Polymorph Function in Ankylosing Spondylitis

by

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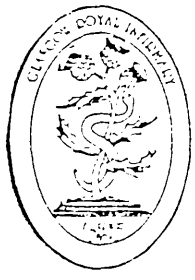

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This thesis is dedicated

to my father

Thomas G.S. Parry

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Summary

The possible role of gram-negative bacteria in the causation of seronegative spondyloarthropathies, and the response of polymorphs to gram-negative bacteria in ankylosing spondylitis, forms the basis of this study.

Polymorph response to arthritogenic gram-negative bacteria in ankylosing spondylitis patients were examined by comparing them to HLA-B27 positive and negative healthy controls, and to a group of rheumatoid arthritis patients.

Bacteria used in the assays had either a speculative or clear association with the seronegative spondyloarthropathies.

Three different assays were used in this study to evaluate

i) Polymorph motility
ii) Polymorph chemiluminescence response to various stimuli and
iii) Phagocytic ingestion of radio-labelled gram-negative bacteria.

No difference was shown in motility between ankylosing spondylitis and normal polymorphs, although a culture filtrate of K. pneumoniae was shown to have some chemotactic quality. In addition phagocytic ingestion of enteric bacteria by polymorphs from ankylosing spondylitis patients was similar to normals. Therefore no abnormalities were shown in either the motility or phagocytic capacity of ankylosing spondylitis polymorphs.

Initial studies using isolated polymorphs showed no difference in chemiluminescence response between patients and normal controls. A whole blood assay was then refined for use in this study, and chemiluminescence basal response was shown to be significantly increased in ankylosing spondylitis polymorphs compared to normal polymorphs. This indicated a pre-activation of normal polymorphs during the cell separation procedure, in our initial studies; thus concealing any inherent difference in chemiluminescence response between patient and normal polymorphs. Subsequent assays also showed a significantly enhanced chemiluminescence response in ankylosing spondylitis patients polymorphs compared to normals following stimulation with gram-negative bacteria and S. aureus. Contrastingly polymorphs from patients with rheumatoid arthritis showed an increase only to S. aureus. These results could indicate that gram-negative bacteria may contribute to an enhanced chemiluminescence response in ankylosing spondylitis patients, which is not specific. In addition, chemiluminescence response did not differ between HLA-B27 positive and negative normals, suggesting that enhanced activation develops with disease, rather than being inherent.

Conclusions suggest a chronic activation and exposure of ankylosing spondylitis to gram-negative triggers.

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ABBREVIATIONS

AS	: Ankylosing spondylitis
HLA	: Human leucocyte antigen.
RS	: Reiters syndrome
AAU	: Acute anterior uveitis.
OM	: Outer membrane
LPS	: Lipopolysaccharide.
PMN	: Polymorphonuclear leucocyte
LP	: Lymphoprep.
NH_4CL	: Ammonium chloride.
HBSS	: Hanks balanced salt solution.
GH	: Gel Hanks
MH	: Mueller Hinton broth.
CLED	: Cysteine lactose electrolyte deficient medium.
PBS	: Phosphate buffered saline.
CFU	: Colony forming units.
OD	: Optical density.
NPS	: Normal pool serum.
ECA	: Enterobacterial common antigen.
cpm	: Counts per minute.
luminol	: 5-amino -2,3 dyhydro-1,4-phthalazinedione.
O_2	: Superoxide anion.
H_2O_2	: Hydrogen peroxide.
-OH	: hydroxyl radical.
$^1O_2$	: singlet oxygen.
CL	: chemiluminescence
CX	: chemotaxis

MPO	: Myeloperoxidase
FMLP	: n-formyl-1-methionyl-1-leucyl-1-phenylolanine.
PMA	: Phorbol myristate acetate.
SOD	: Superoxide dismutase.
CGD	: Chronic granulomatous disease.
Fc	: Complement - fixing region of immumoglobulin.
ESR	: Erythrocyte sedimentation rate
CRP	: C-reactive protein
Igs	: Immunoglobulin levels
Nsaids	: Non-steroidals

CHAPTER 1 INTRODUCTION

1.1. ANKYLOSING SPONDYLITIS

The term ankylosing spondylitis (AS) is derived from the Greek 'ankylos' meaning crooked and 'spondylos' meaning vertebrae. It is a chronic inflammatory disorder and the pathology is dominated by restricted spinal movement and ultimate ossification in the thoracolumber region (Ball, 1971). Involvement of the sacroiliac joint is the hallmark of the disease, and this becomes immobilised as a result of repeated bouts of inflammation of the entheses (bone-ligament junction) followed by bone deposition. Peripheral joints are involved at some stage of the disease in as many as 35% of patients,(Wilkinson & Bywaters, 1958), namely hip & shoulder joints. Other peripheral joints are relatively infrequently involved in primary AS, and tend to resolve without any residual joint deformity in most patients. The most common extraskeletal involvement in AS patients is acute anterior uveitis which occurs in 25% of patients at some time during the course of the disease (Khan et al 1981). The majority of patients with AS report onset of symtoms in their late twenties, early thirties, with only 5% or so beginning after the age of 50 years. (Wilkinson, and Bywaters 1958).

Management of the disease is directed at the suppression of pain and stiffness with non-steroidal anti-inflammatory drugs and a life-long exercise programme, to prevent or minimise the deformities of the spine, which are inherent in the disease.

1.1.1. Epidemiology

The prevalence of the antigen HLA-B27 in AS patients varies from population to population. The highest known prevelence of B27 in normals is in the Haida Indians of British Columbia, of whom 50% carry the B27 antigen (Gofton et al 1975). Navajo Indians have a 36% frequency of B27 (Rate et al 1980), and in both populations, 10% of the males develop A.S. Where the antigen is rare, as with the Australian aborigines and South American Indians, AS is rarely seen (Khan 1985).

Only 9% of normal caucasian populations possess the antigen B27, and 80% of normal, healthy, B27 positive people do not develop AS. Therefore clearly the presence of the antigen itself is not sufficient to cause the disease.

Ankylosing spondylitis is more predominant in males than in females. Wright and Mall in 1973 suggested a sex ratio of 8/10 males to 1 female. More recent studies suggest that the disease has been grossly underdiagnosed, especially in females, and clinical features of AS are found to evolve more slowly in women than in men, thus male : female ratio may be closer to 7:3 (Hill et al , 1976) and (Van der Linden, 1984).

Familial aggregation has been demonstrated for AS, suggesting the disease is genetically inherited (Calin 1985, Wright 1978), and the relatives of probands with spondylitis have the disease more frequently than would be expected from the normal population. In a

survey of 76 probands with AS 3.6% of 250 first degree relatives age 15 years and over, had clinical AS compared to 0.18% of a control population. The relative risk was 20. (Emery and Lawrence, 1967).

A genetic mechanism alone however could not account for the pathogenesis of AS, as monozygotic twins have been shown to be discordant for the disease. (Eastmond and Woodrow 1977). This indicates environmental factors are also involved.

1.1.2. Genetics and HLA Status

The major histocompatibility complex of humans, a multigene family, known as the human leucocyte antigen (HLA) system, is located on chromosome 6 and codes for three classes of cell surface glycoproteins which control several important immunological functions (fig. 1). Class 1 antigens, HLA-A,B,C, are involved in tissue graft rejection since they are recognised as foreign substances by cytotoxic T-cells (Swartz BD, 1982).

The HLA-D region codes for class II proteins and these are involved in antigen presentation to helper T-cells.

Class III antigens are important components of the complement pathway, and are mediators of acute inflammation.

In 1973 Brewerton et al and Schlosstein et al simultaneously reported an association of HLA-B27 with AS. Although the significance and the molecular basis of this association of HLA-B27 with AS is still not

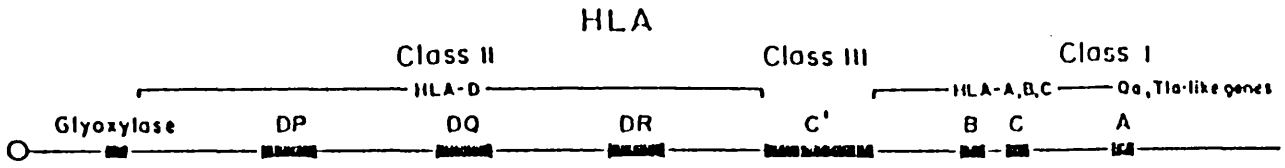


Fig I Molecular map of the HLA complex (taken from Wake 1986).

understood, there are two main theories.

i. The two gene theory which proposes that an associated or linked immune response gene near the B27 locus on chromosome 6, is in 'linkage disequilibrium', rather than randomly associated, with B27 (Benacerraf and McDevitt 1972), (McDevitt and Bodmer 1974). Its gene product somehow mediates the development of AS. This association provides a selective advantage which maintains the genetic link. However no such gene product has been identified. 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. Nor does this theory explain AS patients who do not have the B27 gene.

ii. The one gene theory suggests that the B27 gene product is directly involved in the disease and either resembles an antigen from some environmental agent eg. a micro-organism (cross tolerance hypothothesis) or may act as a receptor for foreign antigens. Interest in the involvement of a micro-organism in the aetiology of AS was stimulated by an initial report from Ebringer et al (1976), which suggested that there was cross-reactivity between HLA-B27 positive (B27+) lymphocytes and several gram-negative bacteria. Klebsiella pneumoniae, they found, could be more frequently isolated from the bowel flora of AS patients than normals, especially when the disease was 'active' (Ebringer et al 1977). Such micro-organisms are thought to initiate the production of antibacterial antibodies that bind to self antigens, activating the complement cascade, thus inducing

inflammatory disease (Ebringer 1983). Kuberski et al (1983) also reported an increase in faecal carriage of K.pneumoniae in 'active' AS and Reiters syndrome (RS).

However some groups have been unable to substantiate these findings e.g. Eastmond et al 1978, and Warren and Breweron 1980 failed to show a significant difference in the isolation rates of K.Pneumoniae in the bowel flora of AS patients to normals. Beaulieu et al 1983 failed to show any cross-reactivity between antisera raised to HLA-B27 lymphocytes and K.pneumoniae. Nevertheless it has been recognised that bacterial infections can trigger spondylarthritis. This is clearly demonstrated in Reiters syndrome (RS) where gram-negative bacteria, including Salmonella (Stein et al 1980) and Yersinia (Chalmers et al 1978) have been clearly implicated in the onset of the disease. The high association of AS and HLA-B27 is also found in many other forms of seronegative inflammatory disease (Ebringer 1980). 60% of patients with RS and 90% who develop arthritis after enteric bacterial infection are HLA-B27 positive. 50% of those with acute anterior uveitis (AAU) and 100% with AAU and AS also possess this antigen. However the strongest association remains between AS and HLA-B27. The true interaction between cell surface structures and micro-organisms is still not understood. Geczy et al in Australia have shown that an antiserum to an isolate of K. pneumoniae is cytotoxic for lymphocytes from AS patients with HLA-B27 positive antigen, but not from HLA B27 negative patients or from HLA B27 positive or negative normals. (Geczy et al, 1980). These cross reactivity experiments have been validated in Sydney, where cells from the UK, Netherlands and New Zealand were correctly identified as being

spondylitic, using Geczy's Klebsiella antisera (Archer et al 1985; Van Rood et al 1985; McGuigan et al 1986a). This cross-reactivity also occurs with other enteric bacteria (Prendergast et al 1983, 1984; McGuigan et al 1986). From their results Geczy suggested that several species of enteric bacteria share a common factor which is related to an HLA-B27 associated cell surface structure on AS patient's cells. Welsh et al 1980 confirmed this theory by showing that serum raised to B27 positive lymphocytes cross reacted with antigens on various bacteria.

An immune response initiated by an invading microbe may also react with a normal host protein, where the linear amino acid sequences are homologous. HLA-B27 has been found to share six consecutive amino acids with K. pneumoniae nitrogenase by comparing the sequence of HLA-B27 with bacteria pathogens in the Dayhoff protein sequence data bank. (Schwimmbeck et al, 1987). They also showed a significant correlation between B27 positive individuals with AS and the production of antibodies to the homologous area, common to both HLA-B27 and K. pneumoniae. This was not the case with healthy B27 positive individuals. These results suggest that an immune response, directed initially against a Klebsiella infection, would also react against homologous HLA-B27 sequences in the host, thus explaining the high incidence of HLA-B27 in AS patients, and providing a potential pathogenic mechanism for the disease.

However, the association between AS and enteric bacteria still remains to be further confirmed by other independent sources before micro-organisms can be said to play a key role in AS pathogenesis.

CHAPTER 1.2 ENTEROBACTERIACEA

1.2.1. Cell Wall Structure

The enterobacteriaceae by definition are gram-negative, non - sporing rods, sometimes motile. They inhabit the gastrointestinal tract of man, either as bowel commensals, or as pathogens, and may or may not be capsulated. The bacterial cell is a rich source of antigenic determinants, and many species exhibit antigenic variation. The most important antigens are those associated with the cell surface, and these form part of the structure of the bacterial membrane, periplasmic space, wall or envelope and are concerned with protection against adverse physiological conditions, such as extremes of pH, dessication, temperature changes, and phagocytosis. In addition, external structures, such as caspular or slime layers, fimbrae and flagella, interact with the environment to allow the bacteria to establish their situation.

The cell wall of gram-negative bacteria is much more complex than that of the gram-positive bacteria, which is a relatively simple structure composed of roughly equal proportions of peptidoglycan, which is responsible for the shape and strength of the cell wall, and an anionic polymer, which is covalently linked to muramic acid residues on the peptidoglycan network.

The gram-negative envelope consist of a thin layer of peptidoglycan, next to the cytoplasmic membrane. Outside this is the outer membrane (OM) consisting of protein, phospholipid and lipopoly-saccharide (LPS)

which is connected to the peptidoglycan layer by covalently linked, low molecular weight lipoproteins (Fig II).

The o-somatic antigen is an endotoxic LPS, which is located in the outer leaflet of the outer membrane. It is the major surface antigen, partially responsible for the strength and shape of the cell wall, and forms the basis for serological classification. The structure has been extensively studied and numerous reviews are available e.g. Rogers et al 1980. Basically it consists of three parts:

1. A hydrophobic lipid A region which remains fairly constant in nature from species to species.

2. A hydrophilic O - specific polysaccharide (O-antigen), which is made up of oligosaccharide repeating units, consisting of a short linear main chain, with or without single hexose branch substitutes, permitting immense antigenic variability.

3. A core oligosaccharide region that connects lipid A and the O-antigen, and shows more variability in structure than that of bacterial lipid A.

1.2.2. Permeability.

Permeability of the OM toward hydrophilic nutrients is due to porins (Nikaido and Nakae 1979), a special group of proteins which span the OM forming pores or channels, permitting only passage of molecules of a certain size. Their exclusion limit is low in enteric bacteria, and

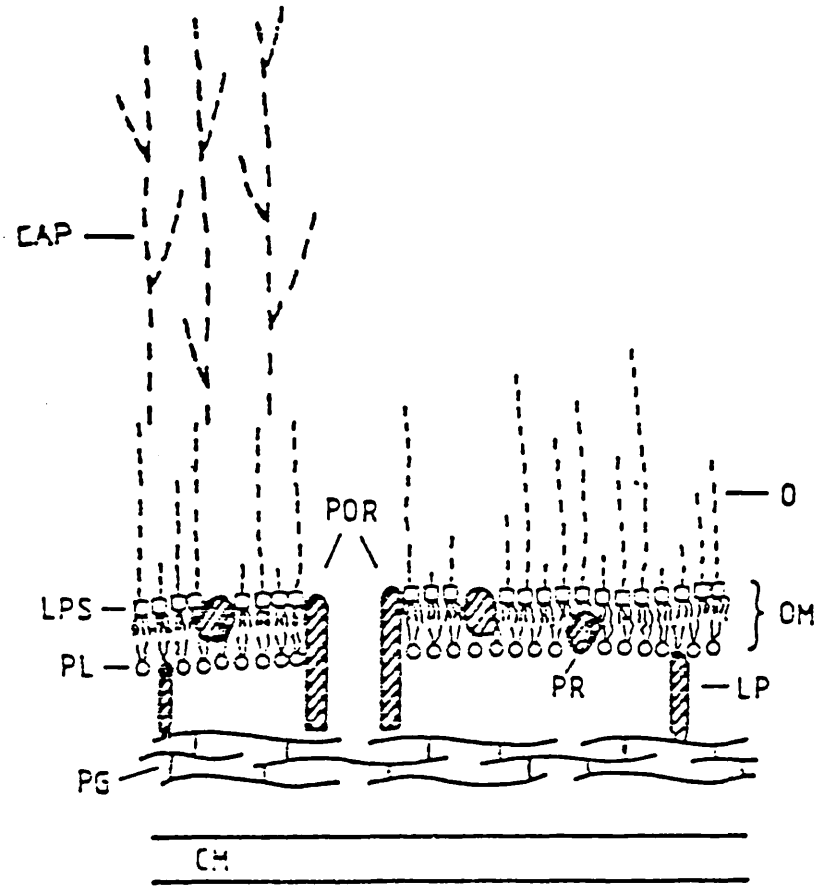


Fig II Cross-section of the envelope of a typical Gram-negative bacterium (taken from Lambert 1983).

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

little is known about factors which control their functional state. There are two pathways by which agents can permeate the OM; a hydrophilic pathway via the aqueous pores, and a hydrophobic pathway involving diffusion across the OM layer. The more hydrophilic the bacterial cell surface, the more resistant is the organism to phagocytosis.

1.2.3. Capsular polysaccharides.

Two types of capsular polysaccharides are found in enteric bacteria. The M antigen or colonic acid is made only under adverse physiological conditions, which indicates that enteric bacteria make it only when situated outside the animal intestinal tract.

The other type is the K-antigen. the classical serotype specific polysaccharide. This provides a highly hydrated gel-like coat around the cell which protects it from phagocytosis and dessication. However it does not prevent the penetration of nutrients. Capsulated bacteria grow as rapidly as non-capsulated strains, and appear to have a central role in adhesion to surfaces (Costerton *et al* 1981).

Polysaccharide capsules are antiphagocytic as a result of their hydrophilic nature. Surface polysaccharides also protect bacterial cells from the bactericidal action of complement and antibody. This is well established for the *vi* antigen of *Salmonella typhimurium* (see Dudman 1977).

1.2.4. Flagella and Fimbrae in Enterobacteriaceae

Flagellar (H) antigens are present in motile species and consist of protein subunit polymers of flagellin. Flagellins from different species are antigenically distinct. Some genera e.g. Proteus, possess a sheath structure, surrounding the flagellar filament, which is antigenic.

Fimbrial antigens enable bacteria to adhere to the surface of cells possessing the appropriate receptors, e.g. enterobacteriaceae with common type 1 Mannose - sensitive fimbiae adhere to many kinds of cells other than erythrocytes. Most animal, plant and fungal cells serve as substrates for such organisms. Certain non- fimbriates of salmonellae are pathogenic, whereas fimbriate strains can be isolated from normal healthy subjects.

1.2.5. Infections

During inflammation, reactions of tissues to an infection may be divided into purulent and granulomatomous forms.

Purulent infections are characterised by short duration, acute course and the accumulation of polymorphonuclear leucocytes (PMNs) at the sites of bacterial invasion. Disease subsides after PMNs and mononuclear phagocytes have phagocytosed the bacteria, and killed them within phagolysosomes. These bacteria are known as extracellular bacteria and include cocci, as well as the gram-negative rods found in many infections.

Faculative intracellular bacteria, the best known being Mycobacterium tuberculosum and Mycobacterium leprae, elicit granulomatomous tissue responses and also include the Yersinia species and Salmonella typhimurium. These bacteria survive after phagocytosis, within the PMNs and initially in the mononuclear phagocytes, and as a result cause chronic, cyclic, systemic disease.

1.3. COMPOSITION OF HUMAN BLOOD.

Normal human blood is a suspension of complex cellular components suspended in an aqueous medium called plasma. When anticoagulated blood is allowed to settle, it separates into three major constituents: plasma, red cells (erythrocytes), white cells (leucocytes) and platelets. The complex nature of blood is reflected in its many diverse functions.

Transport and exchange of gases.
Transport of nutrients, hormones and enzymes.
Removal of waste products.
Maintenance of body temperature.
Provision of defence mechanisms against infection or haemorrhage.

1.3.1. Plasma, Platelets and Red Cells.

Plasma and serum are the aqueous fractions of anticoagulated and clotted blood respectively. They both have essentially the same composition except that fibrinogen and some clotting factors have been removed from the plasma. Platelets are the smallest of the circulating

bodies, are non-nucleated and are usually round or oval in shape. Activated platelets aggregate to help seal a damaged vessel, a reaction which occurs within seconds of an injury. Red cells are the most numerous in blood, and are involved in the transport of oxygen. Red cells are produced from a stem cell in the bone marrow, a process called erythropoiesis. They share this precursor with white cells, whose production is termed lymphopoiesis.

1.3.2. White cells.

White cells consist of five cell types;
Neutrophils, eosinophils, basophils, monocytes and lymphocytes.

Neutrophils, eosinophils and basophils are collectively known as granulocytes, and all contain lobed nuclei.

1.3.2.1. Neutrophils

Neutrophils have the most lobed nuclei and are commonly referred to as polymorphonuclear leucocytes (PMNs). They are the most numerous of the white cells and their major function is the destruction of micro-organisms. They migrate from the blood to sites of inflammation via a process called chemotaxis. Once at an inflammatory site, they engulf the invading organisms in a vacuole formed by invagination of the cell membrane (phagocytosis). Phagocytosed material is then killed and degraded by enzymes produced by this vacuole and the cells cytoplasmic granules. Polymorphonuclear leucocytes spend only a few hours in the blood before migrating into the tissues where they may

survive for 3-4 days. When they are in the blood they can be freely circulating or marginated along blood vessel walls. The total pool of PMN is composed of approximately equal numbers of circulating and marginated cells. Morphology and function will be further discussed in chapter 5.

1.3.2.2. Eosinophils

Eosinophils are much less numerous than PMNs, and are distinguishable by their large reddish brown cycoplasmic granules. Their function is not well understood, but they are involved in IgE immune responses (e.g. allergic disorders), responding to basophil and mast cell degranulation by neutralising the released inflammatory mediators.

1.3.2.3. Basophils

Basophils are the least numerous of the peripheral blood leucocytes, and are identified by large violet staining granules. They are similar to mast cells, and both bind specific IgE to their cell surface. Further exposure to specific antigen results in rapid degranulation, with release of histamine and other mediators involved in hypersensitivity reactions.

1.3.2.4. Lymphocytes

Lymphocytes are the most heterogeneous of the leucocytes; most are small (6-7um) with a high nucleo-cytoplasmic ratio. There are two major types; T cells (Thymus dependent) and B cells (bursa or bone

marrow dependent), which are concerned with cell mediated and humoral immunity respectively. Lymphocytes differ from granulocytes in several respects. For example, many lymphocytes re-circulate (i.e. re-enter the blood from the tissues) and many can live for several years.

1.3.2.5. Monocytes

Monocytes are maturing cells released from the bone marrow and are able to migrate to the tissues where they can survive for long periods, accumulating at the sites of chronic imflammatory reactions. Average diameters of monocytes are around 7-8um, with solid kidney-shaped nuclei. They differ from granulocytes in that they have only small cytoplasmic granules and possess other cytoplasmic organelles (e.g. mitochondria, rough endoplasmic reticulum) which enable them to live in the tissues for long periods as macrophages.

CHAPTER 2. MATERIALS AND METHODS.

2.1. COLLECTION OF SAMPLES.

A velcro fastening rubber tourniquet was applied to the subjects arm, giving gentle pressure to aid the puncture of an antecubital vein.

Using either a 19 or 21 gauge sterile disposable needle (Becton, Dickinson & co., Ltd.) or similar gauge butterfly needle (Abbott Ireland Ltd.) blood was slowly drawn into a sterile plastic syringe (Becton-Dickinson Ltd., U.K.). The whole blood samples were collected into universals (Lab Sales (U.K.) Ltd.) containing preservative free sodium heparin (1,000 units/ml, Leo Labs, U.K.), 50ul being used per 5 ml of blood. Tubes were stoppered and gently mixed. All blood samples were taken between 09.00 and 11.00 hours to minimise the effect of diurnal variation, and samples were processed as soon as possible after collection.

2.2. SEPARATION MEDIA

2.2.1.Dextran 150

A 5% dextran 150 (Fisons UK) solution was made in 0.9% saline (Sodium chloride B.D.H. Ltd. England.) When dissolved this solution was filtered, using a 0.22 micron disposable, sterile filter assembly (Gelman Sciences, USA), into a sterile bottle, and stored at four degrees centigrade.

2.2.2. Lymphoprep.

Lymphoprep (LP) is a ready made sterile solution (Nycomed, Norway) with a density of 1.077 g/ml and an osmolarity of 300 mOsm/Kg.

2.2.3. Ammonium Chloride

Ammonium chloride (NH_4Cl, Sigma) was prepared as an 0.87% solution in distilled water, and stored at 4°C.

2.2.4. Gel Hanks

10ml of Hanks Balanced Salt solution (10x) (HBSS, Gibco Ltd., Scotland) without salts, was added to 80 mls of sterile distilled water. 2 ml of Hepes buffer (1M solution, Gibco) and 1.1ml of Sodium bicarbonate solution (Northumbrian Biologicals Ltd., UK) were added to stabilize the solution.

This was adjusted to PH 7.2 with a Normal sodium hydroxide (BDH Ltd., England) and 1 Normal Hydrochloric acid (BDH, sp:gr. 1.18).

10mls of 1% gelatin (BDH Chemicals, UK) was added to prevent aggregation of cells. This solution (GH) will remain stable for several days.

2.3. SEPARATION OF POLYMORPHONUCLEAR LEUCOCYTES (Pmns)

Polymorphonuclear leucocytes were prepared from heparinised venous blood samples,using a modification of the method of Boyum (1968).

10 ml of blood was mixed with 3ml of the 5% dextran (2.2.1.) in 15 ml conical based centrifuge tubes (Elkay products Ltd., USA), and allowed to gravity - sediment for 30-45 minutes, at ambient room temperature.

The red blood cells settled at the bottom of the tube, while the leucocyte-rich plasma remained at the top. (Fig 111).

This plasma was then layered on to 3ml of lymphoprep (2.2.2.), and centrifuged (Mistral 4L, MSE UK) at 289g for 25 minutes.

After centrifugation the mononuclear cells settled at the interphase, and the PMNs pelleted at the bottom of the tube. (Fig IV)

The supernatent was discarded and NH_4Cl (2.2.3) was added for 20 minutes, to lyse any remaining red blood cells that had not sedimented out in the dextran. Cells then washed with GH, and adjusted to required concentration of cells, as follows.

2.4. COUNTING METHOD

Using a light microscopy method (Dacie and Lewis, 1966), 10 ml of cell suspension is diluted in 90 ml of white cell fluid (2% V/V solution of glacial acetic acid in distilled water, to which a few grains of crystal violet have been added).

The number of cells (Y) in the 4 x outermost corners of 16 x squares, on the grid of a Neubauer chamber was estimated, and the mean calculated. (40 x magnification) Watson microsystem 70). This is

Fig. III Dextran sedimentation of whole blood

Fig. IV Lymphoprep separation of leucocyte-rich plasma.

multiplied by the dilution factor (1:10) and then by 10^4 to give the number of cells per ml present i.e. y x 10 x 10^4 cells per ml. Where whole blood was being used in an assay, a differential white cell count was done, again using a Neubauer chamber, as follows.

A total cell count was done followed by a mononuclear cell count.

Concentration and percentage of polymorphonuclear leucocytes present could thus be calculated.

2.5. BACTERIA

2.5.1. Growth Media

Mueller Hinton broth (MH, oxoid, UK) was prepared by adding 21grams per litre to distilled water, and autoclaving at 121° centigrade for fifteen minutes. Blood agar plates, and cysteine lactose electrolyte deficient medium (CLED) plates were used to maintain the bacteria, single colonies of which were inoculated onto fresh plates every two weeks.

2.5.2. Diluting and Washing Duffer

Phosphate buffered saline tablets (PBS), (Oxoid,UK), were dissolved in distilled water (1 tablet per 100 ml), and autoclaved at 121°C for fifteen minutes and stored at 40°C.

2.5.3. Standardisation of Bacteria

Four gram-negative and one gram-positive bacteria were used in this work, namely,

1. Klebsiella pneumoniae, strain K43
2. Proteus mirabilis
3. Yersinia enterocolitica
4. Salmonella typhimurium
5. Staphyloccocus aureus, Cowan strain

Suspensions of known concentration had to be determined for each bacterium.

This was achieved by preparing a series of culture dilutions, made with a constant dilution factor (Miles and Misra 1938).

A bacterial suspension of known optical density (OD) was prepared in PBS, and 10-fold dilutions from this were made. Spectrophotometer was set at 620nm, slit width 0.1nm.

10ul from each dilution were then placed on an agar plate, and incubated overnight at 37°C. Colony forming units (C.F.U.)were counted the next day and the number of bacteria per ml in the original sample calculated.

i.e. C.F.U. x diluting factor x 100 = required O.D.

This was repeated several times until OD for each bacterium was standardised for concentration of 1×10^7 CFU/ml and 1×10^8 CFU/ml (Fig. V).

	1×10^7cfu/ml	1×10^6cfu/ml	Optimum % opsonin
K. pneumoniae	0.015	0.1	50
P. mirabilis	0.025	0.045	50
Y. enterocolitica	0.01	0.05	10
S. typhimurium	0.01	0.05	10
S. aureus		0.1	10

Fig. V. Optical densities and percentage . . . . . for dilution and opsonisation of each bacteria.

2.5.4. Normal Pool Serum for opsonisation of bacteria.

A normal pool serum (NPS) was established by bleeding 7-10 volunteer healthy individuals. Serum was separated by centrifugation at 2020'g' for 10 minutes, then aliquoted and stored at -70°C.

Several dilutions of NPS were then tested for opsonisation of each bacterium until the optimum percentage of NPS was standardised for both bacterial uptake and chemiluminescent response. (Fig.V).

In our uptake assay, S. typhimurium failed to opsonise with NPS. This was overcome by using a rabbit antiserum raised to a known enterobacterial common antigen (ECA) positive strain of Escherichia coli, which successfully opsonised the bacteria at a concentration of 10%. Enterobacterial common antigen is an amphiphilic glycophospholipid located in the outer membrane of almost all wild type strains of the Enterobacteriaceae, and its expression on the cell surface is readily available to homologous antibody in non-encapsulated rough strains.

2.6. CHEMOTAXIS AND CHEMOKINESIS

The membrane filter technique was first designed by Boyden in 1962 and then Falk in 1980 developed a micro-chem taxis assembly.

These assays were performed on 48-well micro-chemotaxis chambers, supplied by Neuro Probe inc., U.S.A. (See Fig V1)
Nucleopore polycarbonate filters for use in these chambers are

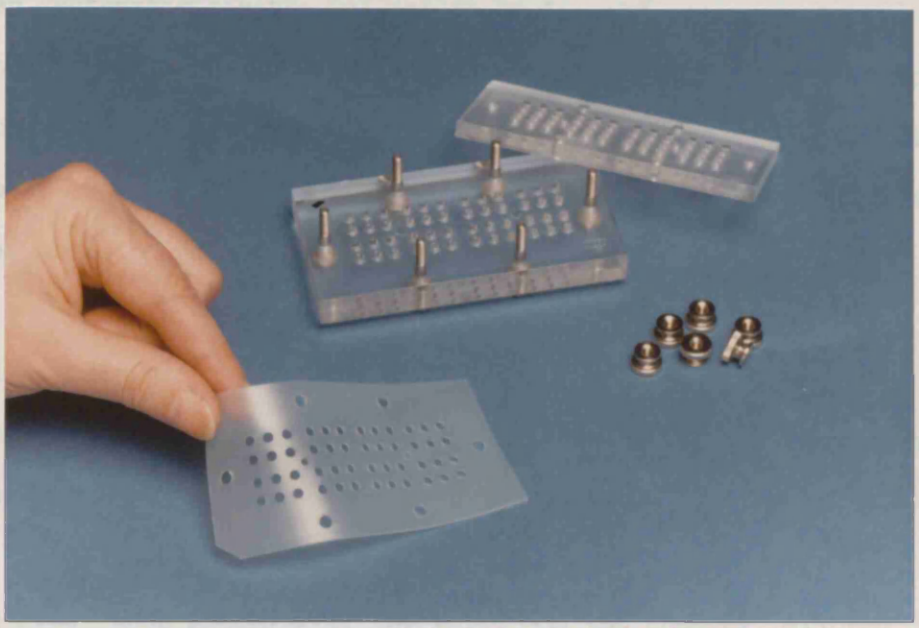


Fig VI Micro-chemotaxis chamber.

available in a range of pore sizes, according to which cells were being examined.

For our purposes 3u pore, 10u thick filters were used for PMN migration.

Various chemotaxins were placed in the lower wells of the chamber, using an automatic pipette pre-set at 26ul, and care was taken to avoid the formation of any air bubbles on the surface. The meniscus should be slightly raised. The membrane was then applied, shiny side down, allowing the centre portion to make contact first. Minor adjustments to the filter position could be made at this point.

The silicon gasket and the top plate were secured in place by maintaining an even pressure until all the screws had been tightened to prevent air being introduced into the system.

A slide was then placed over the wells and the chamber allowed to equilibrate at 37°C in a 5% mixture of CO_2 and air for 10 minutes (water-jacketed incubator, Scotlab Ltd., UK). Cells were prepared to the desired concentration, as described in 2.3, and 50µl were added to the top of the chamber, again carefully avoiding the introduction of any air bubbles by holding the pipette at an angle against the upper part of the well and ejecting the fluid rapidly.

Further incubation of the uncovered chamber followed for 30 minutes. Screws were removed and chamber was inverted on to a paper towel, and the membrane removed; the migrated cells now faced upwards. The lower side was then washed 3xtimes in PBS and excess Pmns were wiped

off, using a filter wiper (Neuro Probe inc., U.S.A.). The membrane was left to dry in air, then fixed in methanol, A.R. quality (James Burrough F.A.I. Ltd., UK) for 2 minutes, and stained with 100% Leishmans stain (Exogen, UK) for 5 minutes. Then 50% Leishmans for a further 5 minutes.

After drying, the membrane filter was mounted in D.P.X. (B.D.H. Ltd., UK) and the number of cells present in each well was estimated in 5 x fields under a 40 x objective. Each test was done in triplicate, and the mean result calculated. Chambers should be placed in sterile distilled water immediately after use, then washed in distilled water and left to air dry.

2.7. PHAGOCYTIC UPTAKE ASSAY

Phagocytosis was measured by a modification of the method of Verhoef et al (1977)

2.7.1. Preparation of bacteria

One colony of bacteria was inoculated into 10ml of Mueller Hinton (MH) broth, containing 20ul 3H adenine (Radiochemical Centre, Amersham). This was incubated overnight at 37° centigrade, where the bacteria incorporate the radioactively-labelled adenine into their own genome. Bacteria were then washed x 3 in Phosphate buffered saline (PBS), and centrifuged at 1200'g' (MSE, Super minor, UK) for 15 minutes.

The bacterial suspension was then diluted until the appropriate optical density (OD) was obtained on a spectrophotometer (Unicam, SP 1700), set at 620nm and slit width 0.1nm (see 2.5.3.).

2.7.2. Opsonisation of bacteria

The bacteria were now ready for opsonisation with a normal pool serum (NPS). Equal volumes of diluted NPS (see 2.5.4.) were added to the bacterial suspensions, and placed on a shaking incubator at 37° centigrade for 15 minutes. These were centrifuged at 1200'g' for 15 minutes. The supernatent was removed and the pellet re-suspended at the original volume of bacteria, in gel-hanks.

2.7.3. Assay

0.1 ml opsonised radio -labelled bacteria and 0.1 ml of pmn suspension (prepared as described in 2.3, at 1 x 10^7/ml) were added in duplicate to two sets of polypropylene scintillation vials (Pony vial, Canberra Packard, UK) and placed on a shaking incubator for 15 minutes at 37°C Phagocytosis was terminated in one set of vials by the addition of 3mls PBS at 4°C and then centrifuged at 1000rpm for 5 minutes. This was repeated three times, and after the last wash 3ml scintillation fluid was added (299 from Canberra Packard, UK). The purpose of these washes was to remove any free bacteria, i.e. non-phagocytosed bacteria, which would not be spin down at 1000rpm, thus only those bacteria which were leucocyte- associated would remain. The vials were counted on a scintillation 3 counter [1216 Rackbeta II (LKB,UK)] for 1 minute each. These counts were referred to as low counts.

The duplicate set of vials were assessed for total bacterial radioactivity, by the addition of 3ml scintillation fluid, immediately after the uptake period (i.e. no washes). These were also counted for one minute per vial and were referred to as high counts.

A further set of duplicated vials, containing 0.1 ml bacteria and 0.1ml Gel Hanks were similarly treated for high and low counts, to give a reading representing non-specific bacterial adherence to the tubes and aggregation. These were referred to as blank values. (FIG VIa).

2.7.4. Calculations for determination of phagocytosis

The % uptake of bacteria was calculated as follows.

Formula 1

$$\% \text{ uptake test} = \frac{\text{counts per min low counts}}{\text{counts per min high counts}} \times 100$$

The above formula was also applied to the blank tubes and the final value for the blanks was subtracted from the test values.

Formula 2

$$\% \text{ uptake} = \% \text{ c.p.m. test} - \% \text{ c.p.m. blank}$$

These formulae gave the final readings for percentage uptake of bacteria by PMNs, and all results were expressed in this manner.

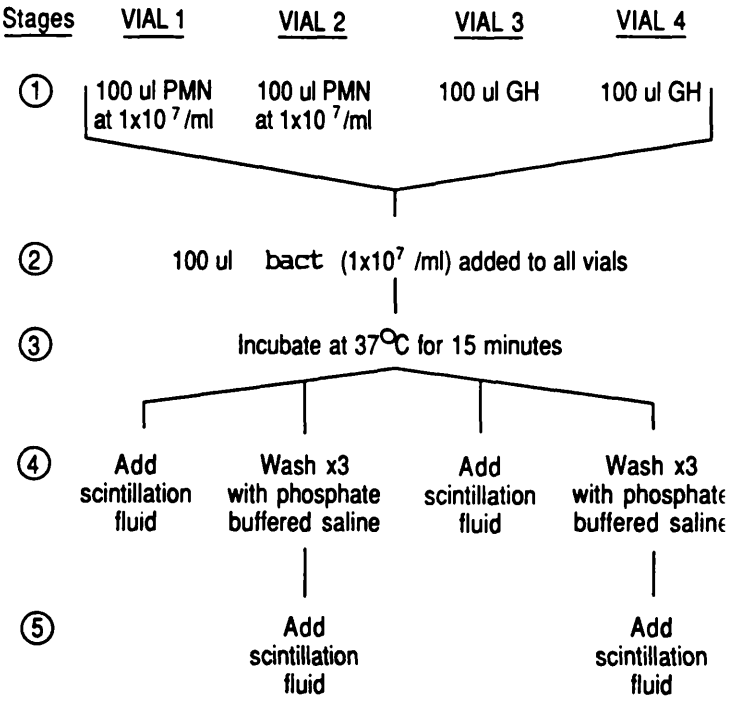


Fig VIa Outline of phagocytic uptake assay.

2.8. CHEMILUMINESCENCE

Chemiluminescence (CL) was measured using a luminometer, Picolite (Packard U.K.), which consists of 2 units, a detector and an analyser. This model is an automatically programmable instrument used to measure single photons of light, emitted by certain biological and chemical reactions. It requires a clean dry air supply that delivers a minimum of 60-80 psi to drive the carousel.

Two types of assays were performed, using luminol (5-amino- 2, 3-dyhydro-1, 4-phthalazinedione) at 10^{-5} as an amplifier, which is converted to an excited aminophthalate ion in the presence of oxidising species like superoxide anion (O_2) hydrogen peroxide (H_2O_2), hydroxyl radical (-OH), and singlet oxygen ($^1O_2$): thus enabling trace amounts of activated oxygen species to be measured (Allen et al 1976).

2.8.1. Purified PMNs

In our first series of assays we looked at purified PMNs, and their reaction to gram-negative bacteria.

Bacteria were prepared as described in 2.5.3.at a concentration of 1 x 10^8 colony forming units (cfu) per ml, and opsonised with appropriate dilution of NPS (2.5.4.). Polymorphonuclear leucocytes were also prepared (2.3) at 1 x 10^7 per ml, i.e. a minimum ratio of 1:10, PMNs to bacteria. The exact ratio was not crucial, as long as it was maintained throughout the assays.

It was important that patients and normal samples were run simultaneously, as results do vary from day to day.

100 ul PMNs were added, in sequence, to the luminometer chambers. These were left for a short time to equilibrate at 37°C - i.e. the temperature within the carousel. Meanwhile the programme was set on the computer, according to length of time between readings, number of readings and automatic injection of the bacteria, if required. Bacteria in these assays were added manually as four types were used in each run. Computer was also primed with sample information and contents of each chamber.

100ul of luminol (Sigma) at 10^{-5} molar was added, following by 100ul of opsonised bacteria.

The CL responses were measured in peak counts per second at $1x10^5$. CL curves and absolute peak responses were produced automatically by the computer.

Peak counts per second were taken for each sample and these results were analysed using a Mann Whitney U Test.

2.8.2. Whole Blood Assay

In this series we used heparanised whole blood samples, rather than isolated PMNs.

A differential white cell count was done on each sample, using a Neubauer chamber, determining number of PMNs and percentage PMNs, present in each sample (2.4).

Opsonised Bacteria was prepared, as before at $1x10^6$/ml.

The Picolite analyser was primed with previously calculated PMNs data and the computer set for a whole blood assay. This time 20ul of whole blood was added to the Picolite chambers followed by 50ul of gel hanks, 100ul bacteria and 100ul of 10^{-5} luminol.

Results again were taken as peak count per second at $1x10^3$/ml, for each sample, and analysed according to Mann-Whitney. See Fig VII for methodology and Fig VIII for example of computer print out of results.

2.9 BLOOD SAMPLES

2.9.1. Ankylosing spondylitis (AS) Patients

Throughout these assays samples were taken from AS patients, who were either attending the Rheumatology clinic, or who were undergoing treatment in the wards. All patients were informed that samples were required for medical research.

Classical AS patients, as described by the New York criteria were used, and a disease scoring activity was devised by rheumatologists from the Centre for Rheumatic Diseases. Other disease parameters were taken into account as well as biochemical and haematological

CHEMILUMINESCENCE

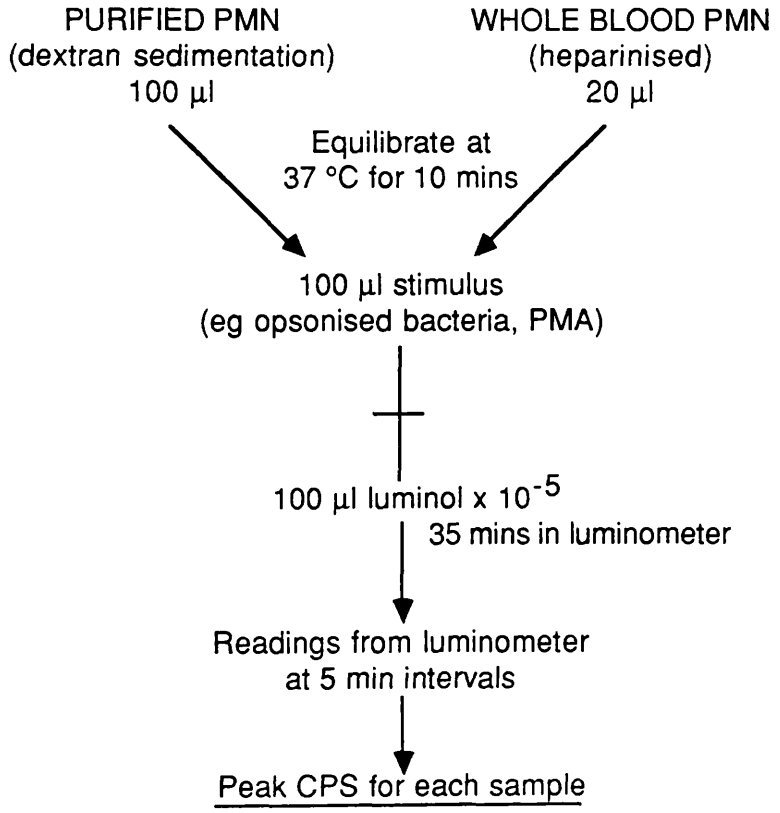
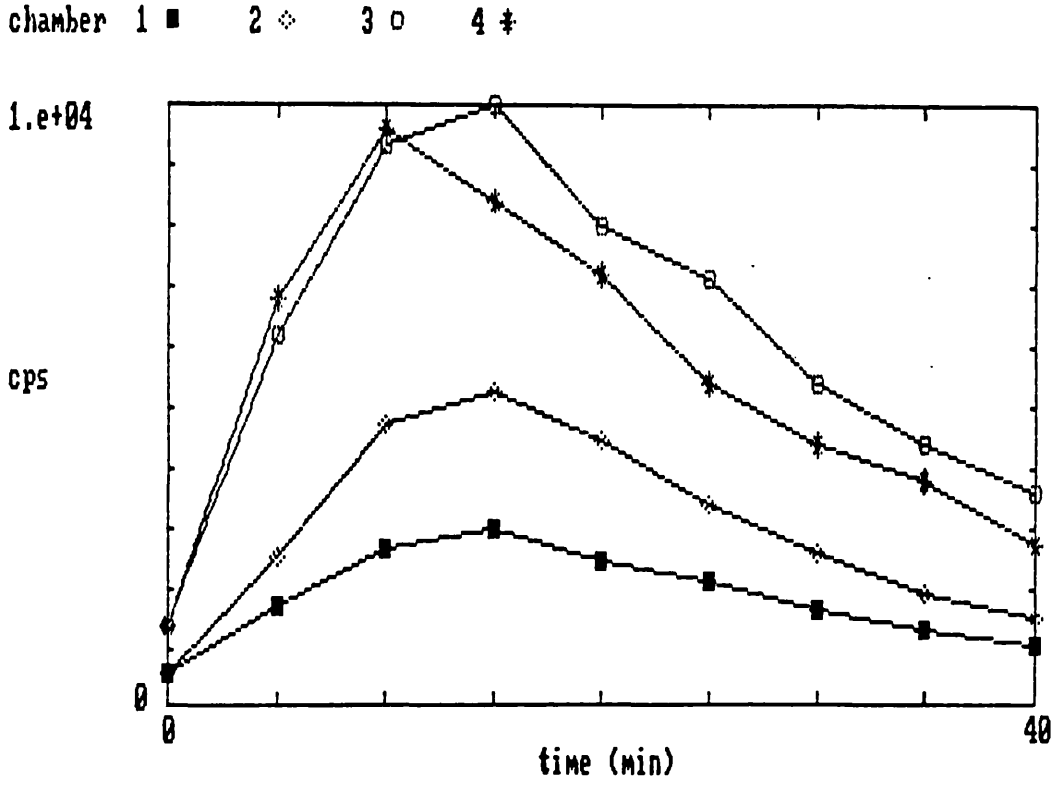


Fig VII Chemiluminescent methodology.

WHOLE BLOOD/K43 21/10/88



Program £4, 9 cycles
5 seconds count time, with 5 minutes count delay

Identification	WBC/ml	%PHC	chamber	peak (min)	peak cps	peak cpsc
N1+K43	6.10e+06	66.0	1	15	2.92e+03	0.04
N2+K43	1.00e+07	66.0	2	15	5.20e+03	0.04
AS1+K43	1.00e+07	70.0	3	15	9.99e+03	0.07
AS2+K43	1.00e+07	74.0	4	10	9.56e+03	0.06

Identification	chamber	area	half-max (min)
N1+K43	1	4.55e+06	30 - 35
N2+K43	2	7.63e+06	25 - 30
AS1+K43	3	1.58e+07	30 - 35
AS2+K43	4	1.42e+07	25 - 30

Fig VIII Example of computer print-out of chemiluminescent results from luminometer.

data, i.e. peripheral joint involvement, drug therapy and the general well-being of the patient. Consequently a more global determination of disease was achieved. Scoring for each section was between zero and two, making a total maximum score of 10. (See Fig. 1X).

2.9.2. Normal Controls

Samples from AS patients were compared at all times, to normal controls, which were obtained from laboratory and medical "volunteer" staff.

AS DISEASE ACTIVITY SCORE

NSAIDS	None/occasional	0
	Regular	1
Peripheral joint involvement	None	0
	Previous	1
	Current	2
Morning back pain/ stiffness	None	0
	<1 hour	1
	>1 hour	2
ESR	<30	0
	30-60	1
	>60	2
CRP	<10	0
	10-50	1
	>50	2
IGS	Normal	0
	Elevated	1

Maximum score = 10

Fig IX Disease activity score.

CHAPTER 3 CHEMOTACTIC RESPONSE OF NORMAL AND ANKOLYSING SPONDYLITIS POLYMORPHONUCLEAR LEUCOCYTES TO VARIOUS CHEMOATTRACTANTS

3.1. CHEMOTAXIS

Chemotaxis (CX) is the directional locomotion of organisms in relation to sources of chemical attractants, (Zigmond, 1974). The capacity of leucocytes to move in response to chemotactic stimuli is an essential part of host defence against infection, (Ward 1974), whereby cells are attracted to sites of tissue injury. Polymorphonuclear leucocytes (Pmns), which show good chemotactic responsiveness, are the most numerous of the leucocytes in blood and are easily obtained in high yield and purity. These cells appear also to be homogenous, behaving alike on stimulation. On addition of a chemotactic factor the cells become polarised into an anterior lamellipodium and posterier tail. This is accompanied by contractile waves which move down the cell from front to back. Receptor redistribution also occurs where the cell contents move to the front end of the cell. (Wilkinson, Michl and Silverstein, 1980). A high degree of cell deformability contributes to the ability of the PMNs to leave the capillary lumen by squeezing between the capillary endothelial cells and moving into the tissue (diapedesis). Polarisation and redistribution are the earliest visible responses of a leucocyte to environmental signals.

Introduction of micro-organisms into tissue results in the production of factors to which the PMNs respond due to high affinity receptors on their surface. These factors are either products of microbial metabolism e.g. formyl peptides, complement proteins, or products of

the phagocytes themselves. (Snyderman and Gaetze, 1981). The complement system is the most important known source of chemotactic factors in vivo, from which is derived C5a fragment and factor Ba (a fragment of factor B resulting from action by factor D). Other plasma- derived mediators with chemotactic activity include the Hageman factor-dependent substances, kallekrein and plasminogen activator, and by-products of arachidonic acid metabolism. Cell-derived chemotactic factors include lymphocyte, monocyte and PMN products, (Ward, 1974). Also in certain tissue, bacterial and viral products contain C3- and C5- cleaving enzymes which can indirectly result in the release of complement - derived chemotactic factors. Chemotactic factors may contribute to deformability of PMNs. (Kawaoka, Miller and Cheung, 1981).

Locomotion is either random or directed. Stimulated random movement (chemokinesis) occurs when a chemoattractant interacts with a leucocyte in the absence of a concentration gradient, whereas chemotaxis is a unidirectional movement in response to the concentration gradient of a chemo-attractant. Chemotactic direction finding in PMNs is very efficient and cells in good gradients move in near straight-line paths towards the gradient source, (Allan and Wilkinson, 1978). Thus chemotaxis is an excellent mechanism for directing cells towards loci of infection or tissue injury.

Studies of locomotion and chemotaxis in monocytes and lymphocytes have advanced at a much slower rate, although both are important in chronic inflammation and in immune reactions. Heterogeneity is a major problem with both cell types, and of course high cell yield and purity

are less easily obtainable than with PMNs.

The first observation of chemotaxis was reported by Leber in 1888, and has been studied *in vitro* on slides or coverslips (Harris, 1953), in capillary tubes (Ketchel and Favour, 1955), in culture wells under agarose (Cutler, 1974) and by a membrane filter technique, first described by Boyden in 1962. Falk in 1980, developed a 48-well micro chemotaxis assembly which had many advantages over other methods, such as time involved in setting up the assay, volume of chemotactic factor needed, and low cell number requirements,(see chapter 2.6)for details of method used in this study.

Despite an initial report that there was no significant difference in polymorph motility between AS and controls (Mowat, 1978), there have been several reports documenting significant increases in PMN mobility in AS. Pease *et al*, in 1984 showed that directed movement of PMNs was significantly increased in AS patients whether they were HLA-B27 positive or negative. Reports from Finland also indicated that not only was the *in vitro* chemotaxis of HLA-B27 positive PMN significantly increased over HLA-B27 negative PMN normals, but that sera from HLA-B27 positive subjects were capable of significantly increasing random mobility, or chemokinesis, of PMN in comparison to HLA-B27 negative sera. (Leirisalo, *et al*, 1980, Repo *et al*, 1982). It has also been shown that increased PMN motility occurs *in vivo* in HLA-B27 positive individuals (Koivaranta *et al* , 1984) using a skin chamber technique. Consequently it has been proposed that such hyperreactive PMNs may trigger a vicious circle of inflammation and thereby contribute to tissue injury in HLA-B27 associated diseases.

3.2. AIMS OF STUDY

To compare the motility of AS PMNs to normal PMNs using various chemotaxins. Results were analysed by a Mann Whitney, two-tailed, non-parametric test, and expressed as medians and inter-quartile ranges.

3.3. PREPARATION OF CHEMOTAXINS

Several preparations were tested for their use as chemotaxins.

3.3.1. Zymosan-Activated Sera

125mg zymosan + 5ml of a normal pool serum were incubated for 60 minutes at 37°C, then centrifuged at 750g for 10 minutes. The supernatant was then inactivated by incubation for 30 minutes at 56°C, and used at a dilution of 1/16 in gel hanks.

3.3.2. Formalin Killed Bacteria

K. pneumoniae and *Y enterocolitica* were cultured in MH broth overnight at 37°C, then washed x 3 in PBS. 1% formalin was added and left at room temperature for 24 hours, when a sample was plated out to test for loss of viability. Optical density was then adjusted to achieve 1 x 10^8/ml dilutions for each bacteria, see chapter 2.5.3.

3.3.3. Sonicated bacteria

1 x 10^8/ml dilutions of K. pneumoniae and Y. enterocolitica were prepared in 10mls of gel hanks. These were centrifuged at 1200'g' for 15 minutes and re-suspended in 1ml gel hanks. This preparation was then sonicated at 12 microns for 10 minutes, and re-suspended in 10mls of gel hanks.

3.3.4. Bacterial culture filtrates

K. pneumoniae, Y. enterocolitica and S. typhimurium were cultured in MH broth overnight at 37°C, then centrifuged at 1200'g' for 15 minutes. The supernatents (culture filtrates) were aliquoted, and stored at -20°C.

3.4. CHEMOKINETIC RESPONSE OF 10 NORMAL AND 11 AS PATIENT PMNS

Chemokinesis, or random chemotaxis, was examined in 10 normals and 11 AS patients to compare random mobility.

3.4.1. Materials and Methods

Samples were collected in heparin, PMNs separated, and prepared at 3.5 x 10^5/ml in gel hanks. These were placed in the bottom wells of the microchemotaxis chamber, and gel hanks was added at the top, so that no gradient was formed. Migration of PMNs were measured on 5 fields per well. The mean result of 3 wells was calculated for each

sample, and these were analysed using a Mann-Whitney test.

Details of samples were as follows:

		Normals	AS
No. of Samples		10	11
Mean Age (yrs) range		32 (25 - 43)	48 (29-77)
Sex	M	7	9
	F	3	2

3.4.2. Results

Results are expressed as medians and interquartile ranges. See Fig X. Patients showed an increased chemokinetic motility when compared to normals, but this increase was not statistically significant.

3.5. FORMALIN KILLED AND SONICATED BACTERIA

No chematactic gradient was achieved when either sonicated or formalin killed bacteria were used as chemotaxins. Therefore no motility was produced and no results obtained.

3.6. BACTERIAL CULTURE FILTRATES

These were prepared with *K. pneumoniae*, *Y. enterocolitica* and *S. typhimurium*, (as described in 3.3.4.).

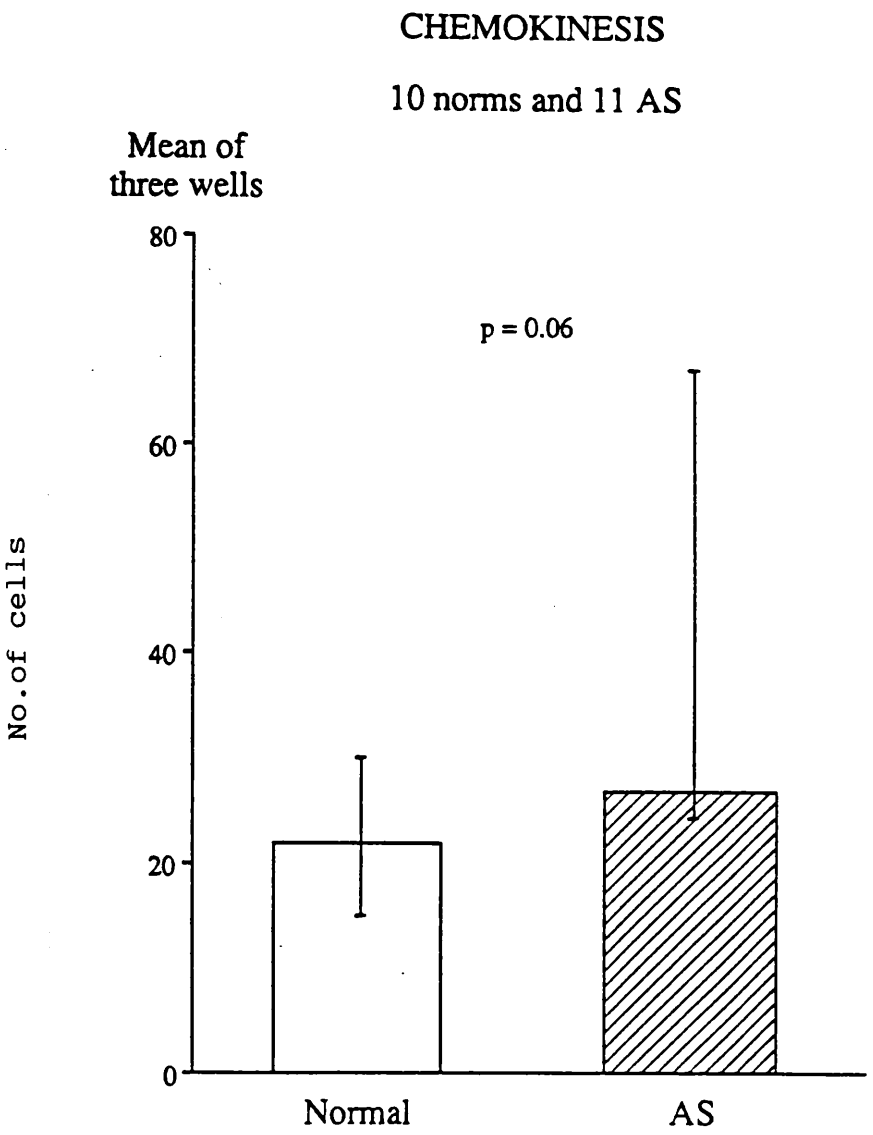


Fig X Chemokinetic response of polymorphonuclear cells from 10 normals and 11 ankylosing spondylitis patients.

Expressed as medians and interquartile ranges.

3.6.1. Materials and Methods

Samples of heparinised-blood were collected from 11 normals and 15 A.S. patients. Cells were separated and prepared at 5 x 10^5/ml, and motility of normal PMNs was compared to AS PMNs. Details of samples were as follows:

		Normal	AS
No. of Samples		11	15
Mean age (yrs) (range)		30 (22-43)	40 (32-59)
Sex	M	8	11
	F	3	4

3.6.2. Results

Results are again expressed as medians and interquartile ranges. No significant difference was seen in motility between normal PMNs and AS patient PMNs when any of the bacterial culture filtrates were used as chemotaxins. The chemotactic values of Y enterocolitica and S. typhimurium were similar to that of MH broth, indicating that these two bacteria on their own had no chemotactic qualities. K. pneumoniae culture filtrate, however, showed a higher chemotactic response than MH broth, suggesting that some product of this bacterium behaved as a chemotaxin to both normals and AS patients. (Fig. XI).

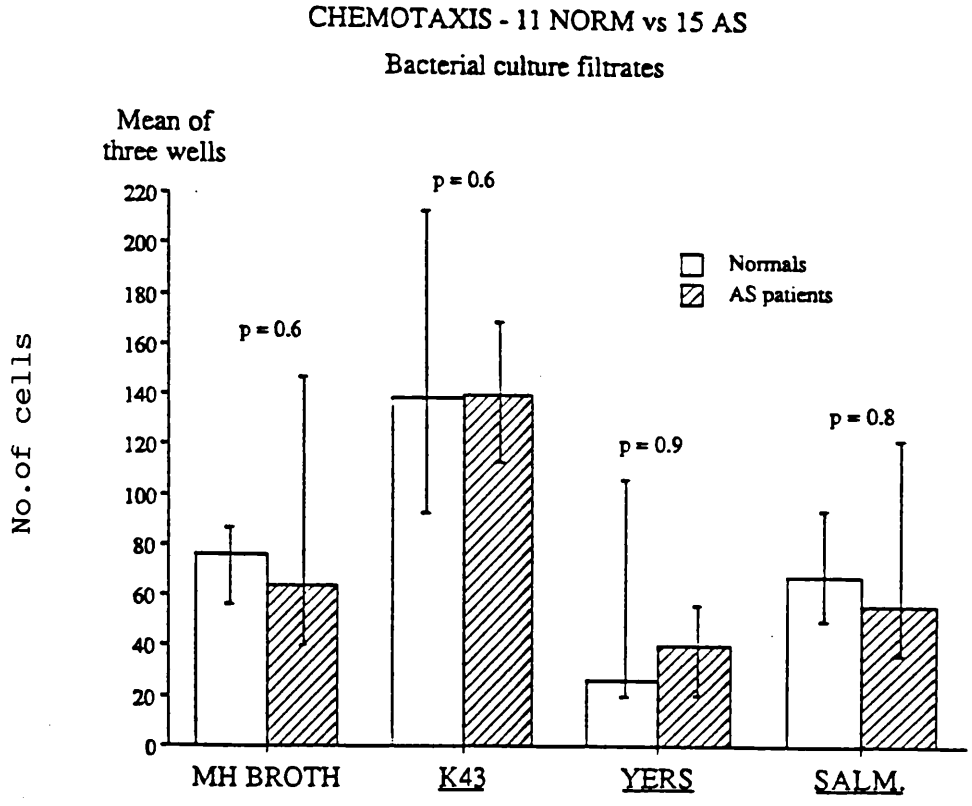


Fig XI Chemotactic response of polymorphonuclear from 11 normals and 15 ankylosing spondylitis patients, to K. pneumoniae (K43), Y. enterocolitica (yers) and S. typhimurium (salm), culture filtrates.

Expressed as medians and interquartile ranges.

3.7. CHEMOTACTIC RESPONSE OF PMNS FROM HLA B27 POSITIVE AND HLA-B27 NEGATIVE INDIVIDUALS WITHOUT DISEASE.

Since more than 90% of AS patients are HLA-B27 positive, it is important also to examine HLA-B27 positive normals. Bacterial culture filtrates were again used as chemotaxins and samples from HLA-B27 positive and HLA-B27 negative normal individuals were collected and PMN motility examined.

3.7.1. Materials and Methods

Culture filtrates from K. pneumoniae, Y. enterocolitica, and S. typhimurium were prepared as desribed in 3.3.4. Samples from nine HLA-B27 positive and nine HLA-B27 negative normal individuals were collected in heparin. Polymorphs were separated by dextran sedimentation and prepared at a dilution of 5×10^5/ml in gel hanks. Both positive and negative HLA-B27 normals were assayed simultaneously using micro-chemotaxis chambers.

3.7.2. Results

No difference in motility was seen between HLA-B27 positive and negative normals when bacterial culture filtrates were used as chemotaxins. Again, as seen with patient and normal groups, only K. pneumoniae appeared to show any chemotactic abilities. Results are expressed as medians and interquartile ranges. (See Fig. X11).

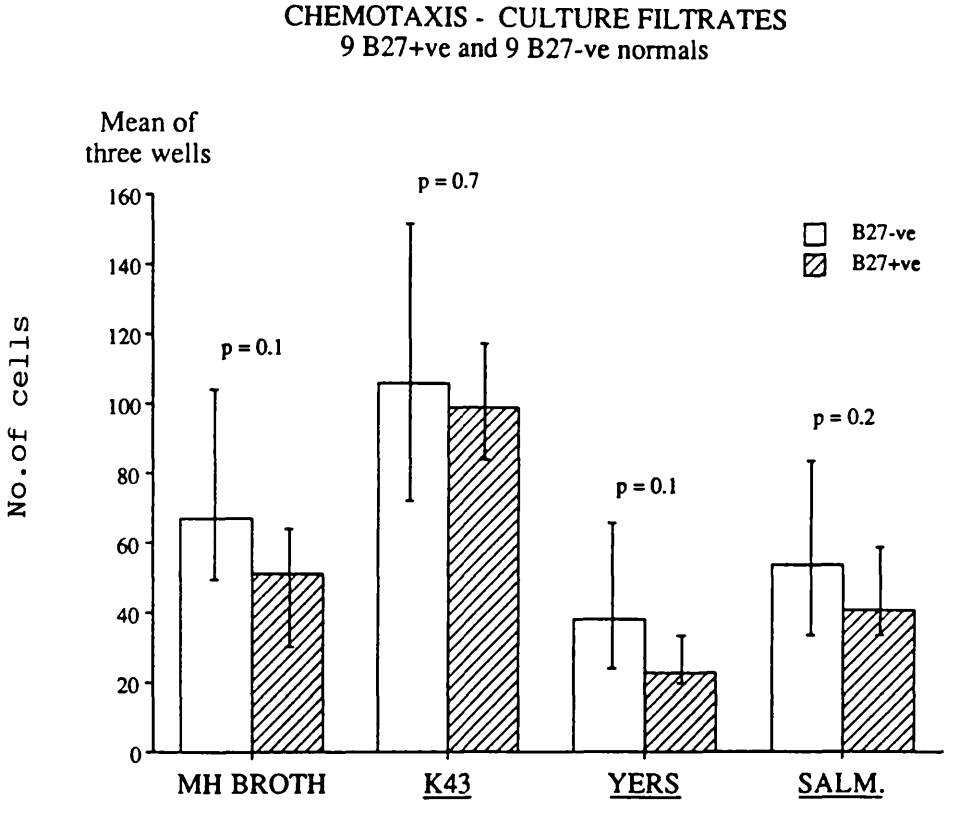


Fig XII Chemotactic response of polymorphonuclear cells from 9 B27 positive and 9 B27 negative normals to K. pneumoniae (K43), Y. enterocolitica (yers) and S. typhimurium (SALM), culture filtrates.

Expressed as medians and interquartile ranges.

3.8. ZYMOSAN - ACTIVATED SERA

The motility of AS and normal were compared using zymosan-activated serum as a chemotaxin.

3.8.1. Materials and Methods

Samples of heparinised blood from both normals and patients were collected and assayed simultaneously. Polymorphs were separated using dextran sedimentation (2.3) and prepared at a dilution of 3.5 x 10^5/ml for chemotaxis, (2.6). Zymosan was prepared at 1/16 (3.3.1.). Details of samples are as follows:

		Normal	AS
No. of Samples		22	27
Mean age (yrs) range		30 (23-43)	44 (27-73)
Sex	M	9	20
	F	13	7

3.8.2. Results.

Results showed no difference in motility between patient and normal PMNs (see Fig X111), and are expressed as medians and interquartile ranges.

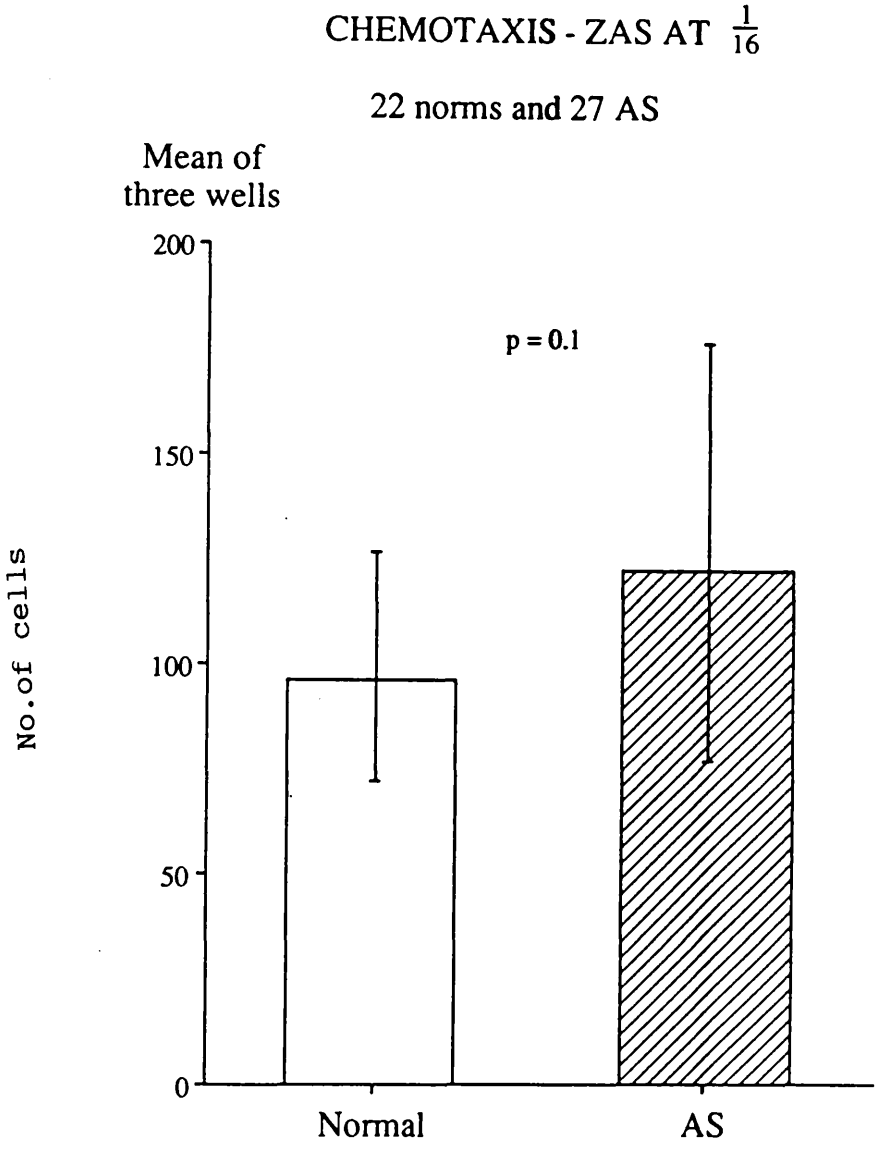


Fig XIII Chemotactic response of polymorphonuclear cells from 22 normal and 27 ankylosing spondylitis to zymosan-activated serum (ZAS).

Expressed as medians and interquartile ranges.

3.9. AGE CORRELATION

Correlation of motility of normal PMNs to age was examined using a Spearman Rank test.

3.9.1. Materials and Methods.

The ages of 20 normal controls were correlated with motility of the PMNs, when zymosan-activated serum was used as a chemotaxin, (3.8.1.).

3.9.2. Results.

A poor correlation was evident, the Spearman rank coefficient being -0.44. We can be confident that this is a good estimate of correlation as the significance level was 0.05. (See Fig. XIV).

3.10 DISEASE ACTIVITY

Correlation of motility of AS PMNs to disease activity using a Spearman rank test.

3.10.1. Materials and Methods

Disease activity from 12 AS patients was scored according to the method described in chapter 2. This was compared to the motility of these AS PMNs when zymosan-activated sera was used as a chemoattractant.

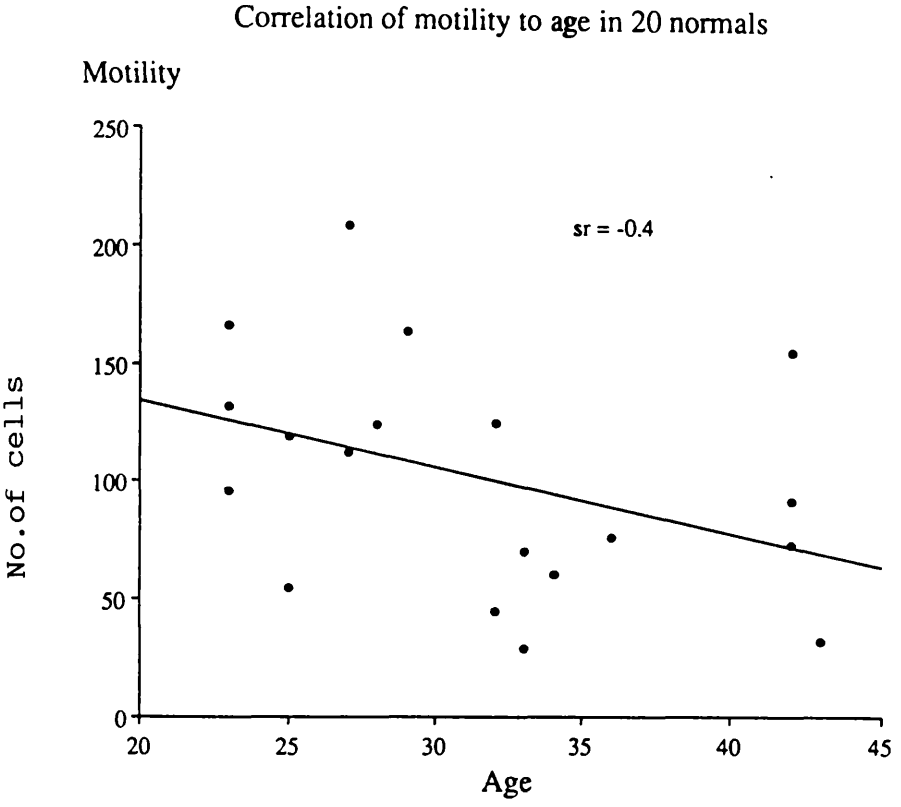


Fig XIV Correlation of polymorphonuclear cells motility to age in 20 normals.

Spearman rank coefficient (sr) = -0.4.

Significance level, P = .05.

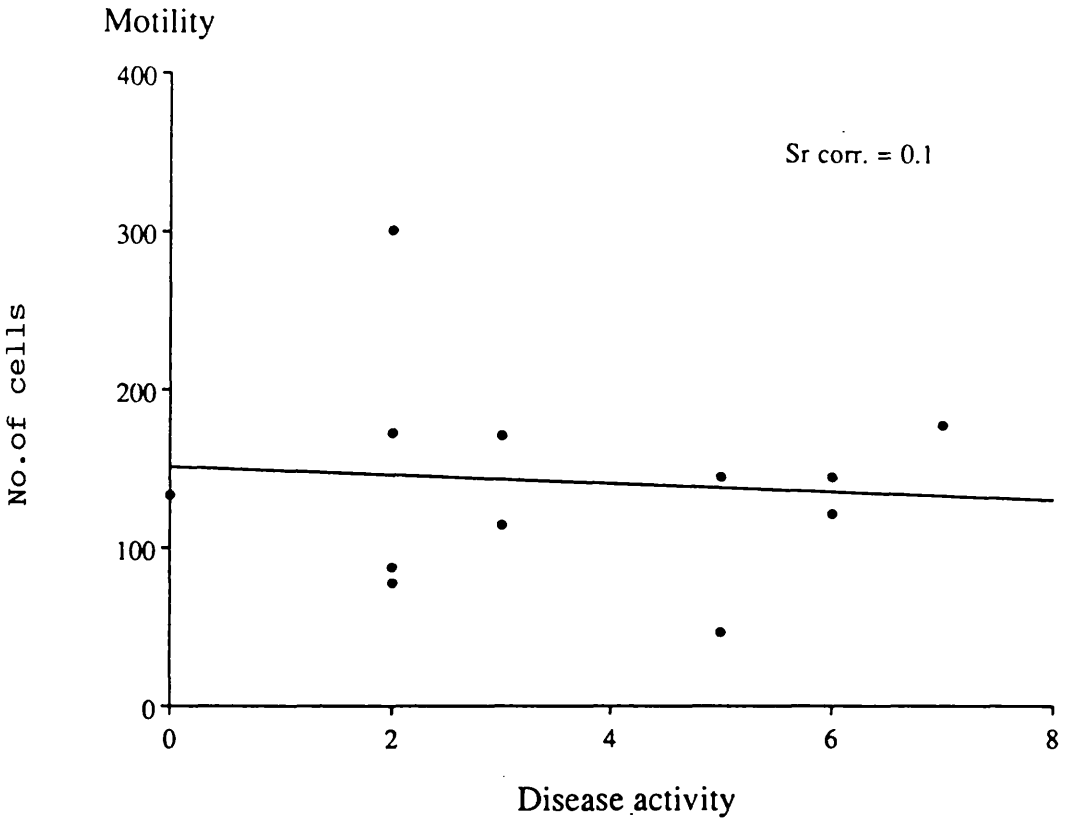


Fig XV Correlation of polymorphonuclear cells motility to disease activity in 12 ankylosing spondylitis patients.

Spearman rank coefficient (Sr. corr.) = 0.1

Significance level, P = 0.7

3.10.2. Results

The Spearman rank correlation was 0.01 indicating no correlation between disease activity and PMN motility.

3.11 CONCLUSIONS

Random motility was increased in AS compared to normal, but this increase did not reach statistical significance (Fig. X).

Both formalin -killed, and sonicated bacteria showed no chemotactic value at all. Also bacterial culture filtrates proved not to be very successful chemotaxins, and only *K. pneumoniae* showed some chemotactic properties. Culture filtrates prepared from *Y. enterocolitica* and *S. typhimurium* showed no chemotactic properties, in fact they appeared to inhibit the chemotactic value of MH broth (Fig. XI). In all cases no difference in motility was shown between AS PMNs and normal PMNs.

Polymorph motility between HLA-B27 positive and HLA-B27 negative normal individuals were similar, and again *K. pneumoniae* showed better chemotactic qualities than the other bacterial culture filtrates (Fig. XII). Only bacterial culture filtrates were examined as chemotaxins in this section, as HLA B27 positive normal samples are difficult to obtain.

When zymosan-activated serum was used as a chemoattractant, again no difference was observed between the motility of AS PMNs to normal PMNs. (Fig. XIII).

No correlation was found between age and PMN motility in normals, nor between disease activity and motility in AS patients.

CHAPTER 4. CHEMILUMINESCENT RESPONSE TO VARIOUS STIMULI OF POLYMORPHONUCLEAR LEUCOCYTES FROM NORMAL SUBJECTS AND FROM PATIENTS WITH ANKYLOSING SPONDYLITIS.

4.1. CHEMILUMINESCENCE

Chemiluminescence was first described by Allen et al in 1972 when he determined that stimulation of the hexose monophosphate shunt correlated with generation of observed light. This phenomenon has become a basic tool for measuring phagocytic action, as well as for assaying substances likely to influence the activity of activated oxygen. When interaction occurs with particulate or chemical stimulation, PMNs respond with a chain of events known as the respiratory burst. Oxygen is taken up from the surrounding media, and under the influence of a membrane-associated pyridine nucleotide oxidase (NADPH/NADH oxidise) is rapidly converted to several active oxygen species (Babior et al, 1981): namely superoxide anion (O_2^-) singlet oxygen ($^1O_2$), hydroxide radical (OH) and most important for microbicidal activity, hydrogen peroxide (H_2O_2), (Klebanoff, 1980), which is a stable, membrane permeable, powerful oxidant. (See Fig XVI).

Human PMNs contain a high concentration of myeloperoxidase (MPO), the enzyme that gives a greenish colour to abscess fluid. Klebanoff and Hamon in 1972, showed that H_2O_2 reacts with MPO, in the presence of a halide (e.g. chloride) to form oxidised chlorine ions which allows destructive halogenation or oxidation of substrates.

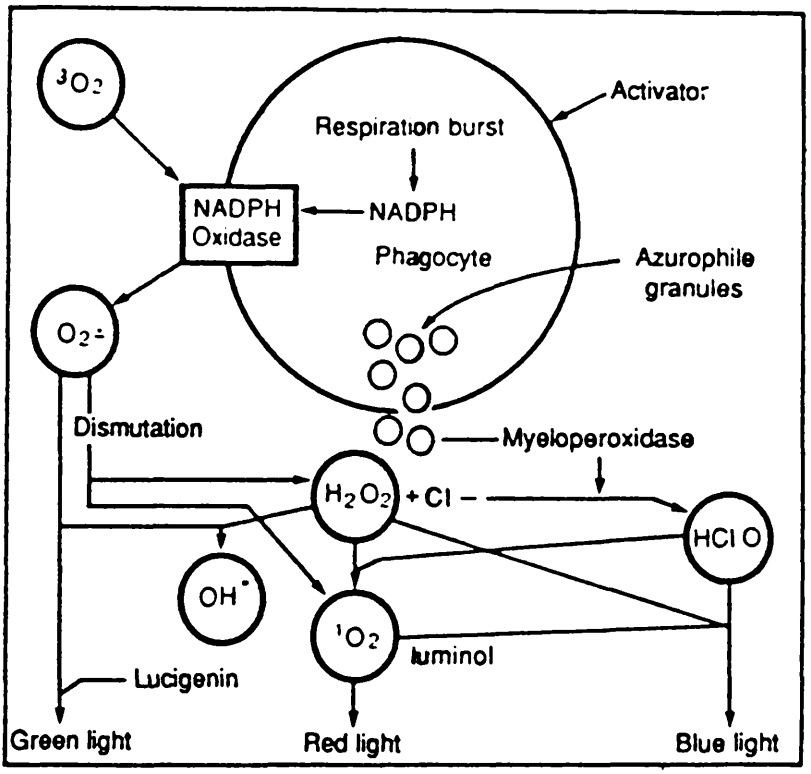


Fig XVI Diagram showing production and reactions of activated oxygen.

Also in the presence of NH_4^+ or amino acids such as taurine, MPO and H_2O_2 can react to form N-chloroamines which appear to act as long lived microbicidal products (Weiss et al, 1983).The H_2O_2 - MPO - halide system is thought to be the major bactericidal peroxidative mechanism in the PMNs. A system of detoxifying substances within each cell serves to protect against its own oxidants.

The relatively poor quantum yield of chemiluminescence (CL) can be amplified by the addition of luminol (5-amino - 2, 3 - dihydro - 1, 4 phthalazinedione), an organic substrate that produces light from post-phagocytic production of excited oxygen, enabling phagocytic oxidative activity to be measured in a sample (Allen and Loose, 1976).

Chemiluminescence has been shown to be quantitatively proportional to bacteria and neutrophil populations in phagocytic systems, and also to the concentration of luminol used (Quie, 1982).

As luminol can diffuse into the cell it can be used to monitor the production of both extracellular and intracellular hydrogen peroxide, Lucigenin, another organic substrate that produces light, behaves in a quite different way, essentially reacting with superoxide radicals, but unlike luminol it is unable to penetrate the cell thus reacting only with extracellular superoxide.

Response of neutrophils to surface stimulation involves several aspects: release of activated superoxide; secretion of granule components (myeloperoxidase containing azurophil granules); phagocytosis; and chemotaxis. Such a multiple response is triggered

by several agents, either particulate or soluble. Neutrophil membranes bear specific receptors for the Fc fragment of immune complexes and for particles opsonised with the C3b complement factor through the classical or alternate pathway. The effect of these physiological agents is mimicked by other agents, such as antibodies to neutrophil surface antigen and lectins (concanavalin A.) Chemotactic peptides (FMLP) stimulate the production and release of oxygen derivatives as well as the secretion of granule material, while other agents circumvent some of the steps involved in the activation of the phagocyte, e.g. ca++ ionophores and phorbol esters.

All of these agents do not necessarily activate the same functions of the neutrophil, so the shape and time-course of the luminescence curve may vary. Zymosan (yeast cell walls) activated with serum through the alternate pathway of complement is probably the best established and most popular activator of phagocytes. Non-phagocytic membrane stimulation by soluble stimulants such as phorbol myristate acetate (PMA) and FMLP are also used on a large scale but show different profiles.

When tested at room temperature with luminol, FMLP induces bimodal peaks. The first peak corresponds to extracellular release of myeloperoxidase (MPO) and hydrogen peroxide, and the second peak is related to the intracellular production of activated superoxide. (Brieheim, 1984).

The diffusion of luminol to the intracellular compartment was thought necessary for the production of the second peak. The finding in some

later work by Dahlgren, 1985 that lucigenin, thought incapable of diffusing into the cell, is associated with only the first peak substantiates this concept.

Microorganisms differ greatly in their susceptibility to killing by oxidants. These differences are due in part to varying levels of intracellular protective enzymes such as superoxide dismutase (SOD), catalase reductases and peroxidases. However it is not known whether any microorganism has sufficiently high levels of such enzymes to escape killing by PMN oxidants. They may be more likely to survive by blocking phagocytosis, inhibiting the respiratory burst or MPO, or by detoxifying agents.

The importance of the NADPH/NADH oxidase and MPO systems in microbicidal killing is exhibited in patients with chronic granulomatous disease (CGD) whose PMNs ingest organisms normally, but unless the organism itself produces hydrogen peroxide (eg pneumococci), it is not killed (Mandell and Hook, 1969). This correlates with the knowledge that the PMNs of these patients do not manifest a respitory burst (Quie et al, 1967). Purified cell preparations are normally used for CL studies, but a CL emission, which is primarily dependent on PMN activity, may be detected using whole blood in microlitre quantities. Kato et al, 1981, and Herberer et al, 1982 developed an assay of unfractionated whole blood CL for a simple and rapid evaluation of phagocytosis - induced metabolic respitory bursts which may rather reflect in vivo phagocytic functions under natural conditions. This method was used by De Chatelet and Shirley in 1981 to evaluate PMN function in C.G.D. patients. They

stimulated the cells by adding PMA, which induces PMNs to behave as they normally do during phagocytosis (De Chatelet et al, 1976), and were able to quantify the luminescent response in microlitre quantities of whole blood and to demonstrate that cells from patients with CGD were not responsive to stimulation. The use of whole blood to detect phagocytosis has also been adapted for looking at the opsonic activity of serum (Stevens, 1981): by adding an appropriate bacterium and luminol to diluted whole blood it is possible to evaluate the opsonic activity of an added serum sample by measuring the light emitted during phagocytosis.

Chemiluminescence of whole blood has also been useful for monitoring patients with bacterial infections. Kohashi et al, 1987 and Tatsuhito Tono-Oka et al, 1983 showed that CL was signficantly elevated in patients with definite bacterial infections after stimulation with zymosan or PMA, compared to CL of blood from patients with viral infections or from normal healthy controls. Background CL was also elevated in patients with bacterial infections suggesting that PMNs were preactivated in vivo.

In this study the CL response of polymorphs, from patients with ankylosing spondylitis, to various stimuli, was compared to that of polymorphs from normal subjects.

All graphical results were expressed as medians and interquartile ranges.

4.2. CHEMILUMINESCENT RESPONSE OF ISOLATED POLYMORPHS TO VARIOUS BACTERIAL STIMULI.

The aim was to isolate PMNs from AS patients, and from normal healthy controls, then to stimulate them by adding various bacteria to see whether or not any variations in CL response were detectable.

4.2.1. Materials and methods

Bacteria were standardised, as described in 2.5.3., prepared at 1 x 10^8/ml and opsonised with N.P.S. (See 2.5.4.). Both patient and normal samples were collected between 9 a.m. and 11 a.m. Cells were separated, prepared at 1 x 10^7/ml (see 2.3.), and both patient and normal samples run simultaneously on the luminometer (see 2.8.1.).

Details of samples are as follows:

		Normal Controls	AS Patients
No. of Samples		19	23
Mean Age (Yrs) range		29 (21-43)	46 (27-63)
Sex	M	9	20
	F	10	3

4.2.2. Results

No significant difference was seen between normals and AS polymorphs with any of the four gram-negative bacteria tested, i.e. *K. pneumoniae*, *P. mirabilis*, *Y. enterocolitica* and *S. typhimurium*.

K. pneumoniae in particular showed a wide range of CL response for both normal and AS polymorphs, and also a higher response than the others. (See Fig. XVII). Results were analysed using a Mann Whitney test, and medians and inter-quartile ranges were calculated.

4.3. CHEMILUMINESCENT RESPONSE OF ISOLATED POLYMORPHS AND WHOLE BLOOD POLYMORPHS TO VARIOUS BACTERIAL STIMULI.

The aim here was to compare results of CL response from both isolated PMNs and whole blood PMNs using samples from AS patients and normal healthy controls, stimulated with bacteria.

4.3.1. Materials and Methods

K. pneumoniae, P. mirabilis, Y. enterocolitica and S. typhimurium were all standardised as described in 2.5.3, prepared at 1 x 10^8/ml and opsonised with N.P.S. (see 2.7.2.). Samples from AS patients and normal controls were collected between 9-11 a.m.;

Details of samples are as follows:-

		Normal Controls	AS Patients
No. of Samples		13	15
Mean Age (yrs) range		35 (25-42)	41 (26-59)
Sex	M	8	12
	F	5	3

A differential count was done (2.4) on the whole blood from each

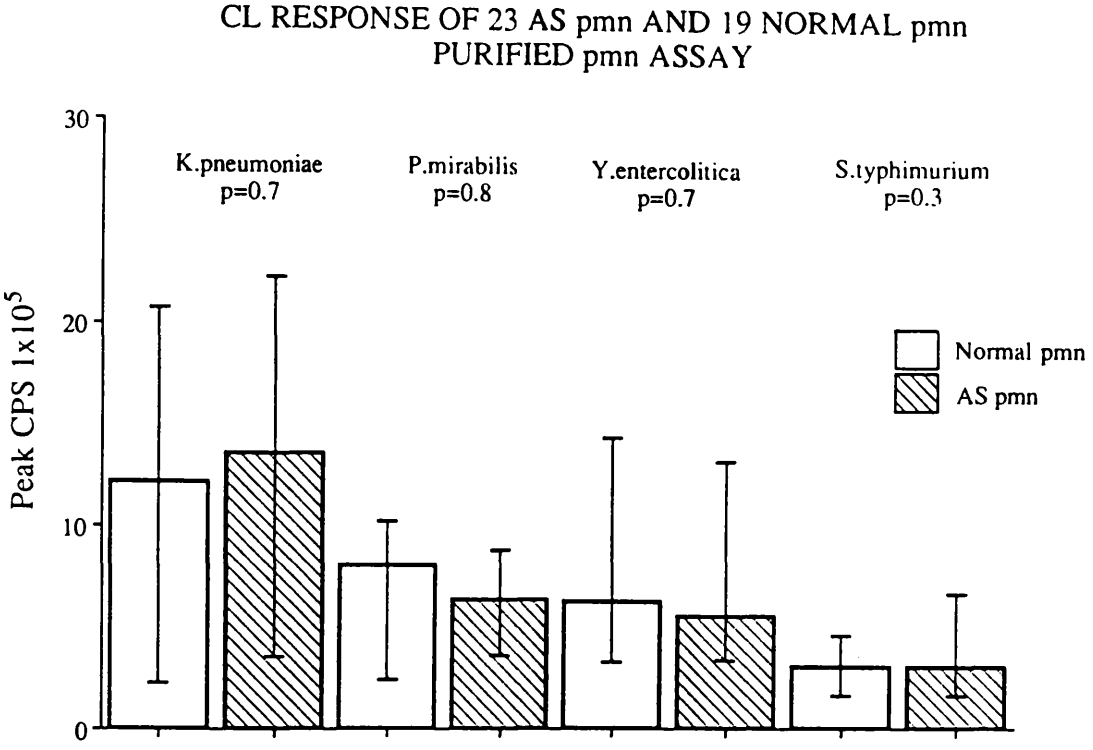


Fig XVII Chemiluminescent response of purified polymorphonuclear cells from 19 normals and 23 ankylosing spondylitis patients to various bacterial stimuli.

Expressed as medians and interquartile ranges.

sample, then both patients and normals were assayed on the luminometer simultaneously. (See 2.8.2.).

Subsequently, purified PMNs from the same patients and normals were separated from the whole blood (see 2.3.), and prepared at a dilution of 1 x 10^7/ml and CL response was estimated for these on the same day.

4.3.2. Results

No difference in CL response was seen, between patient and normal groups, when purified PMNs were stimulated with any of the four gram-negative bacteria, as was previously found. However CL response of the same samples using whole blood PMNs, was significantly enhanced in AS patients, for all bacteria ($P < .05$). Normal control whole blood PMNs appeared to remain inactive during bacterial stimulation. See Fig. XVIII for details. Medians and interquartile ranges were calculated, and a Mann-Whitney test was used to analyse the results.

4.4. CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD POLYMORPHS TO BACTERIAL STIMULI.

Further samples from AS patients and normals were collected and CL response to bacterial stimuli was examined to confirm the initial whole blood assay results.

4.4.1. Materials and Methods

K. pneumoniae, P. mirabilis, Y. enterocolitica and S. typhimurium were all standardised, as previously described in 2.5.3, prepared at 1 x

BACTERIA		SEPARATED PMN (CPS 1 X 10^5)				WHOLE BLOOD PMN (CPS 1 X 10^3)			
	Samples	Median	I.Q. Range	P. Value		Median	I.Q. Range	P.Value	
K. pneumoniae	Controls	2.39	(2.96)			2.95	(1.6)		
	A.S.	3.9	(3.5)	0.4		6.08	(3.55)	0.05	
P. mirabilis	Controls	2.86	(2.07)			2.06	(0.72)		
	AS	2.49	(2.53)	0.7		4.05	(2.18)	0.001	
Y. enterocolitica	Control	3.52	(2.6)			1.61	(0.65)		
	AS	3.33	(2.75)	0.76		2.58	(1.87)	0.002	
S. typhimurium	Controls	1.69	(2.41)			2.01	(0.99)		
	AS	2.2	(2.84)	0.5		3.26	(1.7)	0.002	

Fig. XVIII

Comparison of CL response between separated PMNs from 13 controls and 15 patients and CL response of whole blood PMNs from the same samples, performed on the same day, to various bacterial stimuli.

Measured as "Peak CPS"

10^6/ml and opsonised with NPS (see 2.7.2.). Samples were collected between 9 a.m. and 11 a.m.;

Details of samples were as follows:

		Controls	AS Patients
No. of Samples		17	20
Mean Age (yrs) range		33 (25-42)	43 (26-63)
Sex	M	10	16
	F	7	4

A differential count was done on all samples (see 2.4.), then normal and AS samples were run on the luminometer simultaneously.

4.4.2. Results

Patient and normal controls were again analysed using a Mann Whitney Test and results confirmed our initial findings, that a significantly enhanced CL response was produced from the AS samples compared to normals, when cells were exposed to gram-negative bacteria. P Values, medians and I.Q. ranges were calculated.(See Fig. XIX).

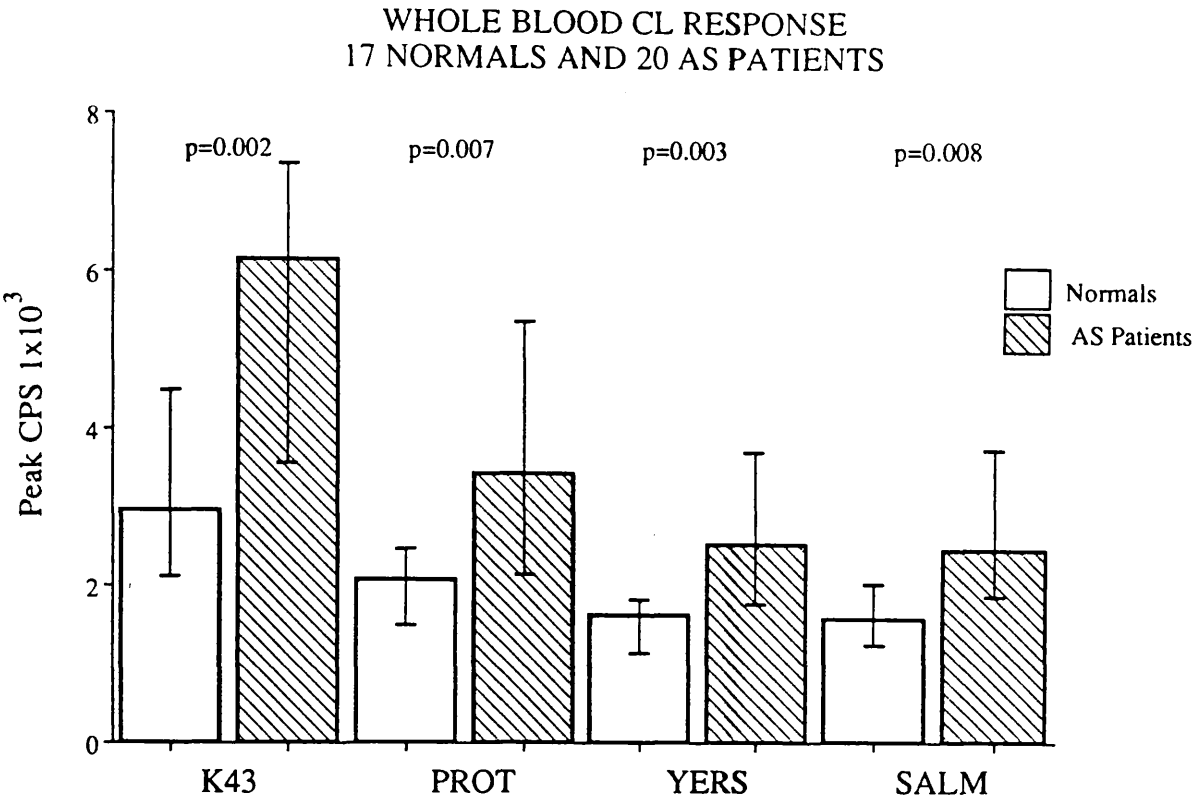


Fig XIX Chemiluminescent response of whole blood polymorphonuclear cells from 17 normals and 20 ankylosing spondylitis patients to various bacterial stimuli, namely

K. pneumoniae (K43)

P. mirabilis (Prot)

Y. enterocolitica (Yers)

S. typhimurium (salm)

4.5. WHOLE BLOOD AND PURIFIED PMN BASAL CL RESPONSE IN NORMALS AND AS PATIENTS.

Unstimulated whole blood and purified PMNs were examined for a basal CL response, to ascertain whether or not there was any variations in CL response between normals and AS patients.

4.5.1. Materials and Methods

Differential counts were done on whole blood samples, (2.4), then patients and normals were assayed simultaneously (2.8.2.).

Purified PMNs from five patients and five normals were subsequently examined for a basal CL response.

4.5.2. Results

Unstimulated whole blood AS PMNs showed a significantly enhanced response compared to normals, (P=.046), see Fig. XX.

Purified PMNs however showed no such enhancement, (see Fig XX1), indicating that the separation technique appeared to pre-activate the normal PMNs.

4.6. CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD POLYMORPHS TO A GRAM-POSITIVE BACTERIAL STIMULUS.

The next step was to see whether or not an enhanced CL response by AS PMN was restricted to stimulation by gram-negative bacteria.

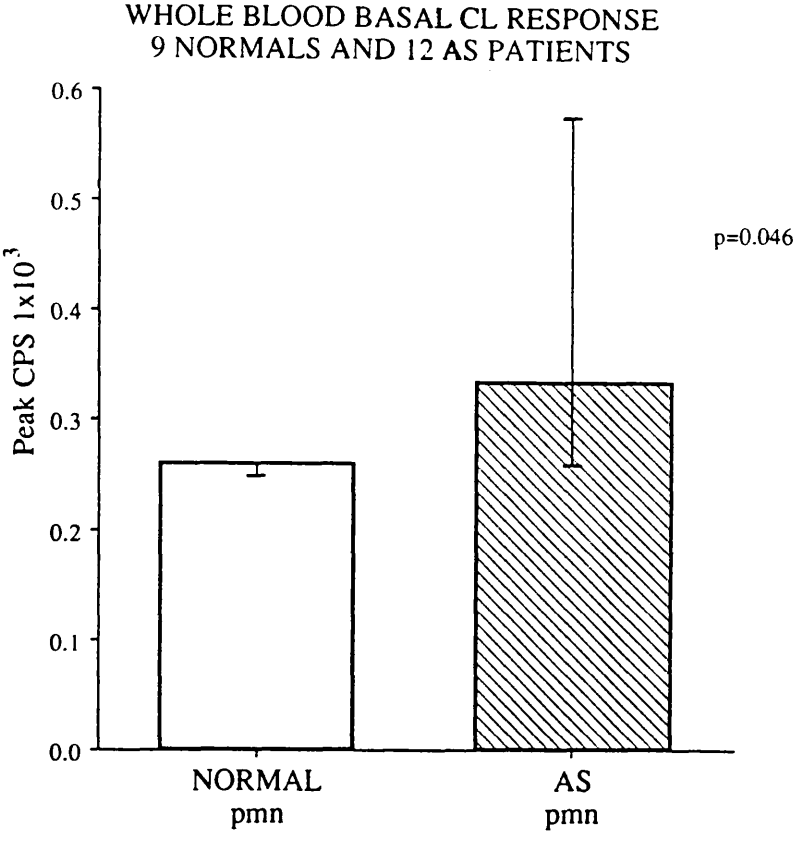


Fig XX Whole blood basal chemiluminescent response of polymorphonuclear cells from 9 normals and 12 ankylosing spondylitis patients.

Median and upper quartile value almost identical in normal polymorphonuclear cells.

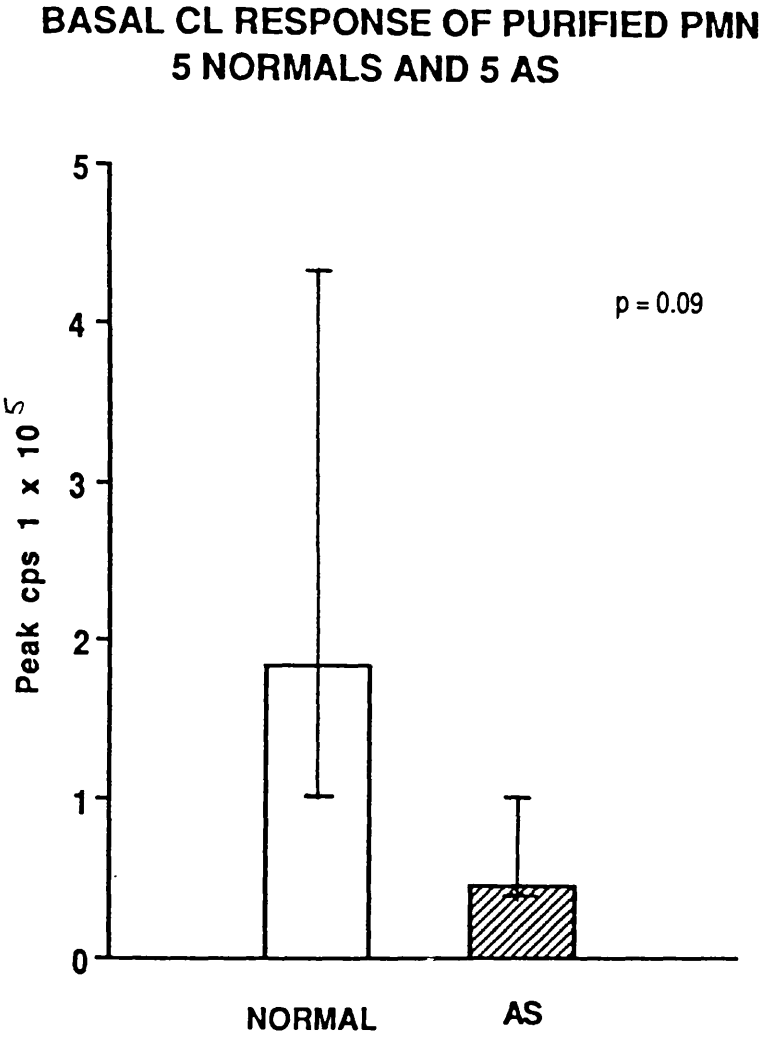


Fig XXI Basal chemiluminescent response of purified polymorphonuclear cells from 5 normals and 5 patients.

Expressed as medians and interquartile ranges.

Therefore whole blood AS and normal PMNs were exposed to Staphylococcus aureus (COWAN strain), a gram-positive bacteria.

4.6.1. Materials and Methods

Samples were collected from normal controls and AS patients, on the same day. Details are as follows:

		Controls	AS Patients
No of Samples		14	18
Mean Age (Yrs) range		31 (25-45)	48 (29-64)
Sex	M	8	13
	F	6	5

S. aureus was adjusted to a concentration of 1 x 10^8/ml, and opsonised with N.P.S. (2.7). Again a differential count was done on each sample so that the percentage of PMNs present in each sample could be calculated. Controls and patient were then run together on the luminometer. (see 2.8.2.). A total of fourteen controls and eighteen AS patients were examined.

4.6.2. Results

Whole blood samples from ankylosing spondylitis patients showed a significantly enhanced CL response when compared to normal controls (P=.002). (See Fig XXI).

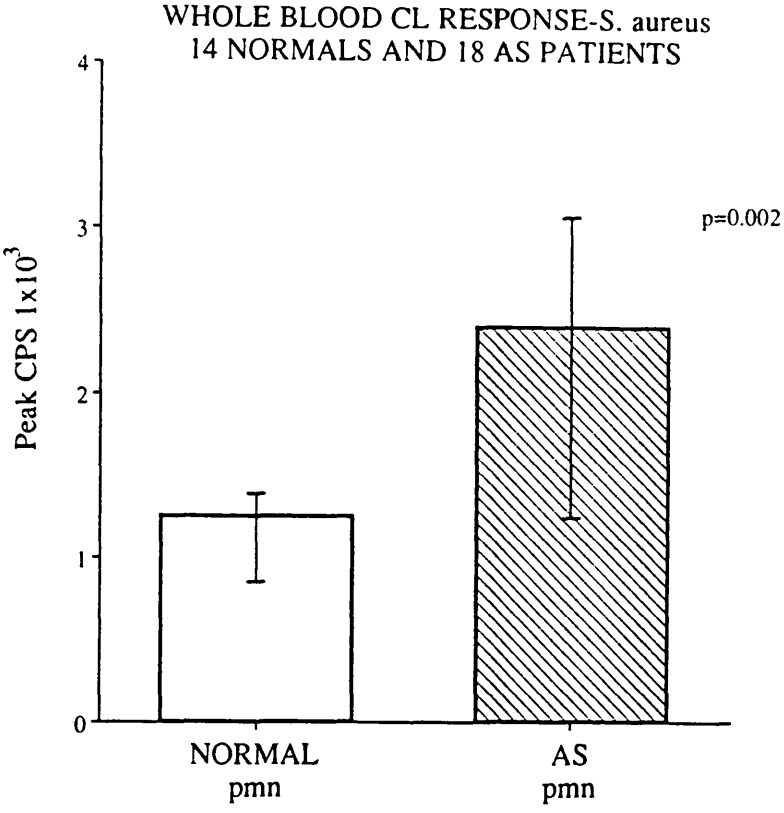


Fig XXI Whole blood chemiluminescent response of polymorphonuclear cells from 14 normals and 18 ankylosing spondylitis patients to S. aureus.

Expressed as medians and interquartile ranges.

4.7. WHOLE BLOOD POLYMORPHONUCLEAR LEUCOCYTES STIMULATED WITH PHORBOL 12-MYRISTATE 13-ACETATE

Phorbol 12- myristate 13- acetate (PMA) is a membrane-active stimulator of oxidative metabolism that is used to study reactive oxygen species production, thus mimicking the action of phagocytosis and bacterial killing by PMNs, (Repine et al, 1974).

4.7.1. Materials and Methods

Phorbol 12-myristate 13- acetate was dissolved in dimethylsulfoxide (DMSO) to give a stock solution of 2.0 mg/ml. An aliquot diluted to the appropriate concentration with phosphate buffered saline (PBS) was prepared immediately prior to use.

A whole blood assay was performed as described in Chapter 2.8., adding 50 nanograms of PMA rather than a bacterial stimulant.

Samples were collected from 24 AS patients and 12 normal controls. Details as follows:

		AS Patients	Normal Controls
No. of Samples		24	12
Mean Age (yrs) range		42 (25-69)	32 (22-43)
Sex	M	18	5
	F	6	7

4.7.2. Results

Results were analysed using a Mann Whitney test, and the CL response of AS whole blood PMNs was found to be significantly enhanced (P=.03) compared to normal controls, when stimulated with PMA. (See Fig XXII).

4.8. CHEMILIMINESCENT RESPONSE OF POLYMORPHS FROM RHEUMATOID PATIENTS,TO A BACTERIAL STIMULUS.

In order to establish whether or not this phenomenon was common to other inflammatory disorders, CL response was examined in rheumatoid patients when whole blood PMNs were stimulated with K. pneumoniae, P. mirabilis and S. aureus.

4.8.1. Materials and Methods

Bacteria were prepared at 1 x 10^8/ml and opsonised with N.P.S. (Chapter 2.7.). Samples from Rheumatoid patients and normal controls were collected between 9 a.m. and 11 a.m. and assayed simultaneously. Details of patients and normal controls are as follows:

		RA Patients	Normal Controls
No. of Samples		14	10
Mean Age (yrs) range		62 (45-70)	35 (24-43)
Sex	M	4	4
	F	10	6

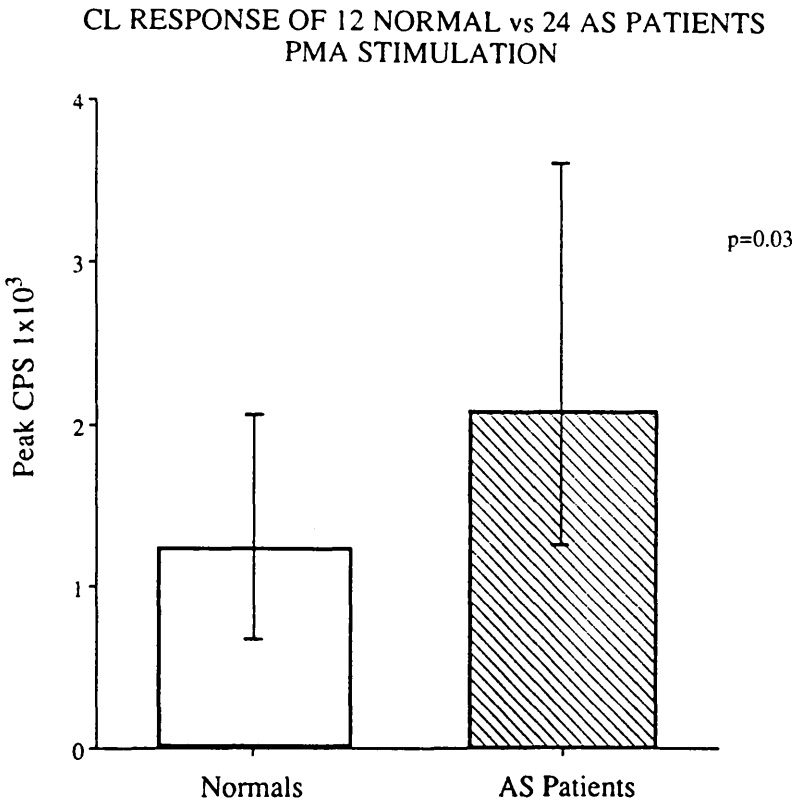


Fig XXII Whole blood chemiluminescent response of polymorphonuclear cells from 12 normal and 24 ankylosing spondylitis patients to Phorbol-myristate-acetate (PMA).

Expressed as medians and interquartile ranges.

A whole blood assay was performed as in Chapter 2.8.

4.8.2. Results

Peak counts per second (CPS) were noted for each sample and results presented graphically. (See Fig. XXIII).

Results were analysed using a Mann Whitney test.

No difference was seen between RA patient and normals when cells were unstimulated indicating no *in vivo* preactivation. Similarly no difference was seen between patients and normals when stimulated with either *K. pneumoniae* or *P. mirabilis*.

However an enhanced CL response was evident in RA patients compared to normal controls (P= .03), when stimulated with *S. aureus*, a gram-positive bacterium often associated with septic arthritis in patients with rheumatoid arthritis.

4.9. CHEMILUMINESCENT RESPONSE OF HLA-B27 POSITIVE AND HLA-B27 NEGATIVE NORMALS TO VARIOUS STIMULI.

CL response was tested in HLA-B27 positive and negative normal individuals to see whether or not enhanced CL enhanced response was related to HLA status.

Fig XXIII Whole blood chemiluminescent response of polymorphonuclear cells from 14 rheumatoid patients and 10 normals to various bacterial stimuli.

Shows basal stimulation.

Expressed as medians and interquartile ranges.

4.9.1. Materials and Methods

A whole blood CL assay was performed as previously described, and cells were stimulated with K. pneumoniae, P. mirabilis, Y. enterocolitica, S. typhimurium and with PMA consecutively.

Unstimulated cells were also examined.

4.9.2. Results

CL response was slightly enhanced in the HLA B27 positive normal samples for all stimulants. These differences however were not statistically significant. See Fig. XXV for details.

4.10. DISEASE CORRELATION

4.10.1. Materials and Methods.

Disease activity was scored in fourteen AS patients, as described in Chapter 2.9.1.

Comparison of the CL response of whole blood PMNs, stimulated with K. pneumoniae was compared with disease activity. A Spearman rank test was used for analysis.

4.10.2. Results

Only a weak correlation was found, the correlation coefficient being

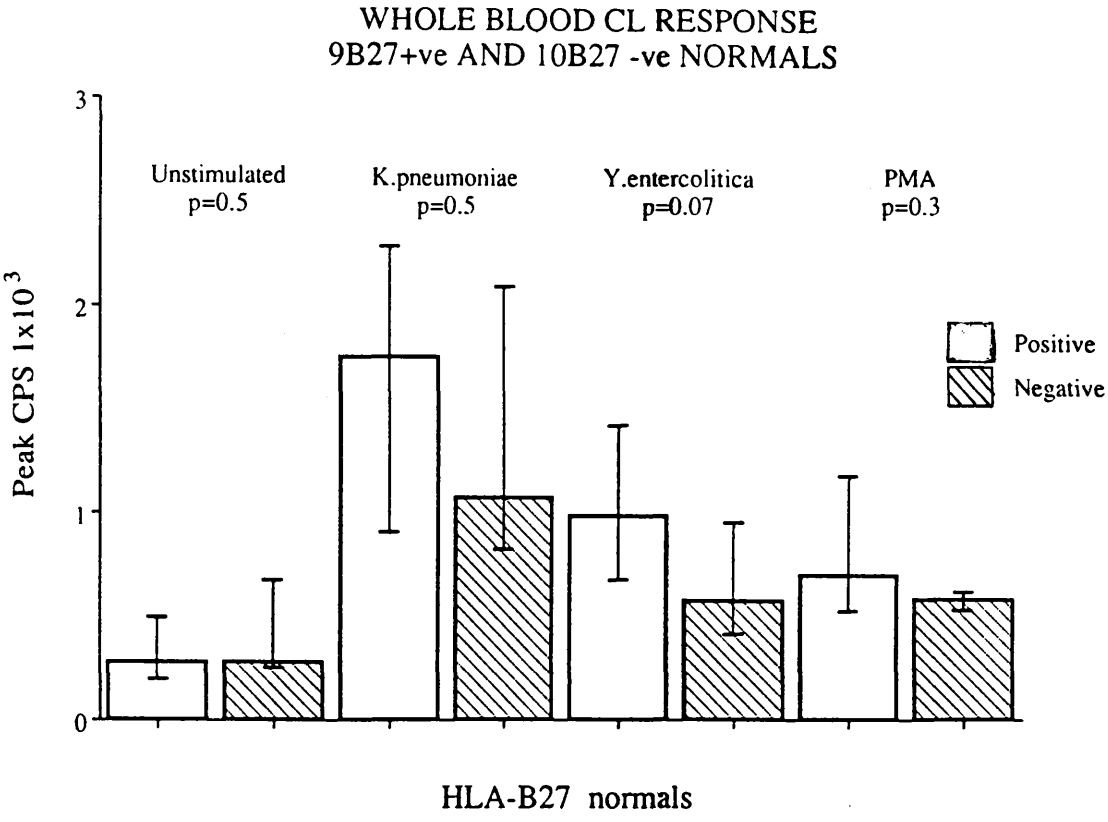


Fig XXIV Whole blood chemiluminescent response of polymorphonuclear cells from HLA-B27 positive and HLA-B27 negative normals to various stimulants.

PMA: Phorbol 12-myristate-13 acetate.

Expressed as medians and interquartile ranges.

CORRELATION OF CL RESPONSE TO DISEASE ACTIVITY IN 14 AS PATIENTS

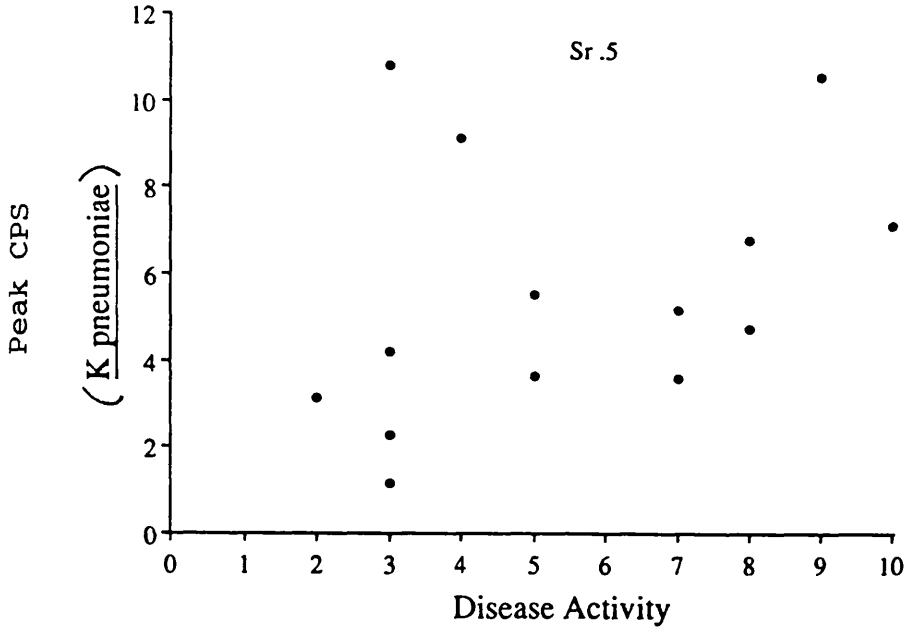


Fig XXV Spearman rank (sr) correlation of chemiluminescent response to disease activity in ankylosing spondylitis patients.

Significance level (P) = .07.

0.5, perhaps due to two outlying values. See Fig. XXV . Significance level or confidence level (P) = .07.

4.11. CONCLUSIONS

Initial results, using purified PMNs, showed no difference in CL response between AS patient and normal subjects. This proved to be a result of the separation technique, which appeared to pre-activate the PMNs before bacterial stimulation, thus masking any enhancement of CL response in AS PMNs.

Comparison of CL response using whole blood PMNs and purified PMNs, from the same samples confirmed that this in fact was the case, and consequently whole blood was used in subsequent assays.

A significantly enhanced CL response was found in whole blood PMNs from AS patients compared to normal PMNs, when stimulated by the gram-negative bacteria, K. pneumoniae, P. mirabilis, Y. enterocolitica and S. typhimurium. The next step was to see whether or not this result was exclusive to gram- negative bacteria. Therefore AS whole blood PMNs were stimulated with S. aureus, (Cowan strain), a gram-positive bacterium which is not arthritogenic. Again AS showed a significantly enhanced response indicating that this phenomenon was not specific to a gram-negative bacterial challenge.

When unstimulated whole blood PMN were examined for basal CL response, AS patients were found to be pre-activated in vivo. This was not the case when PMNs had been separated from the whole blood, as the

separation technique activated the normal controls, thus no difference in basal CL response was seen between patients and normal PMNs. A non-specific activation of PMNs at basal level in AS is evident, the trigger being unclear. An explanation for this could be that as a result of increased intestinal permeability,(Morris, A.J. et al 1989, unpublished) AS patients could be more susceptible to invasion by gram-negative bacteria asssociated with the gut,thus activating host defence mechanisms.

Since RA whole blood PMN do not show basal activation it would appear that this phenomenon is not specific to all chronic inflammatory disorders. Furthermore no enhanced CL response was ascertained with RA patients compared to normals, when whole blood was stimulated with gram- negative bacteria. However, when stimulated with S. aureus, CL response was found to be significantly greater in RA patients compared to normals.

Phorbyl 12- myristate 13- acetate, an active principle of croton oil, causes ultra- structural changes in PMN similar to those observed during phagocytosis (White and Estensen, 1974). When used as a soluble rather than a particulate stimulus, a significantly increased CL response in AS patients was shown, compared to controls.

When HLA-B27 positive and negative normals were examined, no significant difference was found in CL response, although there was a tendency for HLA-B27 positive PMNs to show an increased CL when exposed to gram-negative bacteria or PMA. No difference in basal activation was seen.

Finally only weak correlation was evident between CL response to gram-negative bacteria, and disease activity.

CHAPTER 5 PHAGOCYTIC UPTAKE OF RADIO-LABELLED GRAM-NEGATIVE BACTERIA BY POLYMORPHONUCLEAR LEUCOCYTES.

5.1. POLYMORPHONUCLEAR LEUCOCYTES

In 1883, Elie Metchnikoff injected foreign particles into metazoans, and observed that they were taken up by 'wandering mesodermal cells' that resided in interstitial tissues. He named these 'phagocytes', and twenty years passed before he observed and realised that the phagocytic cell was capable of intracellular killing of bacteria (Metchnikoff, 1905). The predominant phagocytic white cells are the circulating PMNs. Polymorphonuclear leucocytes are produced in the bone marrow from a stem cell pool. The stem cell-pool is believed to be pluripotential, the common ancestor of erythrocytes and platelets (Dunn, 1971). The evolution from stem cell to mature blood PMN takes approximately 13-14 days.

The number of PMNs entering the blood stream each day from the bone marrow is 10^{11}. Half of these are not circulating, but are adherent to the endothelium, a process called margination. The mechanism of adhesion is not precisely known, but involves both net surface negative charges (Hoover *et al,* 1978) and physical contact with the substrate (Malech, Root and Gallin, 1977). The cell surface charge may allow cells to be drawn to each other so that microfilament attachment can occur. Polymorphonuclear leucocytes enter the tissues from the bloodstream where they fulfil their functional role. They have no regenerative capacity, and do not return to the circulation, as do mononuclear phagocytes. The total life span of a PMN is about

15 days. The PMN has a characteristic large multilobal nucleus (Fig XXVI) and numerous cytoplasmic granules, which appear pink using Leishman stain. The azurophil or primary granules appear early in development at the promyelocyte stage, with a rich content of acid hydrolases. The specific or secondary granules appear at a later stage, and are 3 times as numerous as the primary granules. They are called specific since they contain lactoferrin and cobalophilin which are regarded as specific cytochemical markers in PMNs. They are also smaller and lack digestive enzymes and peroxidase. When a particle is phagocytosed the granules are released sequentially into the phagosome, the specific granules being discharged first, followed by the azurophils. Granule contents may also be released by granule fusion with the plasma membrane rather than with a phagosome, which may be important in the destruction of large pathogens which are not easily ingested. This may also have tissue-damaging effects at for example, sites of immune-complex deposition, where released PMN products may be a major cause of tissue destruction.

The PMN is a specialised cell designed for adhesion, movement, ingestion and intracellular killing of microbes. It also behaves as a scavenger cell, removing products of tissue damage and breakdown, and dead or dying cells from the circulation. Its task is primarily migration to the site of injury, where it responds with a significant release of lysosomal constituents and the production of oxygen radicals, therefore having the potential to cause some tissue damage.

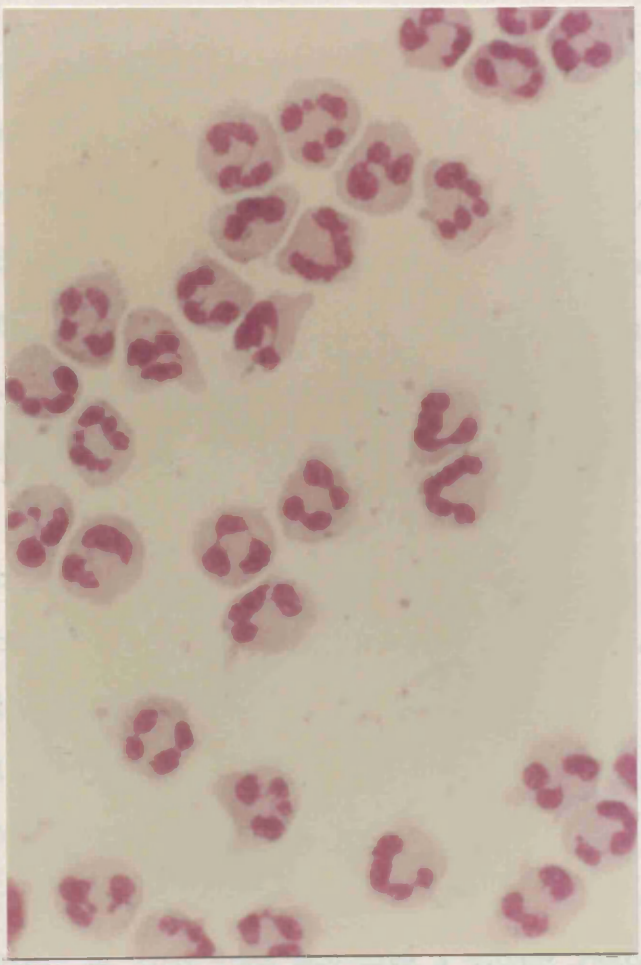


Fig XXVI The multilobed nucleus of the polymorphonuclear leucocyte.

5.2. PHAGOCYTOSIS

Phagocytosis is the process whereby single cells internalise objects that they encounter (Fig. XXVII). The process is a matter of survival for unicellular organisms, which use it to gain nourishment. In higher animals it protects against invasion by micro-organisms in the internal and external environment.

As part of a response to infection there is an increase in PMN production, a leucocytosis. This is associated with acute inflammation and marked in infections with 'cocci', especially if they are generalised (Harkness, 1981). Vigorous exercise and adrenalin are physiological factors, causing leucocytosis by shifting the cells from marginal sites. Such a leucocytosis is not accompanied by an increase in the total number of PMNs in the body.

Chemotaxins attract the circulating PMNs to the infection site (details in chapter 3), whereas monocytes arrive at a slower pace. Greater motility thus results in the prompt response of chemotactic stimulation relative to monocytes. This however could not explain why this reaction stops once the mononuclear cell infiltrate is established. Explanations could be that inhibitors of PMN motility could accumulate, or that PMNs may have a higher response threshold than that of monocytes to chemotaxins. Then as these chemotaxins are absorbed or inactivated by cells in the exudate, their concentrations may fall below that response threshold.

Having arrived at the site of an invading bacterium, phagocytes need

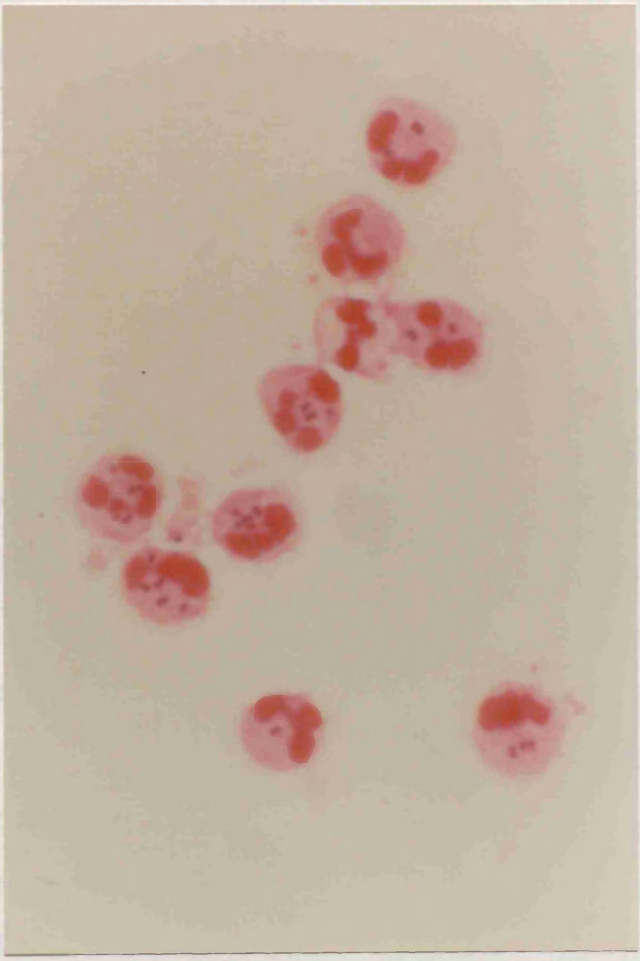


Fig.XXVII Polymorphonuclear leucocytes with internalised Staphylococcus aureus.

to recognise it, i.e. they use specific receptors to bind to the surface of the bacteria. Opsonins coat the bacterium to produce this recognition. Wright and Douglas (1903) originated the term opsonise from the Greek, meaning 'to prepare for dining'. Immunoglobulin G (IgG) and C3b are the primary opsonins which bind PMNs to bacteria and promote ingestion. This is because PMNs and monocytes have receptors for the Fc portion of IgG and for the C3b part of the complement factor (Horwitz, 1982). The subclasses IgG_1 and IgG_3, of the serum IgG antibody are the species which participate in opsonisation. The presence of specific antibody as an opsonin is known as immune opsonisation. Both the Fc portion and the Fab segment of the antibody must be intact for expression of opsonic activity (Quie, 1972). Since the IgG immunoglobulins are heat-resistant, the heat stable opsonic activity of serum is due to the presence of antibodies. Normal serum has little heat-stable opsonic activity, whereas heat-labile opsonic activity can be entirely attributed to the complement-protein C3b. Certain organisms do not require antibody or complement for phagocytosis to occur, namely non-encapsulated pneumococci and many gram-negative bacilli. Other bacteria, particularly encapsulated ones such as klebsiella and virulent strains of S. aureus escape phagocytosis unless antibody and/or complement is present, thus contributing to their pathogenicity.

Attachment of the opsonised particle to the phagocyte induces the formation of pseudopods which surround the particle as a result of interactions between surface receptors, termed the 'zipper mechanism' (Griffin et al, 1975). The pseudopodia then fuse and engulfment is completed with the formation of a phagosome. The action of the

contractile proteins moves the phagosome through the cyloplasm to the region of the Golgi apparatus, where several small vesicles, the primary lysosomes, fuse with the phagosome and form a phagolysosome. This process is called degranulation, a mechanism whereby enzymes are delivered to their operational sites without subjecting the PMN cytoplasm to their potential injurious effects. The phagolysosome contains many hydrolytic enzymes, including proteases, nucleases, acid phosphates and lipases which attack the ingested material. As a result of this metabolic activity and the additions of specific granule contents, the pH inside the phagolysosome increases slightly then falls (Segal et al, 1981), then a series of biochemcal changes occur that result in the killing of ingested material(Root and Cohen, 1981). (See Fig. XXVIII).

The microbicidal activity of PMNs can be divided into 1) oxygen-dependent and 2) oxygen-independent systems.

The oxygen-dependent process involves a coordinated series of metabolic events, dormant in resting cells, whose function is to produce a group of highly reactive microbicidal agents by the partial reduction of oxygen. This 'respiratory burst' is described in detail in chapter 4.

The oxygen-independent mechanisms are responsible for microbial killing in an anaerobic environment or in PMN where the oxidative capacity is deficient or absent e.g. chronic granulomatous disease. These mechanisms are -

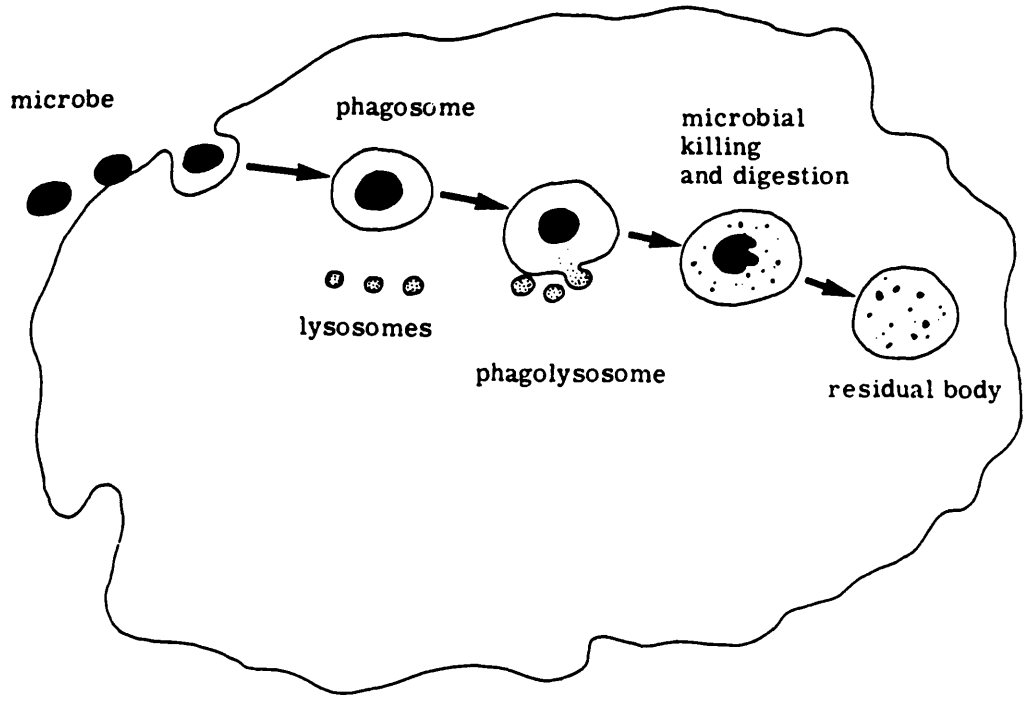


Fig XXVIII Diagram showing phagocytosis and intracellular digestion.

1) The acidic environment of the phagolysosome (pH 4.0 to 6.0).

2) Lactoferrin, an iron-binding protein whose extracellular release can destroy microbes by starving them of iron (Root and Cohen, 1981).

3) The direct action of lysozyme, which attacks the peptidoglycan of some bacterial walls, resulting in the lysis of the microorganisms (Unanue, 1976).

Primary granules are rich in arginine which rapidly affects the ability of bacteria to replicate without destroying their structural integrity. In addition PMNs contain a variety of proteases and hydrolases which have a digestive rather than a microbicidal function (Klebanoff and Clark, 1978).

Oxygen-dependent and oxygen-independent mechanisms tend to be studied separately, and little is known about their interactions. A cooperative attack of oxidants and hydrolytic enzymes may be possible whereby the exposure of bacteria to a free radical-generating system would increase their susceptibility to attack by lysozyme (Thomas, Lehrer and Rest, 1988).

5.2.1. Measurement of phagocytic activity

The process of phagocytosis may be examined *in vivo* and *in vitro*. The latter offers several advantages, namely:

1. Known homogenous populations of phagocytic cells can be separately studied

2. Effect of serum, and of other factors, on phagocytic function can be assessed, and

3) Phagocytic uptake can be evaluated independently of bacterial killing. The use of subjective techniques, such as microscopic examinations of smear preparations and colony counting, have several disadvantages in that they are tedious, time consuming and subject to interobserver error. The radiometric assay, (chapter 2.7), however is an objective and sensitive assay which can be quantitated so that results may be obtained within several hours. There often lacks distinction in these assays between intracellular ingestion and extracellular attachment, therefore care must be taken in interprepation of the results. Polymorphonuclear leucocyte killing can be measured directly by the decrease in number of live bacteria, by radio-active probes or by microbiological methods. The respiratory burst which accompanies PMN phagocytosis is an indirect measurement of the microbicidal activity of these cells. However non-specific activation accompanied by a metabolic burst may not necessarily be accompanied by intracellular killing, therefore this type of measurement would be unsuitable in the estimation of phagocytic ability.

In this study the intention is to evaluate and compare the phagocytic uptake of various gram-negative bacteria by PMNs from patients with

AS, normal controls and from HLA-B27 negative and HLA-B27 positive normal individuals without disease.

5.3 COMPARISON OF HEAT-STABLE AND HEAT-LABILE OPSONINS IN THE UPTAKE OF K. pneumoniae BY POLYMORPHONUCLEAR LEUCOCYTES.

The best described opsonins are antibodies, which are heat stable and thus add an element of specificity to the phagocytic process, and complement, which is heat labile and non- specific. This experiment was devised to establish whether the opsonisation of K. pneumoniae was complement or antibody mediated. The classical complement pathway is activated when a specific antibody, e.g. IgG binds to the bacterium (antigen) by its antibody binding site (Fab fragment), leaving the Fc fragment exposed. The binding of C1q protein to this fragment leads to a subsequent cascade of enzymatic reactions which results in the deposition of certain fragments, C4b, C2a, C3b, C5b, and C6 through C9, on to the bacterial cell wall. Bacteriolysis results, but the precise mechanism is still not known. However there is good evidence that activation of complement components on the cell surface results in the formation of physiological holes in the cell membrane, leading to osmotic lysis of the cell (Provost and Allen, 1976). Activation of the alternate complement pathway does not require whole antibody molecules. Bacterial endotoxins alone can stimulate the late components of complement.

The heat-labile complement, in particular the C3b fragment acts primarily by adhering to the antigen and then binding the antigen to lymphocytes and phagocytic cells by C3b receptors on the cell surface (Stossel,1974), activating the cell and enhancing phagocytosis.

5.3.1. Materials and Methods

Six aliquotes of NPS were prepared, three of which were heated in a waterbath at 56°C for 30 minutes. Heparinised blood was collected from 3 AS patients, and PMNs separated by dextran sedimentation. A radiometric phagocytic uptake assay was performed (2.7), to evaluate percentage uptake, when *K. pneumoniae* was opsonised with NPS, and heat-treated NPS.

5.3.2. Results

When NPS was heat-treated at 56°C for thirty minutes percentage uptake was drastically reduced compared to percentage uptake achieved with unheated NPS, showing the complement to be heat-labile.

NPS	Heat-treated NPS
9.43	5.93
13.28	4.84
12.33	1.88

This clearly showed that opsonisation of the bacterium with complement mediated serum was required to achieve a more efficient phagocytic uptake. This would support the theory that the functional properties of the cleavage products of complement activation are more important than are the actual bacteriolytic reactions, in host defence against infection (Upjohn, 1975).

5.4 COMPARISON OF PHAGOCYTIC UPTAKE OF K. pneumoniae K43 AND P. mirabilis BY POLYMORPHONUCLEAR LEUCOCYTES FROM ANKYLOSING SPONDYLITIS PATIENTS AND FROM NORMAL INDIVIDUALS.

5.4.1. Materials and Methods

Heparinised blood was collected from 16 AS patients and from 10 controls. Details of samples are as follows:

		AS Patients	Normals
No. of Samples		16	10
Mean age (Yrs) range		40 (20 - 62)	29 (19-40)
Sex	M	12	5
	F	4	5

A radiometric assay was used to evaluate percentage uptake of the bacteria, as decribed in chapter 2.7, using purified PMNs (see 2.3) from AS patients and normal controls, opsonised with normal pool serum (see 2.7.2.). A Mann Whitney test was used to analyse the results, which are expressed as medians and interquartile ranges.

5.4.2. Results

A significantly enhanced uptake of K. pneumoniae by AS PMNS was shown, compared to normals (P = .009). No difference was seen between AS PMNs and normal PMNs in the uptake of P. mirabilis (Fig XXIX).

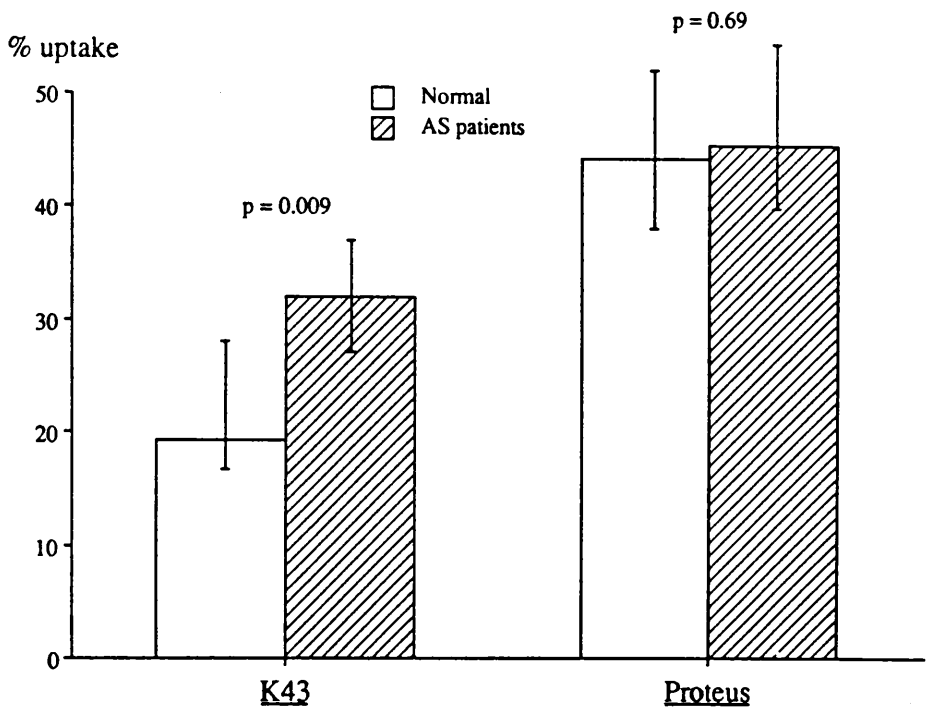


Fig XXIX Comparison of phagocytic uptake of K. pneumoniae (K43) and P. mirabilis (Proteus) by polymorphonuclear cells from AS patients and normals.

Expressed as medians and interquartile ranges.

However not all patient and normal samples were assayed simultaneously, therefore assays were repeated using 30 AS patients and 28 controls, care being taken to assay both patient and normal samples together to minimise any inter-assay variation.

5.5. PHAGOCYTIC UPTAKE OF K. pneumoniae AND P. mirabilis BY PMNS FROM 30 AS PATIENTS AND 28 NORMAL CONTROLS.

5.5.1. Materials and Methods

Samples were collected in heparin, and both AS PMNS and normal PMNS were assayed simultaneously, see 2.7. Details of samples are as follows:

		AS Patients	Normal
No. of Samples		30	28
Mean age (yrs) range		41 (27-69)	29 (24-57)
Sex	M	25	17
	F	5	11

5.5.2. Results

Results are expressed as medians and inter-quartile ranges, and P. values were calculated using a Mann Whitney test, (Fig XXX). No difference was shown in uptake of K. pneumoniae or P. mirabilis between AS PMNs and normal PMNs.

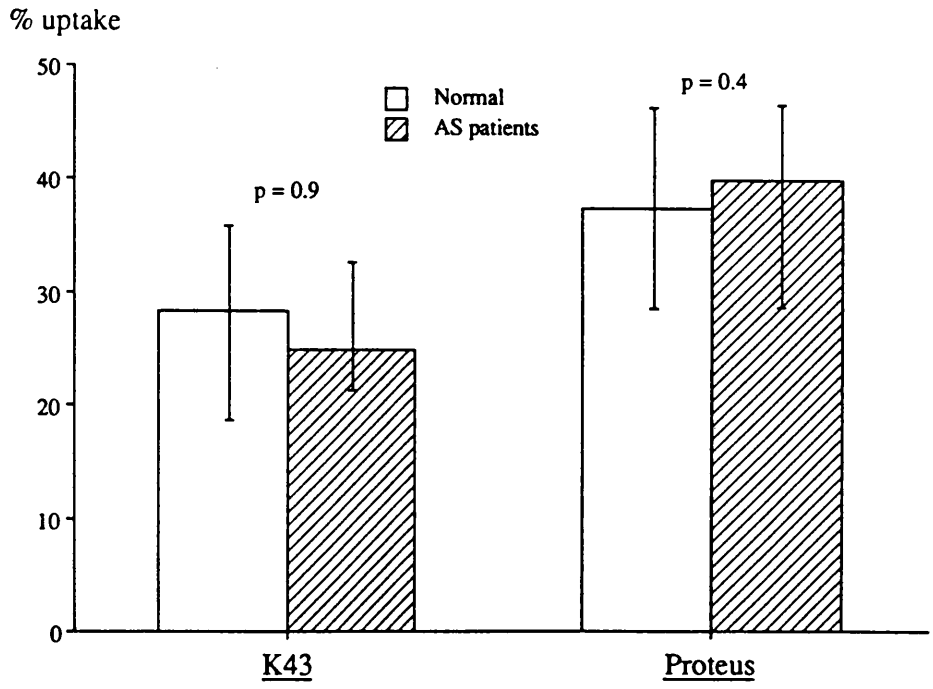


Fig XXX Comparison of phagocytic uptake of K. pneumoniae and P. mirabilis by polymorphonuclear cells from 30 AS patients and 28 normals.

Expressed as medians and interquartile ranges.

Our initial findings of enhanced uptake of K. pneumoniae by AS PMNs to normals was therefore not confirmed, due perhaps to patient and normal samples not being processed together in the first series of assays, and to small numbers.

5.6. PHAGOCYTIC UPTAKE OF Y. enterocolitica AND S. typhimurium BY PMNS FROM AS PATIENTS AND NORMAL CONTROLS.

5.6.1. Materials and Methods

Using the same radiometric assay, the phagocytic uptake of Y. enterocolitica in 18 AS patients was compared to that of 10 normals and the uptake of S. typhimurium in PMNs from 10 AS patients and 10 normals was examined. Details of samples are as follows:

		Y. enterocolitica	S. typhimurium
No. of AS Patients		18	10
Mean age(yrs) range		45 (33-65)	38 (29-59)
Sex	M	14	9
	F	4	1
No. of Normals		13	10
Mean Age (yrs) range		28 (22-55)	28 (22-43)
Sex	M	10	7
	F	3	3

5.6.2. Results

Again, no difference was seen in phagocytic uptake of either Y. enterocolitica or S. typhimurium between AS patient and normal PMNs. (Figs XXXI, XXXII).

5.7. OPSONIC CAPACITY OF AS SERA

Sera was collected from several AS patients, then pooled and frozen in aliquots at -70°C. These aliquots were used to opsonise the bacteria in place of the normal pool serum.

5.7.1. Materials and methods

10ml of peripheral blood was collected in heparin from ten AS patients and from nine controls.

		AS patients	Normals
No. of Samples		10	9
Mean age (yrs) range		41 (23-59)	31 (22-40)
Sex	M	9	5
	F	1	4

Cells were separated and bacteria opsonised with AS pool sera. Phagocytic uptake of K. pneumoniae and P. mirabilis was then evaluated.

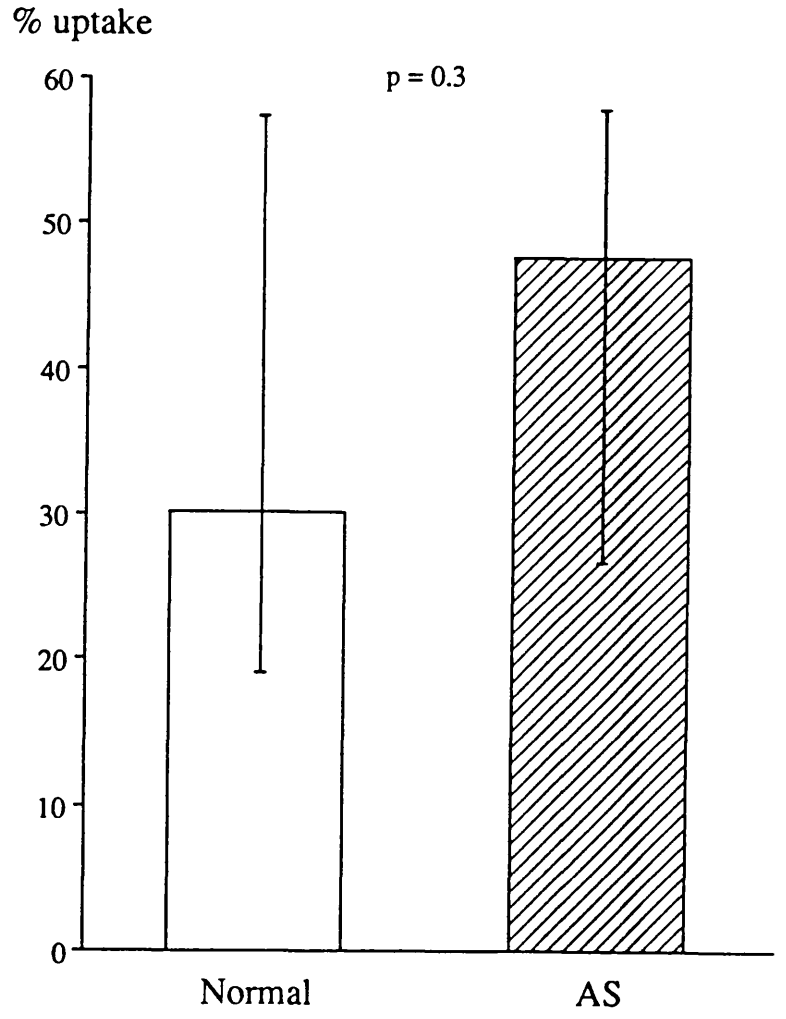


Fig XXXl Comparison of phagocytic uptake of Y. enterocolitica by polymorphonuclear cells from 13 normals and 18 ankylosing spondylitis patients.

Expressed as medians and interquartile ranges.

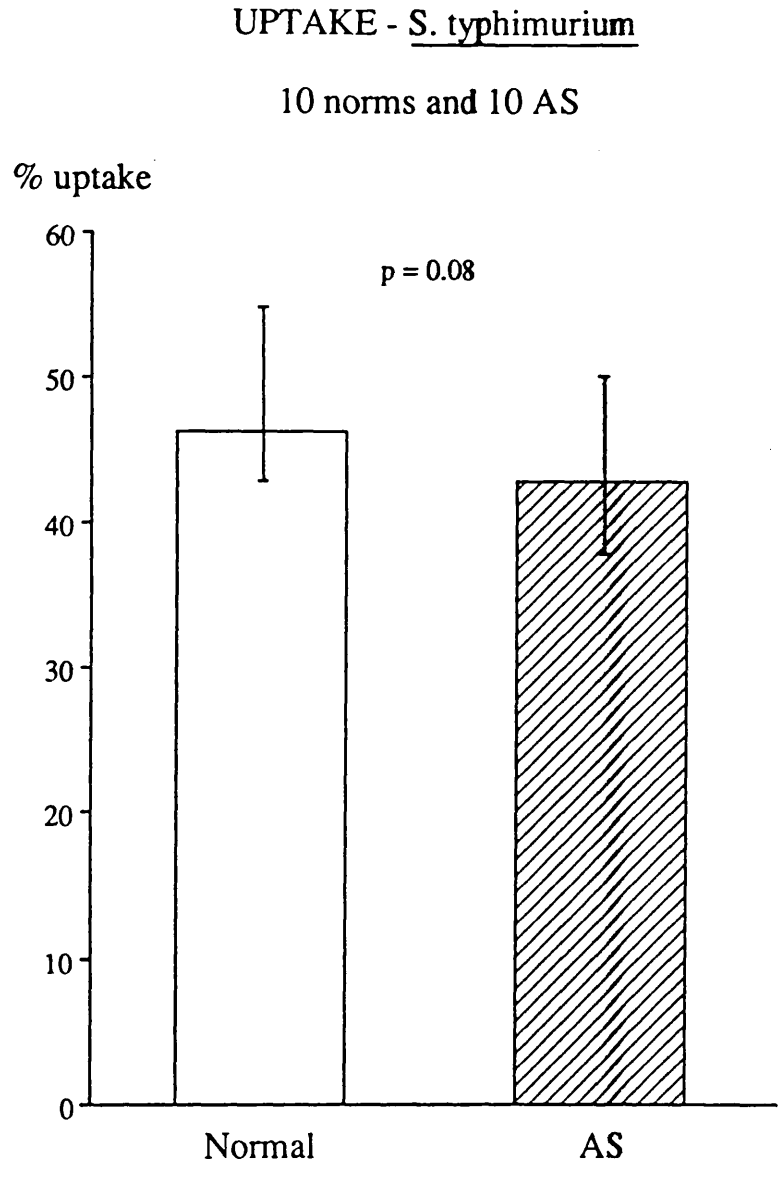


Fig XXXII Comparison of phagocytic uptake of S. typhimurium by polymorphonuclear from 10 normals and 10 ankylosing spondylitis patients.

Expressed as medians and interquartile ranges.

5.7.2. Results

Results were expressed as medians and interquartile ranges (Fig XXXIII). No significant difference was found, in the phagocytic uptake of gram-negative bacteria, between AS patient and normal PMNs, when the bacteria were opsonised with pooled serum prepared from several AS patients rather than a normal pool serum. Also no impairment in the opsonic ability of AS serum was shown as uptake values were comparable to those obtained in previous assays where bacteria were opsonised with a pooled serum from normal individuals.

5.8. COMPARISON OF PHAGOCYTIC UPTAKE OF GRAM-NEGATIVE BACTERIA BETWEEN HLA-B27 POSITIVE AND HLA-B27 NEGATIVE NORMAL INDIVIDUALS.

Because of the HLA-B27 - AS link, it was also important to examine PMNs from HLA-B27 normal individuals.

5.8.1. Materials and methods

Samples of heparinised blood were collected from nine HLA-B27 positive and ten HLA-B27 negative normal volunteers, which were made available via the tissue-typing laboratory, Glasgow Royal Infirmary. PMNs from both B27 positive and B27 negative samples were assayed simultaneously, and phagocytic uptake of *K. pneumoniae*, *P. mirabilis*, *Y. enterocolitica* and *S. typhimurium* was examined. Details of samples as follows.

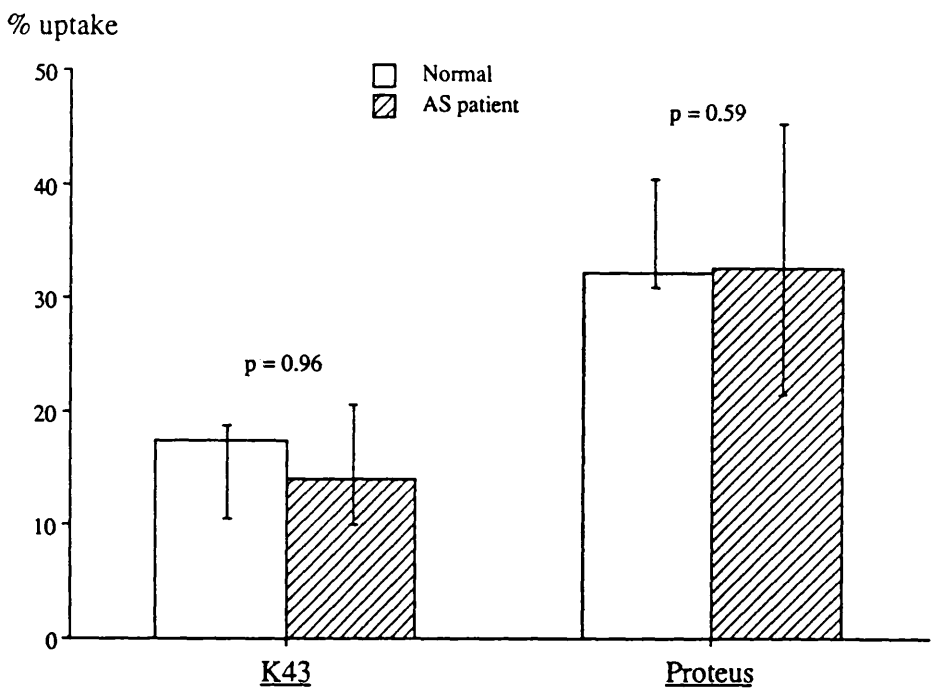


Fig XXXIII Comparison of phagocytic uptake of K. pneumoniae (K43) and P. mirabilis (proteus) by polymorphonuclear cells from 9 normals and 10 AS patients, when bacteria has been opsonised with AS pooled sera.

Expressed as medians and interquartile ranges.

		HLA-B27 + ue	HLA-B27 - ue
No. of Samples		9	9
Mean age(yrs) range		31 (24-43)	35 (29-42)
Sex	M	6	2
	F	3	7

5.8.2. Results

Results were analysed using a Mann Whitney test and expressed as medians and interquartile ranges. Again no difference was expressed between B27 positive and B27 negative PMNs, in the case of all four bacteria. (Fig XXXIV).

5.9. AGE AND UPTAKE CORRELATION

The ages of 19 normals were correlated with the phagocytic uptake of K. pneumoniae, using a Spearman rank correlation test.

5.9.1. Results

The spearman rank correlation coefficient was found to be -0.29, indicating no correlation between age and phagocytic uptake. (Fig XXXV).

Significance level p = .21

UPTAKE - K43, PROT., YERS., SALM.
9 B27 -ve and 9 B27+ve

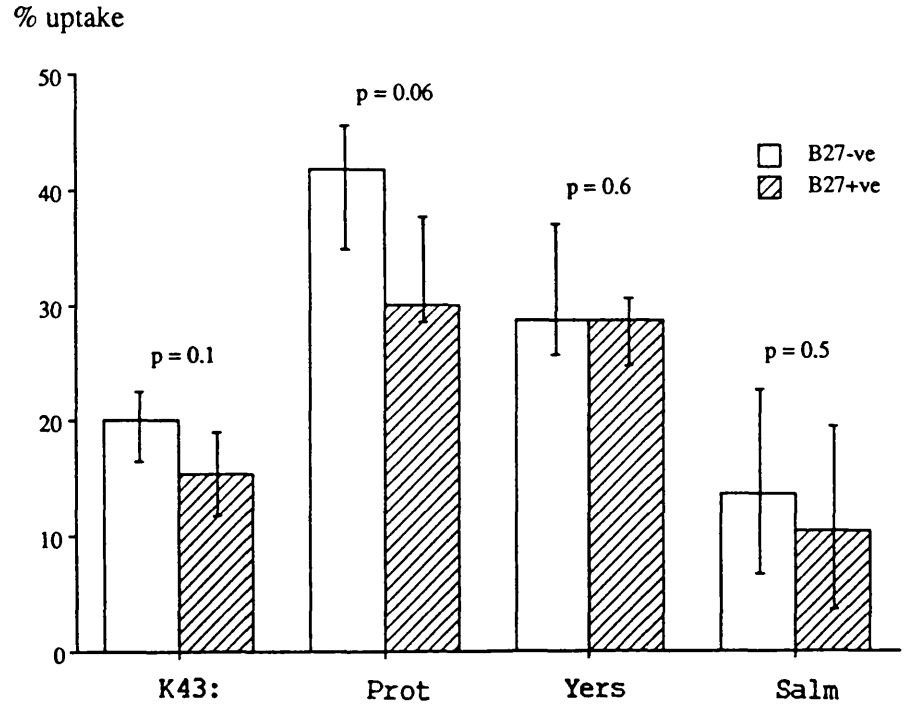


Fig XXXIV Comparison of phagocytic uptake of gram-negative bacteria by polymorphonuclear cells from HLA-B27 positive and negative normals.

K43: K. pneumoniae Prot: P. mirabilis
Yers: Y. enterocolitica Salm: S. typhimurium

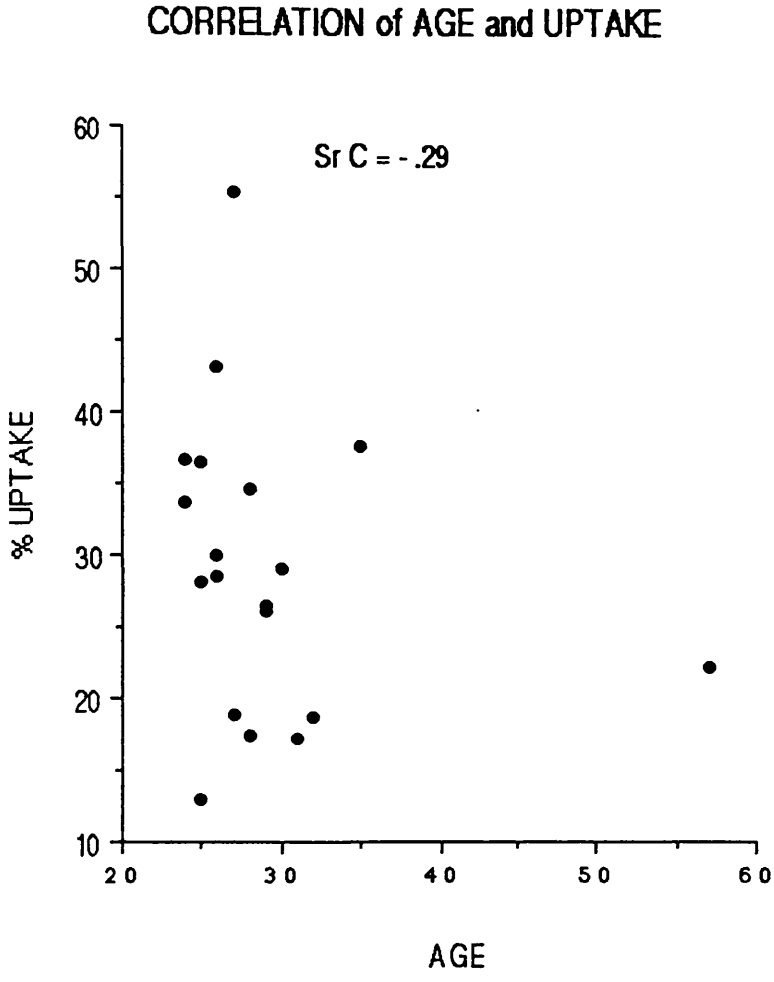


Fig XXXV Correlation of phagocytic uptake and age in 19 normals.

Spearman rank coefficient (SrC) = -.29

Significance level P = .21

5.10 CONCLUSIONS

Despite an initial enhancement of the phagocytic uptake of K. pneumoniae by AS PMNs being shown, this was not confirmed as experimental numbers were increased. This may be a consequence of samples not being assayed simultaneously in the first series of assays. Subsequently both patient and normal samples were examined together to eliminate any interassay variables. As with K. pneumoniae, no difference was shown in the phagocytic uptake pf P. mirabilis, Y. enterocolitica and S. typhimurium between AS patient and normal PMNs. Also no defect was shown in the ability of AS serum to opsonise bacteria in preparation for phagocytosis. Finally HLA-B27 positive samples also showed no defect in phagocytic capacity. Therefore although significant CL activity was shown in AS samples compared to normals in chapter 4, it would appear that this is not indicative of the phagocytic ability of AS PMNs. It may be that the use of purified PMNs in these assays may mask any defect due to pre-activation of PMNs. However defective phagocytic ability has been shown in the PMNs of rheumatoid arthritis patients, using the same radiometric assay (Frame, Ph.D, Thesis 1989). This will be discussed further in chapter 6.

Ankylosing spondylitis is a seronegative inflammatory disorder where more than 90% of patients possess the histocompatability antigen HLA-B27. Although this association is very striking it is not absolute, and studies have suggested that either the B27 antigen in individuals with spondylitis is different from that in individuals without disease, or that an additional factor(s) is also present in the spondylitic families.

Bacterial triggers are thought to play an important role in the aetiopathogenesis of the disease and serological cross-reactivity has been shown between a determinant on HLA-B27 positive spondylitic cells and certain gut bacteria.

One of the principle functions of neutrophils and mononuclear phagocytes, which migrate into the tissues during the inflammatory response, is the internalisation of material by the process of phagocytosis. This process acts as a first line in host defence mechanisms against invasion by microorganisms. Polymorphonuclear leucocytes are the most numerous of phagocytes in the blood and are easy to obtain in high yield and purity. They also appear to be homogeneous, i.e. the majority behave alike under stimulation, rendering them most suitable for *in vitro* study.

Hence this thesis investigated the function of PMNs in ankylosing spondylitis by examining three different aspects of PMN behaviour.

i) Polymorph migration, both random and directed.

ii) Chemiluminescence response to bacterial stimulants.

iii) Ingestion of bacteria.

The results of each aspect of PMN functions are discussed in the same order of presentation throughout this thesis.

Chemotaxis

Polymorphonuclear leucocytes move either randomly, or directionally in response to a chemotactic stimulus, towards a site of injury or imflammation. This activity can be studied *in vitro* by migration under agarose (Nelson *et al*, 1975), or by migration through a polycarbonate membrane filter (Falk *et al*, 1980). The latter has been used by means of a 48-well micro-chemotaxis chamber assembly, a modification of the Boyden chamber. (See chapter 2.).

This method had been previously standardised in our laboratory, and has several advantages over other chemotaxis methods, these include,

i) Reduced time to perform the assay.

ii) Volume of chemotactic factor required is small.

iii) Number of cells needed is low.

iv) Larger numbers of samples may be tested in parallel.

Results show a slight increase in random motility in AS patients compared to normals, but this increase was not statistically different, (Fig X). Pease et al, 1984 used both an agarose plate method and micropore filter method, and found that random motility varies with different techniques.

An increased directional chemotactic response to zymosam-activated serum was noted in our study in AS PMNs compared to normal PMNs. but again this was not statistically significant (Fig XIII). This confirms the results of Mowat, 1978 who also detected no significant differences in motility between AS and normal PMNs, using a filter technique. In contrast to this Pease et al, 1984 and 1989, showed a statistically significant increase in the directional motility of AS PMNs compared to normal PMNs, by means of both filter and agarose plate technique run in parallel.

In results from subsequent work in CL response we showed that normal PMNs were pre-activated during our separation procedure, thus possibly causing any inherent differences between AS and normal PMNs to be masked. In our chemotaxis studies dextran sedimentation was used to isolate PMNs, whereas Pease et al used a one-step density gradient technique which avoids the use of dextran sedimentation and osmotic shock (Ferrante and Thong, 1978), thus perhaps avoiding any basal activation. This may account for differences between the two studies.

However this one-step gradient technique fails to completely purify PMNs from the mononuclear fraction, hence making recovery of pure fractions difficult. Pease et al failed to mention cell purity in their work.

Recently, a modification of the Ferrante and Thong separation technique has been developed in the Department of Medicine, which provides an improved separation of pure sub-fractions. (Lennie et al. 1987). Parallel investigations into different separation techniques and subsequent examination of PMN migration may thus prove useful in future research.

It has been suggested that chemotactic ability may be determined by the presence of the HLA-B27 haplotype (Leirsalo et al, 1980). A study was therefore undertaken to compare B27 positive and B27 negative normal individuals. No difference in PMN migration was observed between these two groups (Fig XII). This was supported by Pease et al who did not find any difference between B27 positive and negative controls. A recent report from Finland suggests that enhancing migratory capacity of HLA-B27 positive sera, decribed by Leirsalo et al, 1980, is not genetically determined, but is acquired secondarily, since the chemokinetic activity of sera from B27 positive children was found to be similar to that of B27 negative children; but in adults migration distances were greater in B27 positive individuals, although not in a statistically different way. (Repo et al. 1988).

Although most groups have used either zymosan-activated serum or

casein chemotaxis, it would seem more appropriate to use bacteria or bacterial products as chemotaxins. We therefore tested several gram-negative bacteria in this respect. Neither formalin-treated nor sonicated bacteria stimulated chemotaxis, and similarly whole bacteria showed no chemotactic qualities. However bacterial culture filtrates did produce a chemotactic gradient, indicating the possible production of a chemotactic substance during the logarithmic growth phase of a bacterium. The chemotactic abilities of culture filtrates of Y. enterocolitica and S. typhimurium were no different from MH broth, the preparation medium. However K. pneumoniae culture filtrate did show a higher chemotactic response than MH broth, suggesting that some product of this bacterium was behaving as a chemotaxin for PMNs from both normal and AS patients. No difference in motility was apparent between normal and AS PMNs, irrespective of the culture filtrate used (Fig XI).

Finally, no correlation was shown between directional PMN motility in AS patients and disease activity (Fig XV), as was similarly reported by Pease et al 1984. Nor was any correlation determined between PMN motility and age (Fig XIV), which has not been previously reported.

Chemiluminescence response

As a result of stimulation of the phagocytic membrane during phagocytosis of micro-organisms, there is an abrupt increase in oxygen consumption followed by the production of various reactive oxygen intermediates. There is also an increase in the oxidation of glucose via the hexose monophosphate shunt pathway. These changes in

via the hexose monophosphate shunt pathway. These changes in oxidative metabolism are collectively known as the 'respiratory burst', and can be measured by oxygen consumption and oxidising radical production.

Purified cell preparations are usually used in chemiluminescent studies, so the intial experiments carried out in this work were done using purified PMNs, and I found no difference in CL response between AS patients and normals, when PMNs were stimulated with opsonised bacteria. An alternative technique was therefore examined to confirm these findings. A CL emission may also be detected using whole blood in microlitre quantities, and an assay was developed by Kato et al, 1981, and Herberer et al, 1982 which gave a rapid evaluation of phagocytosis-induced metabolic respiratory burst which would reflect phagocytic functions in vivo.

A series of assays was therefore run in parallel, examining CL response of AS patients and normals to bacterial stimulation using both purified PMNs, separated by dextran sedimentation, and whole blood PMNs. Results again showed no difference when purified PMNs were used. However a statistically significant difference was shown when whole blood was used from the same samples, i.e. ankylosing spondylitis patients showed a significantly increased CL response to stimulation from a series of gram-negative bacteria. This inferred that something had been happening during the separation procedure, therefore basal CL response was examined, i.e. CL response emitted from PMNs to which no stimulators had been added. A significant difference was shown between whole blood PMNs from AS patients

compared to normals, whereas when purified PMNs were used no difference was detected. This increased basal CL response may reflect an *in vivo* pre-activation of AS whole blood PMNs, a theory supported by Brinch *et al.*, 1982, who showed the complement system in AS patients to be activated. Our separation technique therefore appeared to preactivate normal control PMNs, thus eliminating any difference in CL response. This pre-activation of PMNs has also been shown to occur in patients with a current bacterial infection, (Barbour *et al*, 1980, Kohashi *et al*, 1987), compared to normals, or to patients with a viral infection.

Tatsuhito *et al*, 1983 also showed a basal or background CL to be *elevated* in patients with bacterial infection. Interestingly Mowat and Baum (1971) showed an *impairment* in CX in these patients, suggesting a prior phagocytosis of antibody - antigen complexes by PMNs.

Consequently all our subsequent assays were performed using a whole blood assay and the CL response of PMNs from AS patients, to various stimuli, was compared to normals. Ankylosing spondylitis patients PMNs showed a significantly increased CL response to several arthritogenic gram-negaive bacteria, and also to *P. mirabilis,* a gut bacterium not normally associated with AS. This may indicate a pre-disposition of AS PMNs to these bacteria, which could be explained by an increase of bowel permeability in AS patients (Smith, Gibson and Brooks, 1985).

This phenomenon was shown not to be specific to all chronic inflammatory disorders, since PMNs from rheumatoid arthritis patients showed neither any increased basal CL response nor any increased CL

response to gram-negative bacteria. Stimulation with the gram-positive bacterium S. aureus however produced a significantly enhanced CL response from patients with rheumatoid arthritis compared to normals. This is not wholly surprising in view of there being an increased incidence of S. aureus infection in rheumatoid arthritis patients. Ankylosing spondylitis PMNs also showed an increased CL response when stimulated with S. aureus, indicating that gram-negative bacterial triggers, in vivo, may contribute to an enhanced CL response in AS patients, which is non-specific.

These were in contrast to results by El Abbouyi et al (1988) who found a reduced CL response in AS PMNs compared to controls, when opsonised zymosan was used as a stimulant. However they examined only 12 patients and used separated PMNs in their study.

When unstimulated PMNs from HLA-B27 positive and B27 negative normals were examined, followed by stimulation with K. pneumoniae, Y enterocolitica and finally with PMA, B27 positive normal PMNs showed a slightly increased CL response, but this was not statistically significant. This suggests that enhanced activation develops with disease, rather than being associated with HLA status.

Phagocytes possess externally disposed plasma membrane receptors for many substances, and the binding of agonists to these receptors is the initial step in the series of actions that result in chemotaxis, phagocytosis and activated oxygen radical production. It is possible that this enhancement of CL response in AS PMNs may be due to an abnormality of membrane receptors, located on the cell membrane. A

abnormality of membrane receptors, located on the cell membrane. A clue to the involvement of HLA-B27 may lie in its possible role as a receptor for some environmental agent. However since an increased CL response was also found in AS PMNs when PMA was used as an activator, this abnormality may also be due to extrinsic factors, as PMA does not act through specific cell surface receptors, but reacts directly with Protein Kinase C at a point distal to the cell surface via a different activation pathway, (Nishihira and O'Flaherty, 1985).

Future research might include incubation of AS PMNs in normal serum and vice versa before examination of CL response, to see whether or not this abnormality shown may be due to either, a modification of membrane receptors or to a disorder in the transduction of the activating signal.

Phagocytic Uptake

Phagocytosis is the act of internalisation and the subsequent sequestration, into phagosomes,of extracellular particulate matter.

In vitro cell assay systems were utilised throughout this study in the assessment of phagocytic uptake of radio-labelled bacteria by both AS and normal PMNs. Advantages over the in vivo methods include:

1. Elimination of some of the clinical and immunological variables inherent in patient-orientated studies.

2. Known homogenous granulocyte cell populations can be used; and

3. The process of phagocytosis and intra-cellular killing can be assessed independently of each other.

This work measured only phagocytic uptake, although current work in this department is now underway, examining the killing ability of PMNs from various patient groups.

This study clearly showed that phagocytic uptake required that bacteria were opsonised with complement-mediated serum (chapter 5.3), supporting the theory that functional properties of the cleavage products of complement activation are more important than the actual bacteriolytic reactions, in host defence against infections (Upjohn, 1975).

No correlation was found between age and phagocytic ingestion, confirming a large study which revealed that PMNs from older subjects were no less efficient phagocytes than controls (M. Frame, Phd thesis, 1989).

A total of 58 AS patients were examined for phagocytic uptake of various gram-negative bacteria; neither an enhancement nor a decrease in the phagocytic ability of AS PMNs was shown, compared with normals. This may be due to the use of purified PMNs in these assays, concealing any inherent defect, as a result of pre-activation of the PMNs, determined in our subsequent studies in chemiluminescence response.

Other studies, which utilised separation of PMNs by dextran sedimentation, attested that PMNs from rheumatoid arthritis patients revealed intrinsic cellular defects including impaired phagocytic ingestion, compared to normals, using a radiometric assay, (M. Frame Phd thesis, 1989). However, preactivation of PMNs would be unlikely to mask any visible inherent defect in rheumatoid patients, as it does enhancement of activity in AS patients.

No abnormality was found in the opsonic ability of AS serum (Fig XXXIII). Polymorphonuclear leucoytes from HLA-B27 positive and negative normals were also compared for phagocytic uptake efficiency, and again no difference was determined (Fig XXXIV).

No other large study has examined phagocytic uptake in ankylosing spondylitis patients.

An abnormality in phagocytic function may have been expected due to the pre-activation of AS PMNs shown in the CL study. Having established this pre-activation it might be appropriate to repeat some uptake work, using either a different PMN separation procedure, or attempting to modify the radio-metric assay to use whole blood fractions, thus preventing any in vitro stimulation of normal PMNs.

Ankylosing spondylitis affects predominantly the sacroiliac joints and the spine, although other joints may be involved in up to 35% of patients, mainly hips or shoulders. Spread of the disease to the more peripheral joints e.g. the knees, occurs much less frequently. Therefore data on synovial fluid from AS patients is difficult to

obtain. Kendall et al, 1973 found the lymphocyte count and immunoglobulin levels were significantly higher in AS patients compared to a group with rheumatoid disease and to a second group with osteoarthritis. Current work is being undertaken in this laboratory, examining the CL response of synovial fluid PMNs from AS patients, compared to Rheumatoid patients and to another group of sero-negative patients e.g. psoriatic arthritis, reactive arthritis, to various stimuli. Initial results indicate no significant difference in CL response between AS synovial fluid PMNs and PMNs from either of the other two groups. An increase in the lymphocytes however was determined in the differential white cell counts in AS synovial fluid, as indicated by Kendal et al, 1973, compared to the white cell counts in the rheumatoid patient group, but numbers are too small to determine whether or not this increase is statistically different. These initial results suggest a vigorous lymphocyte response at sites of inflammation, which could in turn reflect what is occurring in the sacroiliac joints in AS patients.

In summary this thesis has shown that an initial membrane excitation of PMNs from AS patients is significantly greater than from normal PMNs, following bacterial stimulation. In contrast, PMNs from rheumatoid arthritis patients showed no abnormal reactions when stimulated with gram-negative bacteria, although S. aureus did produce an increased CL response, due probably to this bacteria being a common cause of minor infections in these patients. This may indicate some gut-inflammation in AS patients, rendering them more susceptible to invasion by gram-negative bacteria associated with the gut, thus

activating host defence mechanisms and producing pre-activation of PMNs.

Granfors et al, 1989 demonstrated that microbial antigens can be found in the synovial fluid cells of patients with reactive arthritis after yersinia infection and suggested that as bacterial polysaccharides are relatively resistant to digestion, they may persist within phagocytic cells for long periods. Therefore studies in synovial fluids from AS patients should be part of a subsequent research project, where PMNs could be examined for the presence of bacterial antigens.

This thesis supports a role for gram-negative bacteria in the aetiopathogenesis of ankylosing spondylitis, but as yet this role is undetermined.

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