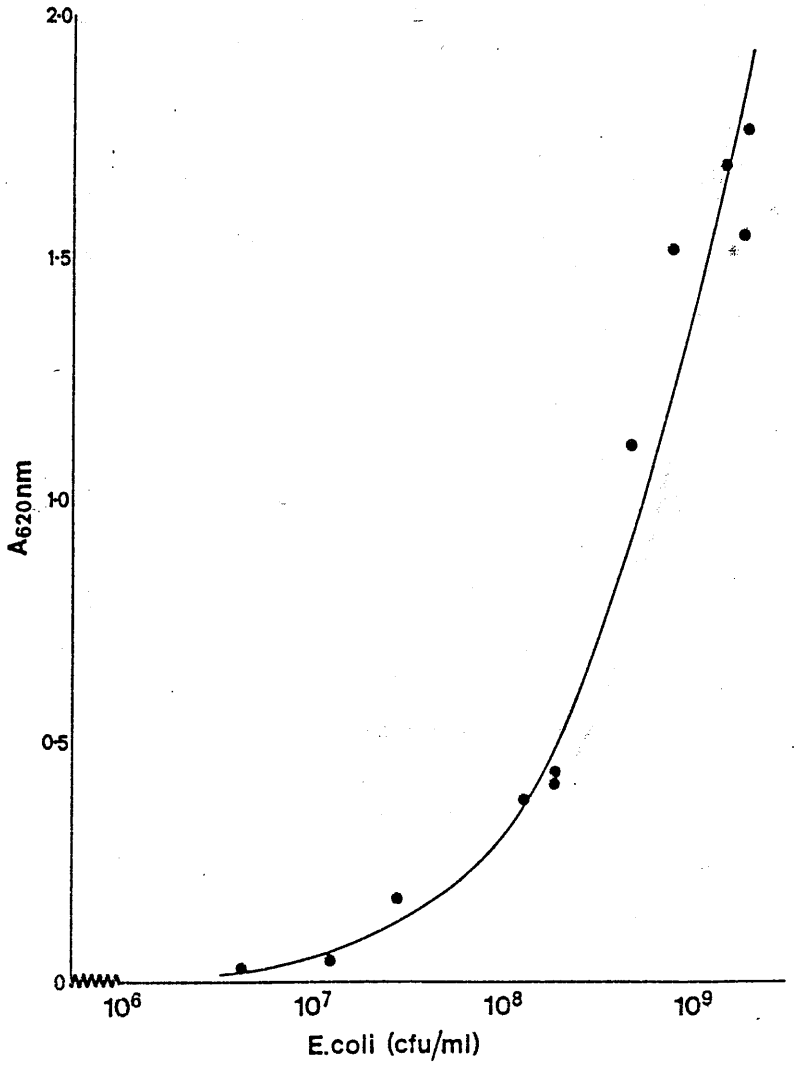



Fig XXXIX Correlation between absorbance at 620nm and concentration of Escherichia coli.

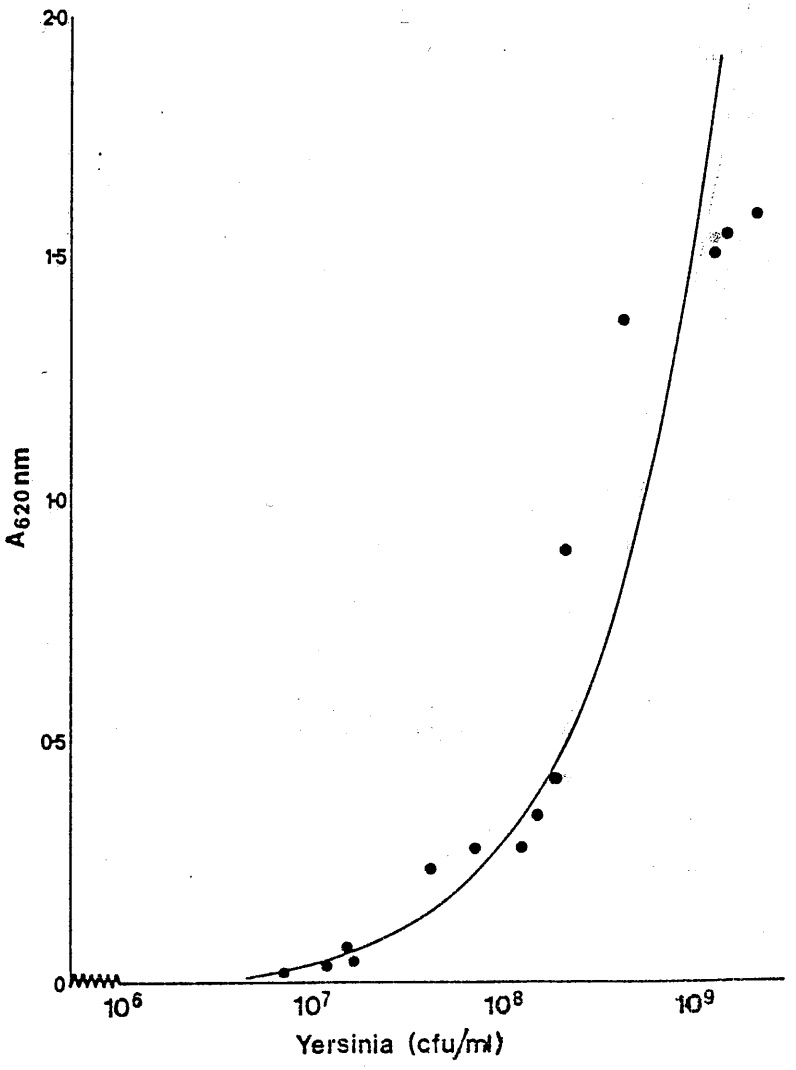



Fig XL Correlation between absorbance at 620nm and concentration of Yersinia enterocolitica.

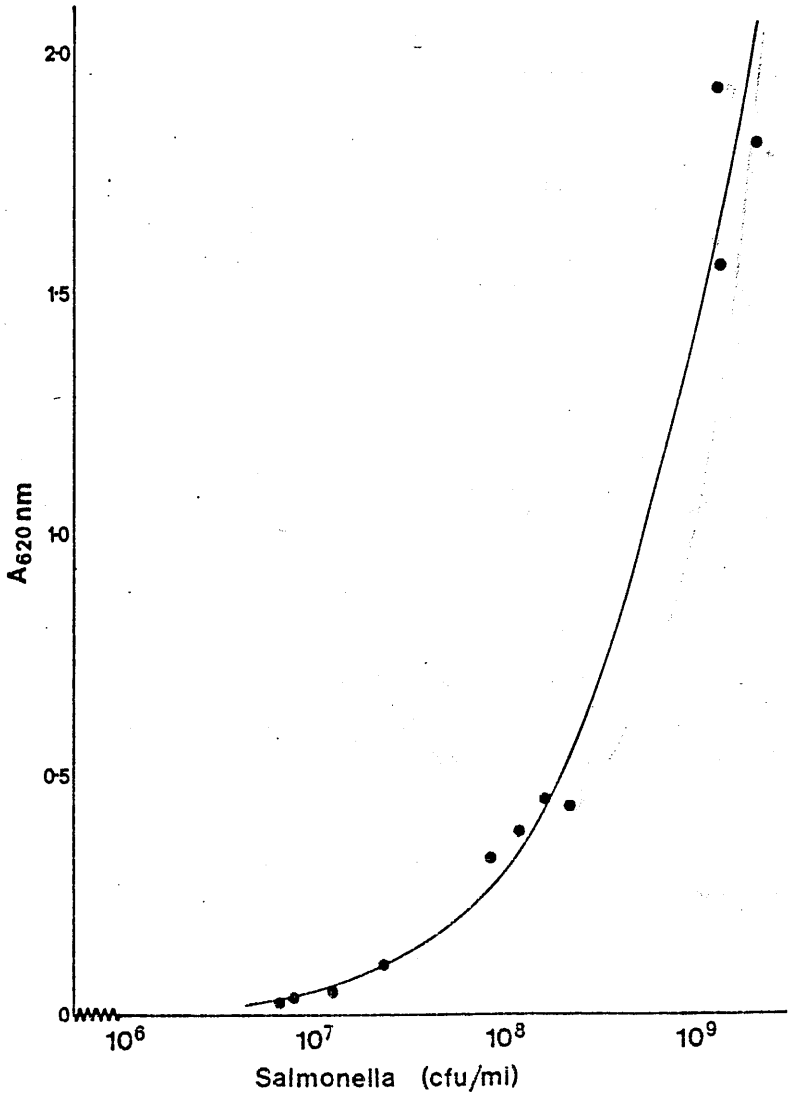



Fig XLI Correlation between absorbance at 620nm and concentration of Salmonella typhimurium.

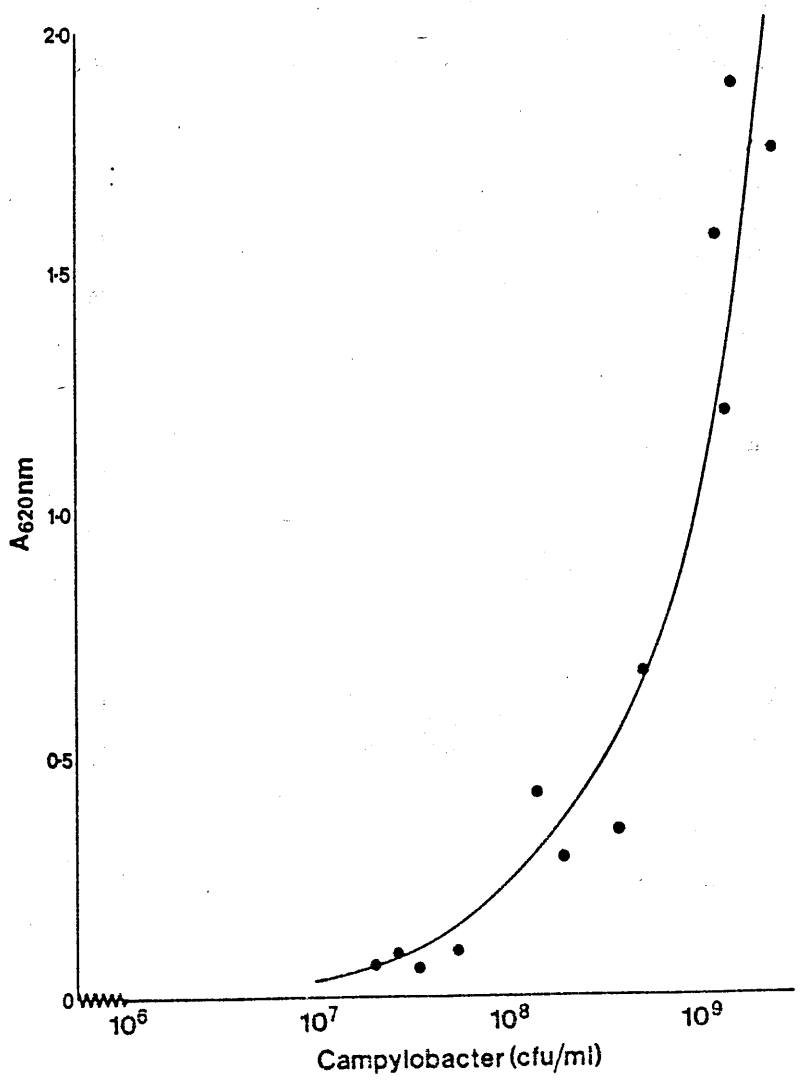



Fig XLII Correlation between absorbance at 620nm and concentration of Campylobacter jejuni.

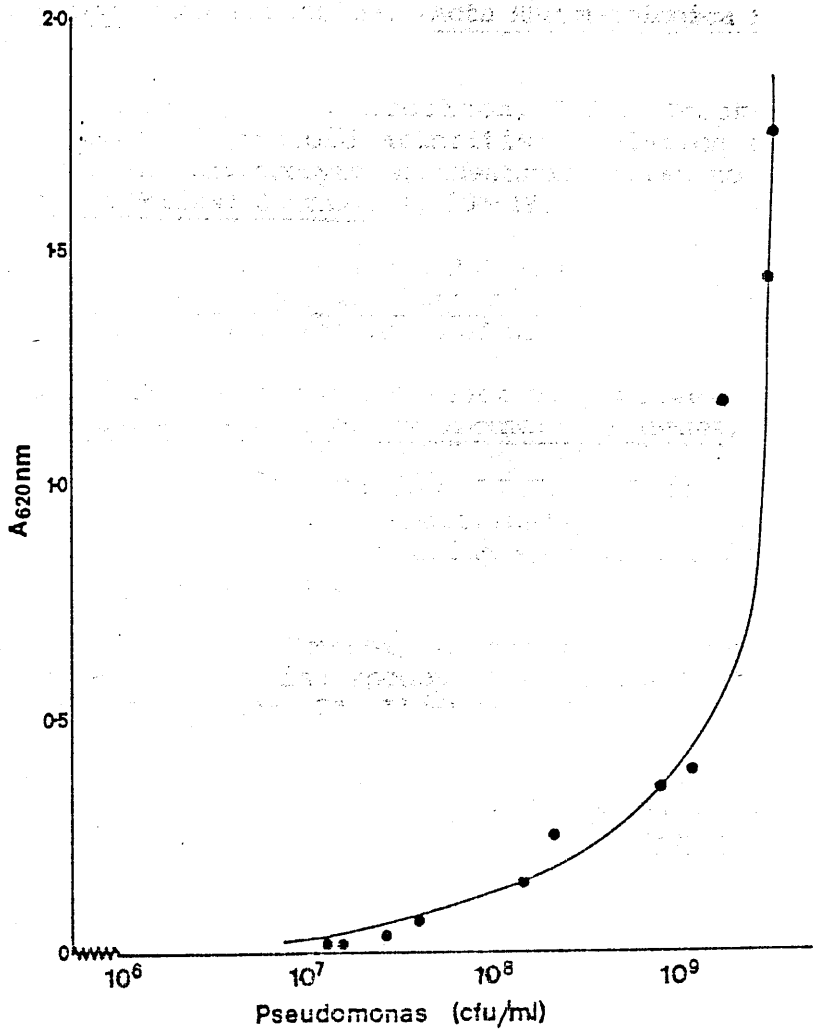



Fig XLIII Correlation between absorbance at 620nm and concentration of Pseudomonas aeruginosa.

## References


Aho, K., Ahvonen, P., Lassus, A., Sievers, K. & Tiilikainen, A. (1974) HL-A 27 in reactive arthritis. A study of Yersinia arthritis and Reiter's disease. Arthritis and Rheumatism, 17, 521-526.

Aho, K., Leirisalo-Repo, M & Repo, H. (1985) Reactive arthritis. Clinics in Rheumatic Diseases, 11, 25-40.

Ahvonen, P., Sievers, K. & Aho, K. (1969) Arthritis associated with Yersinia enterocolitica infection. Acta Rheumatologica Scandinavica, 15, 232-253.

Amos, R.S., Constable, T.J., Crockson, R.A., Crockson, A.P. & McConkey, B. (1977) Rheumatoid arthritis: relation of serum C-reactive protein and erythrocyte sedimentation rates to radiographic changes. British Medical Journal, 1, 195-197.

Arbuthnott, J.P., Owen, P. & Russell, R.J. (1984) Bacterial antigens. In Principles of bacteriology, virology and immunology, ed. Wilson, G. & Dick, H. Vol. 1, Ch. 13. Arnold: London.

Archer, J. (1981) Search for cross-reactivity between HLA-B27 and Klebsiella pneumoniae. Annals of the Rheumatic Diseases, 40, 400-403.

Archer, J.R., Stubbs, M.M., Currey H.L.F. & Geczy, A.F. (1985) Antiserum to Klebsiella K43 BTSI specifically lyses lymphocytes of HLA-B27 positive patients with ankylosing spondylitis from a London population. Lancet, 1, 344-345.

Arellano, J., Vallejo, M., Jimenez, J., Mintz, G. & Kretschmer, R.R. (1984) HLA-B27 and ankylosing spondylitis in the Mexican Mestizo population. Tissue Antigens, 23, 112-116.

Avakian, H., Welsh, J., Ebringer, A. & Entwistle, C.C. (1980) Ankylosing spondylitis, HLA-B27 and Klebsiella. II Cross-reactivity studies with human tissue typing sera. British Journal of Experimental Pathology, 61, 92-96.

Banck, G. & Forsgren, A. (1978) Many bacterial species are mitogenic for human blood B lymphocytes. Scandanavian Journal of Immunology, 8, 347-354.

Bartnik, W. & Kaluzewski, S. (1979) Cellular and humoral response to Kunin antigen in ulcerative colitis and Crohn's disease. Archivum Immunologiae et Therapiae Experimentalis, 27, 531-538.

Beaulieu, A.D., Rousseau, F., Israël-Assayag, E. & Roy, R. (1983) Klebsiella related antigens in ankylosing spondylitis. Journal of Rheumatology, 10, 102-105.

Bjarnason, I., So, A., Levi, A.J., Peters, T.J., Williams, P., Zanelli, G.D., Gumpel, J.M. & Ansell, B. (1984a) Intestinal permeability and inflammation in rheumatoid arthritis: effects of non-steroidal anti-inflammatory drugs. Lancet, 2, 1171-1174.

Bjarnason, I., Ward, K. & Peters, T.J. (1984b) The leaky gut of alcoholism: possible route of entry for toxic compounds. Lancet, 1, 179-182.

Bjarnason, I., Williams, P., Smethurst, P., Peters, T.J. & Levi, A.J. (1986) Effect of non-steroidal anti-inflammatory drugs and prostaglandins on the permeability of the human small intestine. Gut, 27, 1292-1297.

Brewerton, D.A., Caffrey, M., Nicholls, A., Walters, D. & James, D.C.O. (1973a) Acute anterior uveitis and HL-A27. Lancet, 2, 994-996.

Brewerton, D.A., Hart, F.D., Nicholls, A., Caffrey, M., James, D.C.O. & Sturrock, R.D. (1973b) Ankylosing spondylitis and HL-A27. Lancet, 1, 904-907.

Brewerton, D.A., Nicholls, A., Oates, J.K., Caffrey, M., Walters, D. & James, D.C.O. (1973c) Reiter's disease and HL-A27. Lancet, 2, 996-998.

Brewerton, D.A. (1976) HLA-B27 and the inheritance of susceptibility to rheumatic disease. Arthritis and Rheumatism, 19, 656-668.

Bruneau, C. & Bonin, H. (1983) Evidence for a disease specific antigen in circulating immune complexes in ankylosing spondylitis. Clinical and Experimental Immunology, 53, 529-535.

Bull, M.D. & Ignaczak, T.F. (1973) Enterobacterial common antigen-induced lymphocyte reactivity in inflammatory bowel disease. Gastroenterology, 64, 43-50.

Byrom, N.A., Hobbs, J.R., Timlin, D.M., Campbell, M.A., Dean, A.J., Webley, M. & Brewerton, D.A. (1979) T and B lymphocytes in patients with acute anterior uveitis and ankylosing spondylitis, and in their household contacts. Lancet, 2, 601-603.

Calguneri, M., Swinburne, L., Shinebaum, R., Cooke, E.M. & Wright, V. (1981) Secretory IgA: immune defence pattern in ankylosing spondylitis and klebsiella. Annals of the Rheumatic Diseases, 40, 600-604.

Calin, A. & Fries, J. (1976) An "experimental" epidemic of Reiter's syndrome revisited. Follow up evidence on genetic and environmental factors. Annals of Internal Medicine, 84, 564-566.

Calin, A., Marder, A., Becks, E. & Burns, T. (1983) Genetic differences between B27 positive patients with ankylosing spondylitis and B27 positive healthy controls. Arthritis and Rheumatism, 26, 1460-1464.

Calin, A. (1985) Ankylosing spondylitis. Clinics in Rheumatic Diseases, 11, 41-60.

Cameron, F.H., Russell, P.J., Sullivan, J. & Geczy, A.F. (1983) Is a klebsiella plasmid involved in the aetiology of ankylosing spondylitis in HLA-B27 positive individuals? Molecular Immunology, 20, 563-566.

Cameron, F.H., Russel, P.J., Easter, J.F., Wakefield, D. & March, L. (1987) Failure of Klebsiella pneumoniae antibodies to cross-react with peripheral blood mononuclear cells from patients with ankylosing spondylitis. Arthritis and Rheumatism, 30, 300-305.

Caruso, I. & Bianchi, P.G. (1980) Gastroscopic evaluation of anti-inflammatory agents. British Medical Journal, 280, 75-78.

Cavender, D. & Ziff, M. (1986) Anti-HLA-B27 antibodies in sera from patients with gram-negative bacterial infections. Arthritis and Rheumatism, 29, 352-357.

Christiansen, F.T., Hawkins, B.R. & Dawkins, R.L. (1978) Immune function in ankylosing spondylitis and their relatives: influence of disease and HLA-B27. Clinical and Experimental Immunology, 33, 270-275.

Cleland, L.G., Hay, J.A.R. & Milazzo, S.C. (1975) Absence of HLA-27 and of ankylosing spondylitis in Central Australian Aboriginals. Scandanavian Journal of Rheumatology, 8, supp Abstract 30-05.

Cohen, M.L. (1985) Antigenic characterization of enterobacteriaceae associated with Reiter's syndrome. In Advances in Inflammation Research, ed Ziff, M. & Cohen, S.B. Vol. 9., pp 173-177.

Cowling, P., Ebringer, R., Cawdell, D., Ishii, M. & Ebringer, A. (1980a) C-reactive protein, ESR and klebsiella in ankylosing spondylitis. Annals of the Rheumatic Diseases, 39, 45-49.

Cowling, P., Ebringer, R. & Ebringer, A. (1980b) Association of inflammation with raised serum IgA in ankylosing spondylitis. Annals of Rheumatic Diseases, 39, 545-549.

Cupps, T.R. & Fauci, A.S. (1982) Corticosteroid-mediated immunoregulation in man. Immunological Reviews, 65, 134-155.

Eastmond, C.J. & Woodrow, J.C. (1977) Discordance for ankylosing spondylitis in monozygotic twins. Annals of the Rheumatic Diseases, 36, 360-364.

Eastmond, C.J., Cooke, E.M. &Wright, V. (1978) Klebsiella pneumoniae. Annals of the Rheumatic Diseases, 37, 298-299.

Eastmond, C.J., Willshaw, H.E., Burgess, S.E.P., Shinebaum, R., Cooke, E.M. & Wright, V. (1980) Frequency of faecal Klebsiella aerogenes in patients with ankylosing spondylitis and controls with respect to individual features of the disease. Annals of the Rheumatic Diseases, 39, 118-123.

Eastmond, C.J., Calguneri, M., Shinebaum, R., Cooke, E.M. & Wright, V. (1982) A sequential study of the relationship between faecal Klebsiella aerogenes and the common clinical manifestations of ankylosing spondylitis. Annals of the Rheumatic Diseases, 41, 15-20.

Ebringer, A. (1978a) The link between genes and diseases. New Scientist, 79, 865-867.

Ebringer, A. (1982) The cross-tolerance hypothesis in IR-gene systems, rheumatic fever and ankylosing spondylitis. Rivista di Biologia, 75, 197-229.

Ebringer, A. (1983) The cross-tolerance hypothesis, HLA-B27 and ankylosing spondylitis. British Journal of Rheumatology, 22 (supp 2), 53-66.

Ebringer, A., Baines, M., Childerstone, M., Ghuloom, M. & Ptaszynska, T. (1985a) Etiopathogenesis of ankylosing spondylitis and the cross-tolerance hypothesis. In Advances in Inflammation Research, ed. Ziff, M. & Cohen, S.B. Vol. 9, pp 119-128. New York: Raven Press.

Ebringer, A., Baines, M. & Ptaszynska, T. (1985b) Spondyloarthritis, uveitis, HLA-B27 and Klebsiella. Immunological Reviews, 86, 101-116.

Ebringer, A., Corbett, M., MacAfee, Y., Baron, P., Ptaszynska, T., Wilson, C., Avakian, H. & James, D.C.O. (1985c) Antibodies to proteus in rheumatoid arthritis. Lancet, 2, 305-207.

Ebringer, R., Cooke, D., Cawdell, D.R., Cowling, P. & Ebringer, A. (1977) Ankylosing spondylitis: klebsiella and HLA-B27. Rheumatology and Rehabilitation, 16, 190-195.

Ebringer, R.W., Cawdell, D.R., Cowling, P. & Ebringer, A. (1978b) Sequential studies in ankylosing spondylitis. Association of Klebsiella pneumoniae with active disease. Annals of the Rheumatic Diseases, 37, 146-151.

Ebringer, R.W. (1980) HLA-B27 and the link with rheumatic diseases: recent developments. Clinical Science, 59, 405-410.

Ebringer, R. & Ebringer, A. (1981) Ankylosing spondylitis: host parasite interaction in the production of rheumatological disease. In Recent Advances in Rheumatology, ed. Buchanan, W.W. & Dick, W.C. Vol. 2, pp 107-120. New York: Churchill Livingstone.

Edmonds, J., MacAuley, D., Tyndall, A., Liew, M., Alexander, K., Geczy, A. & Bashir, H. (1981) Lymphocytotoxicity of anti-klebsiella antisera in ankylosing spondylitis and related arthropathies. Arthritis and Rheumatism, 24, 1-7.

Edmonds, J. (1984) Reactive arthritis. Australian and New Zealand Journal of Medicine, 14, 81-88.

Enlow, R.W., Bias, W.B., Bluestone, R. & Arnett, F.C. (1982) Human lymphocyte response to selected infectious agents in Reiter's syndrome and ankylosing spondylitis. Rheumatology International, 1, 171-175.

Escanilla, F., Alepa, F.P. & Reefe, W. (1970) Impaired lymphocyte responses in ankylosing spondylitis. Arthritis and Rheumatism, 13, 314.

Fan, P.T., Clements, P.J., Yu, D.T.Y., Opelz, G. & Bluestone, R. (1977) Lymphocyte abnormalities in ankylosing spondylitis. Annals of the Rheumatic Diseases, 36, 471-473.

Foley, J.A. & Mathews, J.A. (1984) Reactive arthritis due to Yersinia enterocolitica. Clinical Rheumatology, 3, 385-387.

Ford, D.K., da Roza, D.M. & Shah, P. (1981) Cell-mediated immune responses of synovial mononuclear cells to sexually transmitted, enteric and mumps antigens in patients with Reiter's syndrome, rheumatoid arthritis and ankylosing spondylitis. Journal of Rheumatology, 8, 220-232.

Ford, D.K., da Roza, D.M. & Schulzer, M. (1982) The specificity of synovial mononuclear cell responses to microbial antigens in Reiter's syndrome. Journal of Rheumatology, 9, 561-567.

Ford, D.K. (1983) Infectious agents in Reiter's syndrome. Clinical and Experimental Rheumatology, 1, 273-277.

Fournie, G.J., Lambert, P.A. & Miescher, P. (1974) Release of DNA in circulating blood and inductions of anti-DNA antibodies after injection of bacterial lipopolysaccharides. Journal of Experimental Medicine, 140, 1189-1206.

Franssen, M.J.A.M., van de Putte, L.B.A. & Gribnau, F.W.J. (1985) IgA serum levels and disease activity in ankylosing spondylitis: a perspective study. Annals of the Rheumatic Diseases, 44, 766-771.

Froebel, K., Sturrock, R.D., Dick, W.C. & MacSween, R.N. (1975) Cell mediated immunity in the rheumatoid diseases. I Skin testing and mitogenic responses in seronegative arthritides. Clinical and Experimental Immunology, 22, 446-452.

Fudenberg, H.H., Stites, D.P., Caldwell, J.L. & Wells, J.V. (1976) Basic and Clinical Immunology. California: Lange Medical Publications.

Geczy, A.F., Alexander, K. & Bashir, H.V. (1980a) A factor(s) in klebsiella culture filtrates specifically modifies an HLA-B27-associated cell-surface component. Nature, 283, 782-784.

Geczy, A.F., Seager, K., Bashir, H.V., de Vere-Tyndall, A. & Edmonds, J. (1980b) The role of Klebsiella in the pathogenesis of ankylosing spondylitis. II Evidence for a specific B27-associated marker on the lymphocytes of patients with ankylosing spondylitis. Journal of Clinical and Laboratory Immunology, 3, 23-28.

Geczy, A.F., Alexander, K., Bashir, H.V., Edmonds, J.P., Upfold, L.I. & Sullivan, J. (1983) HLA-B27, klebsiella and ankylosing spondylitis: biological and chemical studies. Immunological Reviews, 70, 23-50.

Geczy, A.F., McGuigan, L.E., Sullivan, J.S. & Edmonds, J.P. (1986) Cytotoxic T lymphocytes against disease-associated determinant(s) in ankylosing spondylitis. Journal of Experimental Medicine, 164, 932-937.

Georgopoulos, K., Dick, W.C., Goodacre, J.A. & Pain, R.H. (1985) A reinvestigation of the cross-reactivity between Klebsiella and HLA-B27 in the aetiology of ankylosing spondylitis. Clinical and Experimental Immunology, 62, 662-671.

Gofton, J.P., Chalmers, A., Price, G.E. & Reeve, C.E. (1975) HL-A27 and ankylosing spondylitis in B.C. Indians. Journal of Rheumatology, 2, 314-318.

Goodwin, J.S. & Webb, D.R. (1980) Regulation of the immune response by prostaglandins. Clinical Immunology and Immunopathology, 15, 106-122.

Goodwin, J.S., Ceuppens, J.L. & Rodriguez, M.A. (1983) Administration of non-steroidal anti-inflammatory agents in patients with rheumatoid arthritis. Journal of the American Medical Association, 250, 2485-2488.

Granfors, K. (1979) Measurement of immunoglobulin M (IgM), IgG and IgA antibodies against Yersinia enterocolitica by enzyme-linked immunosorbent assay: persistence of antibodies during disease. Journal of Clinical Microbiology, 9, 336-341.

Granfors, K., Viljanen, M., Tiilikainen, A. & Toivanen, A. (1980) Persistence of IgM, IgG and IgA antibodies to Yersinia in Yersinia arthritis. Journal of Infectious Diseases, 141, 424-429.

Granfors, K. & Toivanen, A. (1986) IgA anti-yersinia antibodies in yersinia triggered reactive arthritis. Annals of the Rheumatic Diseases, 45, 561-565.

Greenwood, M. & Hooper, W.L. (1987) Human carriage of Yersinia spp. Journal of Medical Microbiology, 23, 345-348.

Hall, R. & Malia, R.G. (1984) Erythrocyte sedimentation rate and plasma viscosity. In Medical Laboratory Haematology. Vol. 7, pp 182-190. London: Butterworths.

Hammarström, L., Smith, E., Primi, D. & Möller, G. (1976) Induction of autoantibodies to red blood cells by polyclonal B cell activators. Nature, 263, 60-66.

Hickling, P., Turnbull, L. & Dixon, J.S. (1982) The relationship between disease activity, immunoglobulins and lymphocyte sub-populations in ankylosing spondylitis. Rheumatology and Rehabilitation, 21, 145-150.

Hill, H.F.H., Hill, A.G.S. & Bodmer, J.G. (1976) Clinical diagnosis of ankylosing spondylitis in women and relation to presence of HLA-B27. Annals of the Rheumatic Diseases, 35, 267-270.

Hodgson, H.J.F., Wands, J.R. & Isselbacher, K.J. (1978) Decreased suppressor cell activity in inflammatory bowel disease. Clinical and Experimental Immunology, 32, 451-458.

Hofstra, H. & Dankert, J. (1980) Major outer membrane proteins: common antigens in Enterobacteriaceae species. Journal of General Microbiology, 119, 123-131.

Hunter, T., Harding, G.K.M., Kaprove, R.E. & Schroeder, M-L. (1981) Faecal carriage of various Klebsiella and Enterobacter species in patients with active ankylosing spondylitis. Arthritis and Rheumatism, 24, 106-108.

James, S.P., Fiocchi, G., Graeff, A.S. & Strober, W. (1986) Phenotypic analysis of lamina propria lymphocytes-predominance of helper-inducer and cytolytic T-cell phenotypes and deficiency of suppressor-inducer phenotypes in Crohn's disease and control patients. Gastroenterology, 91, 1483-1489.

Jayson, M.I.V., Salmon, P.R. & Harrison, W.J. (1970) Inflammatory bowel disease in ankylosing spondylitis. Gut, 11, 506-511.

Jenkins, R.T., Rooney, P.J., Jones, D.B., Bienenstock, J. & Goodacre, R.L. (1987) Increased intestinal permeability in patients with rheumatoid arthritis: a side-effect of oral non-steroidal anti-inflammatory drug therapy? British Journal of Rheumatology, 26, 103-107.

Jennette, J.C., Ferguson, A.L., Moore, M.A. & Freeman, D.G. (1982) IgA nephropathy associated with seronegative spondylarthropathies. Arthritis and Rheumatism, 25, 144-149.

Keat, A.C., Thomas, B.J., Taylor-Robinson, D., Pegrum, G.D., Maini, R.N. & Scott, J.T. (1980) Evidence of Chlamydia trachomatis infection in sexually acquired reactive arthritis. Annals of the Rheumatic Diseases, 39, 431-437.

Keat, A. (1982) HLA-linked disease susceptibility and reactive arthritis. Journal of Infection, 5, 227-239.

Keat, A. (1983) Reiter's syndrome and reactive arthritis in perspective. New England Journal of Medicine, 309, 1606-1615.

Kekomäki, R., Granfors, K., Leino, R., Penttinen, K., Lindström, P-L. & Wager, O. (1983) Clinical correlates of circulating immune complexes in patients with recent yersiniosis. Journal of Infectious Diseases, 148, 223-229.

Khan, M.A., Braun, W.E., Kushner, I., Grecek, D.E., Muir, W.A. & Steinberg, A.G.G. (1979) HLA-B27 in ankylosing spondylitis: differences in frequency and relative risk in American Blacks and caucasians. Journal of Rheumatology, 4 (supp 3), 39-43.

Khan, M.A. (1985) Spondyloarthropathies in non-caucasian populations of the World. In Advances in Inflammation Research, ed. Ziff, M. & Cohen, S.B. Vol. 9, 91-99. New York: Raven Press.

Kinsella, T.D., Espinoza, L. & Vasey, F.B. (1975) Serum complement and immunoglobulin levels in sporadic and familial ankylosing spondylitis. Journal of Rheumatology, 2, 308-313.

Kinsella, T.D., Lanteigne, C., Fritzler, M.J. & Lewkonia, R.M. (1984) Absence of imparied lymphocyte transformation to Klebsiella spp in ankylosing spondylitis. Annals of the Rheumatic Diseases, 43, 590-593.

Kinsella, T.D., Fritzler, M.J. & Lewkonia, R.M. (1986) Normal anti-Klebsiella lymphocytotoxicity in ankylosing spondylitis. Arthritis and Rheumatism, 29, 358-362.

Kivilaakso, E., Valtonen, V.V., Malkamäki, M, Palmu, A., Schröder, T., Nikki, P., Mäkelä, P.H. & Lempinen, M. (1984) Endotoxaemia and acute pancreatitis: correlation between the severity of the disease and the anti-enterobacterial common antigen antibody titre. Gut, 25, 1065-1070.

Kono, D.H., Park, M.S., Yu, D.T.Y., Granfors, K. & Toivanen, A. (1984) Absence of lymphotoxic antibodies in patients with Yersinia-induced arthritis, Reiter's syndrome and ankylosing spondylitis. Clinical and Experimental Rheumatology, 2, 303-307.

Kono, D.H., Ogasawara, M., Effros, R.B., Waldord, R.L. & Yu, D.T.Y. (1985) Ye-1, a monoclonal antibody that cross-reacts with HLA-B27 lymphoblastoid cell lines and an arthritis causing bacteria. Clinical and Experimental Immunology, 61, 503-508.

Kosunen, T., Kauranen, O., Martio, J., Pitkänen, T., Pönkä, A., Hortling, L., Aittoniemi, S., Mutru, O., Penttilä, O. & Koskimies, S. (1980) Reactive arthritis after Campylobacter jejuni enteritis in patients with HLA-B27. Lancet, 1, 1312-1313.

Kuberski, T.T., Morse, H.G., Rate, R.G. & Bonnell, M.D. (1983) Increased recovery of klebsiella from the gastrointestinal tract of Reiter's syndrome and ankylosing spondylitis patients. British Journal of Rheumatology, 22 (Supp 2), 85-90.

Lahesmaa-Rantala, R., Granfors, K., Kekomäki, R. & Toivanen, A. (1987) Circulating yersinia specific immune complexes after acute yersiniosis: a follow up study of patients with and without reactive arthritis. Annals of the Rheumatic Diseases, 46, 121-126.

Lambert, P.A. (1983) The bacterial surface and drug resistance. In Medical Microbiology, ed. Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W. & Lambert, P.A. Vol. 3. Ch. 1. London: Academic Press.

Lance, E.M. & Knight, S.C. (1974) Immunologic reactivity in rheumatoid arthritis. Arthritis and Rheumatism, 17, 513-520.

Laurent, M.R. & Panayi, G.S. (1983) Acute-phase proteins and serum immunoglobulins in ankylosing spondylitis. Annals of the Rheumatic Diseases, 42, 524-528.

Leach, C. (1983) Introduction to Statistics. Chichester: John Wiley & Sons.

Leino, R., Vuento, R., Koskimes, S., Viander, M. & Toivanen, A. (1983) Depressed lymphocyte transformation by yersinia and Escherichia coli in yersinia arthritis. Annals of the Rheumatic Disease, 42, 176-181.

Leirisalo, M., Skylv, G., Kousa, M., Voipio-Pulkki, L-M., Suoranta, H., Nissilä, M., Hvidman, L., Nielsen, E.D., Svejgaard, A., Tiilikainen, A. & Laitinen, O. (1982) Follow up study on patients with Reiter's disease and reactive arthritis, with special reference to HLA-B27. Arthritis and Rheumatism, 25, 249-259.

Lipsky, P.E. & Ziff, M. (1977) Inhibition of antigen- and mitogen-induced human lymphocyte proliferation by gold compounds. Journal of Clinical Investigation, 59, 455-466.

Lockshin, M.D., Eisenhauer, A.C., Kohn, R., Weksler, M., Block, S. & Mushlin, S.B. (1975) Cell mediated immunity in rheumatic diseases. II Mitogen responses in RA, SLE, and other illnesses: correlation with T- and B-lymphocyte populations. Arthritis and Rheumatism, 18, 245-250.

McConkey, B., Crockson, R.A. & Crockson, A.P. (1972) The assessment of rheumatoid arthritis - A study based on measurements of the serum acute phase reactants. Quarterly Journal of Medicine, 41, 115-125.

McConkey, B., Crockson, R.A., Crockson, A.P. & Wilkinson, A.R. (1973) The effects of some anti-inflammatory drugs on the acute-phase proteins in rheumatoid arthritis. Quarterly Journal of Medicine, 42, 785-791.

McDevitt, H.O. & Bodmer, W.F. (1974) HL-A, immune response genes, and diseases. Lancet, 1, 1269-1275.

McGuigan, L.E., Geczy, A.F. & Edmonds, J.P. (1985) The immunopathology of ankylosing spondylitis - a review. Seminars in Arthritis and Rheumatism, 15, 81-105.

McGuigan, L.E., Geczy, A.F., Prendergast, J.K., Edmonds, J.P., Hart, H.H. & Bashir, H. V. (1986a) HLA-B27 associated cross-reactive markers on the cells of New Zealand patients with ankylosing spondylitis. Annals of the Rheumatic Diseases, 45, 144-148.

McGuigan, L.E., Prendergast, J.K., Geczy, A.F., Edmonds, J.P. & Bashir, H.V. (1986b) Significance of non-pathogenic cross-reactive bowel flora in patients with ankylosing spondylitis. Annals of the Rheumatic Diseases, 45, 566-571.

MacKenzie, A.R. & Williamson, A.R. (1983) The impact of monoclonal antibodies on rheumatoid arthritis. In Recent Advances in Rheumatology, ed. Dick, W.C. & Moll, J.M.H. Vol. 3, pp 29-48. Churchill Livingstone: New York.

MacKintosh, P. & Pease, P. (1982) HLA-B27, ankylosing spondylitis, and some normal rabbit sera. Annals of the Rheumatic Diseases, 41, 433-434.

Madhok, R., MacKenzie, J.A., Lee, F.D., Bruckner, F.E., Terry, T.R. & Sturrock, R.D. (1986) Small bowel ulceration in patients receiving non-steroidal anti-inflammatory drugs for rheumatoid arthritis. Quarterly Journal of Medicine, 225, 53-58.

Manicourt, D.H. & Orloff, S. (1981) Immune complexes in polyarthritis after Salmonella gastroenteritis. Journal of Rheumatology, 8, 613-620.

Mann, D.L., Popovic, M., Savin, P., Murray, C., Reitz, M.S., Strong, D.M., Haynes, B.F., Gallo, R.C. & Blanttner, W.A. (1983) Cell lines producing human T-cell lymphoma virus show altered HLA expression. Nature, 305, 58-59.

Männel, D. & Mayer, H. (1978) Isolation and chemical characterization of enterobacterial common antigen. European Journal of Biochemistry, 86, 361-370.

Mawle, A.C., Hobbs, J.R., Warren, R.E. & Brewerton, D.A. (1978) Lymphocyte transformation to bacterial antigens in ankylosing spondylitis. Annals of the Rheumatic Diseases, 38, 197-200.

Mielants, H. & Veys, E.M. (1985a) Non-steroidal anti-inflammatory drugs and the leaky gut. Lancet, 1, 218-219.

Mielants, H., Veys, E.M., Cuvelier, C., de Vos, M. & Botelberghe, L. (1985b) HLA-B27 related arthritis and bowel inflammation. Part 2 Ileocolonoscopy and bowel history. Journal of Rheumatology, 12, 294-298.

Mogensen, H.H. (1979) Salmonella typhi-induced stimulation of blood lymphocytes from persons with previous typhoid fever. Acta Pathologica Microbiologica Scandinavica (C), 87, 41-45.

Morris, R.I., Metzger, A.L., Bluestone, R. & Terasaki, P.I. (1974) HL-AW27 - a useful discriminator in the arthropathies of inflammatory bowel disease. New England Journal of Medicine, 290, 1117-1119.

Nikbin, B., Brewerton, D.A., Byrom, N., James, D.C.O., Malka, S., McLeod, L., Slater, L., Warren, R.E. & Hobbs, J.R. (1975) Lymphocyte function in ankylosing spondylitis. Annals of the Rheumatic Diseases, 34 (supp), 49-52.

Nilsson, E. & Biberfeld, G. (1980) Subpopulations of T lymphocytes in patients with ankylosing spondylitis. Annals of the Rheumatic Diseases, 39, 566-569.

Noer, H.R. (1969) An "experimental" epidemic of Reiter's syndrome. Journal of the American Medical Association, 197, 693-698.

Nossal, G.J.V. (1987) The basic components of the immune system. New England Journal of Medicine, 316, 1320-1325.

Ogasawara, M., Kono, D.H. & Yu, D.T.Y. (1986) Mimicry of human histocompatibility HLA-B27 antigens by Klebsiella pneumoniae. Infection and Immunity, 51, 901-908.

Orban, P., Sullivan, J.S., Geczy, A.F., Upfold, L.I., Coulits, N. & Bashir, H.V. (1983) A factor shed by lymphoblastoid cells lines of HLA-B27 positive patients with ankylosing spondylitis, specifically modifies the cells of HLA-B27 positive normal individuals. Clinical and Experimental Immunology, 53, 10-16.

Pepys, M.B. (1981) C-reactive protein fifty years on. Lancet, 1, 653-657.

Petit, J.C. & Unanue, E.R. (1974) Effects of bacterial products on lymphocytes and macrophages: their possible role in natural resistance to Listeria infection in mice. Journal of Immunology, 113, 984-992.

Prendergast, J.K., Sullivan, J.S., Geczy, A., Upfold, L.I., Edmonds, J.P., Bashir, H.V. & Reiss-Levy, E. (1983) Possible role of enteric organisms in the pathogenesis of ankylosing spondylitis and other seronegative arthropathies. Infection and Immunity, 41, 935-941.

Prendergast, J.K., McGuigan, L.E., Geczy, A.F., Kwong, T.S.L. & Edmonds, J.P. (1984) Persistence of HLA-B27 cross-reactive bacteria in bowel flora of patients with ankylosing spondylitis. Infection and Immunity, 46, 686-689.

Raeman, F., Decock, W., De Beukelaar, T., Decree, J. & Verhaegen, H. (1981) Enumeration of T lymphocytes and T lymphocyte subsets in autoimmune disease using monoclonal antibodies. Clinical and Experimental Immunology, 45, 475-479.

Ramia, S., Kuhn, H-M., Mayer, H. & Neter, E. (1983) Production by various pseudomonas species of a factor modifying the enterobacterial common antigen (41689). Proceedings of the Society for Experimental Biology and Medicine, 173, 574-578.

Räsänen, L., Karhumäki, E., Majuri, R. & Aruilommi, H. (1980) Polyclonal activation of human lymphocytes by bacteria. Infection and Immunity, 28, 368-372.

Rate, R.G., Morse, H.G., Bonnell, M.D. & Kuberski, T.T. (1980) 'Navajo arthritis' reconsidered: relationship to HLA-B27. Arthritis and Rheumatism, 23, 1299-1302.

Robinson, S. & Panayi, G.S. (1983a) The binding to human lymphocytes of arthritogenic and non-arthritogenic bacteria. Clinical and Experimental Rheumatology, 1, 211-214.

Robinson, S., Panayi, G.S., Marsal, L. & Wollheim, F.A. (1983b) The attachment of certain gram negative bacteria to buccal epithelial cells from patients with Yersinia arthritis. Clinical and Experimental Rheumatology, 1, 207-210.

Rødahl, E. & Iversen, O-J. (1986) Analysis of circulating immune complexes from patients with ankylosing spondylitis by gel electrophoresis and immunoblotting using antiserum against a psoriasis associated retrovirus-line particle. Annals of the Rheumatic Diseases, 45, 892-898.

Rubinstein, A., Das, K.M., Melamed, J. & Murphy, R.A. (1978) Comparitive analysis of systemic immunological parameters in ulcerative colitis and idiopathic proctitis: effects of sulfasalazine in vivo and in vitro. Clinical and Experimental Immunology, 33, 217-224.

Ryder, L.P., Andersson, E. & Svejgaard, A. (eds) (1979) HLA and Disease Registry. Third Report. Copenhagen: Munksgaard.

Sachar, D.B., Taub, R.N., Brown, S.M., Present, D.H., Korelitz, B.I. & Janowitz, H.D. (1973) Impaired lymphocyte responsiveness in inflammatory bowel disease. Gastroenterology, 64, 203-209.

Sampson, J.A. & Cope, J.B. (1984) Enteritis and Reiter's syndrome associated with Yersinia enterocolitica. Australian and New Zealand Journal of Medicine, 14, 864-865.

Sanders, K.M., Hertzman, A., Escobar, M.R. & Littman, B.H. (1987) Correlation of immunoglobulin and C-reactive protein levels in ankylosing spondylitis and rheumatoid arthritis. Annals of the Rheumatic Diseases, 46, 273-276.

Schlosstein, L., Terasaki, P.I., Bluestone, R. & Pearson, C.M. (1973) High association of an HLA antigen, W27, with ankylosing spondylitis. New England Journal of Medicine, 288, 704-706.

Scott, D.G.I., Ring, E.F.J. & Bacon, P.A. (1981) Problems in the assessment of disease activity in ankylosing spondylitis. Rheumatology and Rehabilitation, 20, 74-80.

Seager, K., Bashir, H.V., Geczy, A.F., Edmonds, J. & de Vere-Tyndall, A. (1979) Evidence for a specific B27-associated cell surface marker on lymphocytes of patients with ankylosing spondylitis. Nature, 277, 68-70.

Selby, W.S. & Jewell, D.P. (1983) T lymphocyte subsets in inflammatory bowel disease: peripheral blood. Gut, 24, 99-105.

Sheehan, N.J., Slavin, B.M., Donovan, M.P., Mount, J.N. & Mathews, J.A. (1986) Lack of correlation between clinical disease activity and erythrocyte sedimentation rate, acute phase proteins and protease inhibitors in ankylosing spondylitis. British Journal of Rheumatology, 25, 171-174.

Sheldon, P.J. & Pell, P.A. (1985) Lymphocyte proliferative responses to bacterial antigens in B27-associated arthropathies. British Journal of Rheumatology, 24, 11-18.

Shinebaum, R., Cooke, E.M., Siegerstetter, J. & Wright, V. (1981) Effect of klebsiella capsular antisera on lymphocytes from patients with ankylosing spondylitis. Journal of Medical Microbiology, 14, 451-456.

Shinebaum, R., Neumann, V., Hopkins, R., Cooke, E.M. & Wright, V. (1984) Attempt to modify klebsiella carriage in ankylosing spondylitis by diet: correlation of klebsiella carriage with disease activity. Annals of the Rheumatic Diseases, 43, 196-199.

Shorter, R.G. (1981) $T_G$ cells and non-specific concanavalin-A-induced suppressor cell activity in vitro in colonic inflammatory bowel disease, and in colorectal carcinoma. In Developments in Gastroenterology, ed. Pena, A.S., Weterman, I.T., Booth, C.C. & Strober, W. Vol. 1, pp 448-458. The Hague: Martinus-Nijhoff.

Silvoso, R.R., Ivey, K.J., Butt, J.H., Lockard, O.O., Holt, S.D., Sisk, C., Baskin, W.N., MacKercher, P.A. & Hewett, J. (1979) Incidence of gastric lesions in patients with rheumatic diseases on chronic aspirin therapy. Annals of Internal Medicine, 91, 517-520.

Simon, D.F., Kaslow, R.A., Rosenbaum, J., Kaye, R. & Calin, A. (1981) Reiter's syndrome following epidemic Shigellosis. Journal of Rheumatology, 8, 969-973.

Simpson, L.O. (1985) Non-steroidal anti-inflammatory drugs and the leaky gut. Lancet, 1, 218-219.

Singh, B., Milton, J.D. & Woodrow, J.C. (1986) Ankylosing spondylitis, HLA-B27, and klebsiella: a study of lymphocyte reactivity of anti-Klebsiella sera. Annals of the Rheumatic Diseases, 45, 190-197.

Smith, M.D., Gibson, R.A. & Brooks, P.M. (1985) Abnormal bowel permeability in ankylosing spondylitis and rheumatoid arthritis. Journal of Rheumatology, 12, 299-305.

Sotnik, D. (1979) T cells in ankylosing spondylitis. Annals of Rheumatic Diseases, 38, 199.

Stein, H.B., Abdullah, A., Robinson, H.S. & Ford, D.K. (1980) Salmonella reactive arthritis in British Columbia. Arthritis and Rheumatism, 23, 206-210.

Stodell, M.A., Butler, R.C., Zemelman, V.A., Henry, K. & Brewerton, D.A. (1984) Increased numbers of IgG-containing cells in rectal lamina propria of patients with ankylosing spondylitis. Annals of the Rheumatic Diseases, 43, 172-176.

Struthers, G.R., Andrews, D.J., Wilson, R.J.C., Reynolds, G.A. & Low-Beer, T. (1985) Intestinal permeability. Lancet, 1, 587-588.

Sturges, H.F. & Krone, C.L. (1973) Ulceration and stricutre of the jejunum in a patient on long-term indomethacin therapy. American Journal of Gastroenterology, 59, 162-169.

Sturrock, R.D., Froebel, K., MacSween, K.N.M. & Dick, W.C. (1975) Evidence of impaired cell mediated immunity in the seronegative arthritides. Annals of the Rheumatic Diseases, 34, 203.

Sullivan, J., Upfold, L., Geczy, A.F., Bashir, H.V. & Edmonds, J.P. (1982) Immunochemical characterization of Klebsiella antigens which specifically modify an HLA-B27-associated cell-surface component. Human Immunology, 5, 295-307.

Svejgaard, A. & Ryder, L.P. (1976) Interation of HLA molecules with non-immunological ligands as an explanation of HLA and disease associations. Lancet, 2, 547-549.

Svejgaard, A., Morling, N., Platz, P., Ryder, L.P & Thomsen, M. (1981) HLA and disease associations with special reference to mechanisms. Transplantation Proceedings, 13, 913-917.

Swinscow, T.D.V. (1983) Statistics at square one. London: British Medical Association.

Trapani, J.A. & McKenzie, I.F.C. (1985) Klebsiella 'modifying factor': binding studies with HLA-B27+ and B27- lymphocytes. Annals of the Rheumatic Diseases, 44, 169-175.

Trull, A.K., Ebringer, R., Panayi, G.S., Colthorpe, D., James, D.C.O. & Ebringer, A. (1983a) IgA antibodies to Klebsiella pneumoniae in ankylosing spondylitis. Scandanavian Journal of Rheumatology, 12, 249-253.

Trull, A.K. & Panayi, G.S. (1983b) Serum and secretory IgA immune response to Klebsiella pneumoniae in ankylosing spondylitis. Clinical Rheumatology, 2, 331-337.

Trull, A., Ebringer, A., Panayi, G., Ebringer, R & James, D.C.O. (1984) HLA-B27 and the immune response to enterobacterial antigens in ankylosing spondylitis. Clinical and Experimental Immunology, 55, 74-80.

Turunen, U., Malkamäki, M., Valtonen, V.V., Larinkari, U., Pikkarainen, P., Salaspuro, M.P. & Mäkelä, P.H. (1981) Endotoxin and liver diseases. High titres of enterobacterial common antigen antibodies in patients with alcoholic cirrhosis. Gut, 22, 849-853.

Upfold, L.I., Sullivan, J.S., Prendergast, J.K. & Geczy, A.F. (1985) HLA-B27: Speculations on the nature of its involvement in ankylosing spondylitis. Progress in Allergy, 36, 177-189.

Upfold, L.I., Sullivan, J.S. & Geczy, A.F. (1986) Biochemical studies on a factor isolated from Klebsiella K43-BTS1 that cross-reacts with cells from HLA-B27 positive patients with ankylosing spondylitis. Human Immunology, 17, 224-238.

Urman, J.D., Zurrier, R.B. & Rothfield, N.F. (1977) Reiter's syndrome associated with Campylobacter fetus infection. Annals of Internal Medicine, 86, 444-445.

Van Bohemen, C.G., van Alphen, A.J.W., Zanen-Lim, O.G., Dekker-Saeys, A.J. & Zanen, H.C. (1983) Antibodies against cell envelope antigens of Yersinia enterocolitica in reactive arthritis and ankylosing spondylitis. British Journal of Rheumatology, 22 (supp 2), 83-84.

Van Bohemen, C.G., Grumet, F.C. & Zanen, H.C. (1984) Identification of HLA-B27 M1 and M2 cross-reactive antigens in Klebsiella, shigella and yersinia. Immunology, 52, 607-609.

Van Bohemen, C.G., Nabbe, A.J.J.M., Dekker-Saeys, A.J., Goei The, H.S., van der Linden, J.M.J.P., Cats, A. & Zanen, H.C. (1985) Antibodies to enterobacterial cell envelope antigens in ankylosing spondylitis. In Advances in Inflammation Research, ed. Ziff, M. & Cohen, S.B. Vol. 9, pp 165-171. New York: Raven Press.

Van Bohemen, C.G., Nabbe, A.J.J.M., Goei The, H.S., Dekker-Saeys, A.J. & Zanen, H.C. (1986a) Antibodies to enterobacteriaeceae in ankylosing spondylitis. Scandanavian Journal of Rheumatology, 15, 143-147.

Van Bohemen, C.G., Nabbe, A.J.J.M., Zanen, H.C. & Goei The, H.S. (1986b) Serology and bacteriology in reactive arthritis. Annals of the Rheumatic Diseases, 45, 262-263.

Van de Putte, L.B.A., Berden, J.H.M. Boerbooms, A.T.T., Muller, W.H., Rasker, J.J., Reynvaan-Groendijk, A. & van der Linden, S.M. (1980) Reactive arthritis after Campylobacter jejuni enteritis. Journal of Rheumatology, 7, 531-535.

Van der Linden, S.M., Valkenburg, H.A. & Cats, A. (1985) In Advances in Inflammation Research, ed. Ziff, M. & Cohen, S. Vol. 9, pp 83-89. New York: Raven Press.

Van Kregten, E., Westerdaal, N.A.C. & Willers, J.M.N. (1984) New, simple medium for selective recovery of Klebsiella pneumoniae and Klebsiella oxytoca from human feces. Journal of Clinical Microbiology, 20, 936-941.

Van Rood, J.J., van Leeuwen, A., Ivanyi, P., Cats, A., Breur-Vriesendorp, B.S., Dekker-Saeys, A.J., Kiljstra, A. & van Kregten, E. (1985) Blind confirmation of Geczy factor in ankylosing spondylitis. Lancet, 2, 943-945.

Vartiainen, N.J. & Hurri, L. (1964) Arthritis due to Salmonella typhimurium: report of 12 cases of migratory arthritis in asscciation with Salmonella typhimurium infection. Acta Medica Scandinavica, 175, 771-776.

Veys, E.M. & van Laere, M. (1973) Serum IgG, IgM and IgA levels in ankylosing spondylitis. Annals of the Rheumatic Diseases, 32, 493-496.

Veys, E.M., Verbruggen, G., Hermanns, P., Mielants, H., Van Bruwaene, A., De Brabanter, G., De Landsheere, D. & Immesoete, C. (1983) Peripheral blood T lymphocytes subpopulations in HLA-B27 related rheumatic diseases: Ankylosing spondylitis and reactive synovitis. Journal of Rheumatology, 10, 140-143.

Victorino, R.M.M. & Hodgson, H.J.F. (1981) Spontaneous suppressor cell function in inflammatory bowel disease. Digestive Diseases and Sciences, 26, 801-806.

Vilppula, A.H., Ylo-Kerttula, U.I., Ahlroos, A.K. & Jerho, P.E. (1981) Chlamydial isolations and serology in Reiter's syndrome. Scandanavian Journal of Rheumatology, 10, 181-185.

Vinje, O., Doblong, J.H., Førre, O., Møller, P. & Mellbye, O.J. (1982) Immunoregulatory T cells in the peripheral blood of patients with Bechterew's syndrome. Annals of the Rheumatic Diseases, 41, 41-46.

Vinje, O., Møller, R. & Mellbye, J. (1984) Immunological variables and acute-phase reactants in patients with ankylosing spondylitis (Bechterew's syndrome) and their relatives. Clinical Rheumatology, 3, 501-514.

Vuento, R., Leino, R., Viander, M. & Toivanen, A. (1983) In vitro lymphoproliferative response to Yersinia: depressed response in arthritic patients years after Yersinia infection. Clinical and Experimental Rheumatology, 1, 219-224.

Vuento, R., Leino, R., Viander, M. & Toivanen, A. (1984) Lymphocyte transformation response to gram negative bacteria after Yersinia and Salmonella infection: the importance of entereobacterial common antigen for the response. Journal of Rheumatology, 11, 369-372.

Wake, C.T. (1986) Molecular biology of the HLA Class I and Class II genes. Molecular Biology and Medicine, 3, 1-11.

Warren, C.P.W. (1970) Arthritis associated with Salmonella infections. Annals of the Rheumatic Diseases, 29, 483-487.

Warren, R.E. & Brewerton, D.A. (1980) Faecal carriage of klebsiella by patients with ankylosing spondylitis and rheumatoid arthritis. Annals of the Rheumatic Diseases, 39, 37-44.

Weir, W., Keat, A.C., Welsby, P.D., Brear, G. (1979) Reactive arthritis associated with Campylobacter infection of the bowel. Journal of Infection, 1, 281-284.

Welsh, J., Avakian, H., Cowling, P., Ebringer, A., Wooley, P., Panayi, G. & Ebringer, R. (1980) Ankylosing spondylitis, HLA-B27 and Klebsiella. I Cross reactivity studies with rabbit antisera. British Journal of Experimental Pathology, 61, 85-91.

Winblad, S. (1975) Arthritis associated with Yersinia enterocolitica infections. Scandanavian Journal of Infectious Diseases, 7, 191-195.

Woodrow, J.C. (1985) Genetic aspects of the spondyloarthropathies. Clinics in Rheumatic Diseases, 11, 1-24.

Wright, V. (1978) Seronegative polyarthritis. A unified concept. Arthritis and Rheumatism, 21, 619-633.

Young, C.R., Ebringer, A. & Archer, J.R. (1978) Immune response inversion after hyperimmunisation. Possible mechanism in the pathogenesis of HLA-linked diseases. Annals of the Rheumatic Diseases, 37, 152-158.

Yu, D.T.Y. (1985) Immune response of patients with Reiter's syndrome to Yersinia enterocolitica. In Advances in Inflammation Research, ed. Ziff, M. & Cohen, S.B. Vol. 9, pp 179-187. New York: Raven Press.

Zinkernagel, R.M. & Doherty, P.C. (1974) Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocyte choriomeningitis. Nature, 251, 547-548.