ANTIBACTERIAL HOST DEFENCE MECHANISMS IN RHEUMATOID ARTHRITIS

by

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Dedicated with love and respect to my parents: Elizabeth and James Frame

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SUMMARY

The present in vitro studies employed a radioassay, incorporating tritiated [^{3}H] thymidine, to measure phagocytic uptake of Staphylococcus aureus (S. aureus) by polymorphonuclear leucocytes (PMNs) of patients with rheumatoid arthritis (RA) and healthy controls.

An intrinsic defect was detected in the phagocytic capacity of peripheral blood (PB) PMNs from patients with RA. Serum from RA patients and normals had a similar inhibitory effect on PMN phagocytosis. However, synovial fluid (SF) from RA patients was found to impair phagocytic ability by acting directly on the PMN. Furthermore, these SF significantly impaired PMN uptake as compared to SF from patients with various other arthritides. No correlation was found between defective phagocytosis and age, disease activity, drug therapy, the amount of circulating immune complexes (ICs) or rheumatoid factor (RF). Also, the lack of correlation between clinical parameters such as titres of RF and complement components, suggests factors other than ICs may be involved in this inhibitory activity.

Complement levels and heat-labile opsonic activity were lower in SF from RA patients than SF from other forms of arthritis or normal serum. Nevertheless, none of the RA sera or SF tested lacked opsonic activity.

Interleukin 1 (IL-1) production by monocytes of RA patients and normal subjects were studied both spontaneously and after lipopolysaccharide (LPS) and S. aureus stimulation. Interleukin 1 activity was measured using the LBRM TG-6/HT2 bioassay system. Rheumatoid arthritis monocytes spontaneously produced more IL-1 than the controls and this difference was maintained after

stimulation. When paired PB and SF monocytes were compared, SF cells produced less IL-1 than corresponding PB monocytes, but more than normal PB monocytes. This was similarly observed after stimulation. These findings suggest that this line of host defence is not impaired in RA patients. However, a high concentration of IL-1 may be detrimental to the PMNs migrating into the SF from the PB.

Hydrocortisone sodium phosphate was found to inhibit IL-1 production. This is not true for the other anti-rheumatic drugs studied.

Thus, the findings of these studies suggest that the increased incidence of bacterial infection in RA patients is the consequence of impaired PMN phagocytosis. The mechanism underlying this defect could be intrinsic to the cell, and amplified by extracellular inhibitory factors present in SF, as yet to be characterised.

Abbreviations

Ab	antibody
Ag	antigen
APP	acute phase protein
APR	acute phase response
5-ASA	5-aminosalicylic acid
c.f.u.	colony forming units
CO_2	carbon dioxide
CRP	C-reactive protein
DNA	deoxyribonucleic acid
EP	endogenous pyrogen
ESR	erythrocyte sedimentation rate
FCS	fetal calf serum
GH	gel-hanks (hanks' balanced salt solution containing 0.1% gelatin)
$[^3H]$	tritiated
HBSS	hanks' balanced salt solution
HCL	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid
HLA	human leucocyte histocompatibility antigen
H_2O_2	hydrogen peroxide
ICs	immune complexes
Ig	immunoglobulin
IL-1	interleukin 1
IU	international units
K/EDTA	ethylenediaminetetra-acetic acid di-potassium salt
LAF	lymphocyte activating factor
LPS	lipopolysaccharide
MCP	metacarpophalangeal

MPO	myeloperoxidase
MTP	metacarpophalangeal
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NH_4Cl	ammonium chloride
NSAIDs	non-steroidal anti-inflammatory drugs
$^1O_2$	singlet oxygen
O_2^-	superoxide anion
OD	optical density
OpD	opsonic differential
OH^-	hydroxyl radical
P	probability
PB	peripheral blood
PBS	phosphate buffered saline
PD	phagocytic differential
PEG	polyethylene glycol
PG	prostaglandin
PHA	phytohaemagglutinin
PI	phagocytic index
PIP	proximal interphalangeal
P. mirabilis	Proteus mirabilis
PMNs	polymorphonuclear leucocytes
r	recombinant
RA	rheumatoid arthritis
RF	rheumatoid factor
RIA	radioimmunoassay
RNA	ribonucleic acid

r_s	spearman rank correlation coefficient
SASP	sulphasalazine
S. aureus	Staphylococcus aureus
SF	synovial fluid
SOD	superoxide dismutase
SP	sulphapyridine
TNF	tumour necrosis factor
Tris	tris (hydroxymethyl) aminomethane

OBJECTIVES

1. To investigate the possible intrinsic phagocytic defect in peripheral blood polymorphonuclear leucocytes from patients with rheumatoid arthritis.

2. To investigate the effect of serum upon phagocytic uptake by normal and rheumatoid arthritis peripheral blood polymorphonuclear leucocytes.

3. To investigate the effect of synovial fluid, from patient with and without rheumatoid arthritis, upon phagocytic uptake by normal peripheral blood polymorphonuclear leucocytes.

4. To investigate the opsonic activity of serum and synovial fluid from patient with rheumatoid arthritis.

5. To establish optimum in vitro conditions for production of interleukin 1 supernantants, to be used as a routine procedure in the laboratory.

6. To study interleukin 1 production by peripheral blood and synovial fluid monocytes from patients with rheumatoid arthritis, both spontaneously and in response to stimulation by different stimuli; lipopolysaccharide (as soluble stimulus) and Staphylococcus aureus (as particulate stimulus).

7. To investigate the effect of anti-rheumatic drugs on polymorphonuclear leucocyte phagocytosis and monocyte interleukin 1 production.

8. To relate host susceptibility to infection to the presence or absence of cellular dysfunction in vitro.

CHAPTER ONE - GENERAL INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

1.1.1 Historical and clinical aspects

The term 'rheumatoid arthritis' was coined by Sir Alfred Baring Garrod in 1876. One of the most intriguing aspects of the disease is its apparent scarcity prior to the 18th century, in contrast to osteoarthritis, gout, and infectious arthritis, which are diseases of antiquity. Because of the lack of convincing historical evidence, Boyle and Buchanan (1971) postulated that RA is a disease of the modern era. The first convincing description of RA is that of Landre-Beauvais in 1800 (Parish, 1963).

Rheumatoid Arthritis is a chronic systemic disease manifested most prominently by an inflammatory, symmetric polyarthritis but it can begin in a single joint (monoarticular RA). The onset is generally between 20 and 60 years of age, with peaks in the 5th and 6th decades of life. It shows a predilection to involve the proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, shoulder, ankle, knee, hip, metatarsophalangeal (MTP) and tempromandibular joints (Fig I). Also involvement of the cervical spine is more common than generally recognised (Martel, 1977). The most frequent clinical manifestation of cervical spine involvement is painful limitation of neck movement.

The principal extra-articular manifestation is subcutaneous nodules. These nodules tend to occur over traumatised areas such as elbows, extensor surface of arms, knees, knuckles, heels, bridge of nose and buttocks. Approximately 20% to 25% of all RA patients manifest such nodules eventually. They are

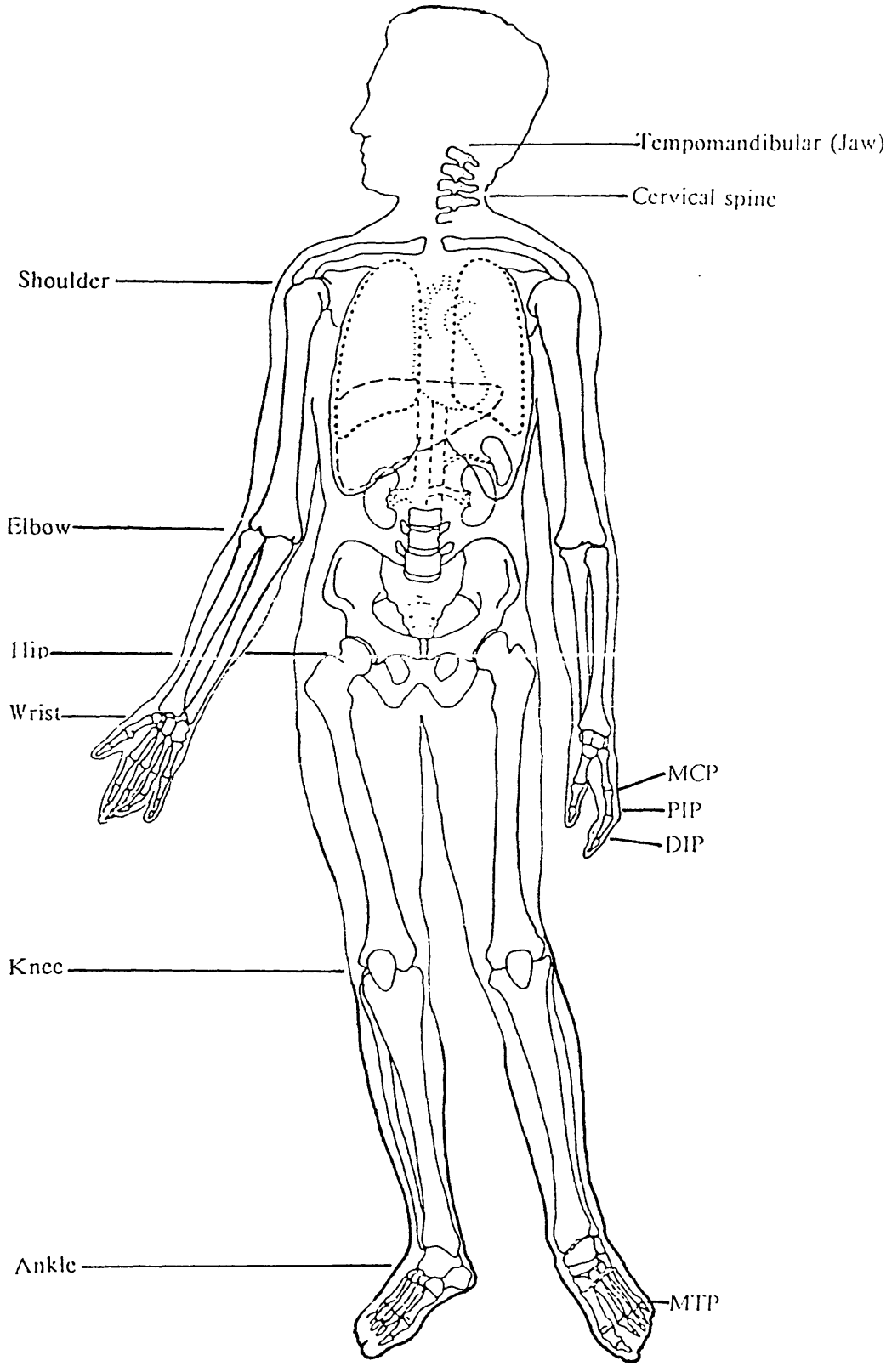


Fig. 1 Rheumatoid arthritis - distribution of target joints.

generally firm, non-tender, mobile and may spontaneously vary in size. Other manifestations, such as vasculitis, scleritis and neuropathy do occur, although less frequently than the aforementioned nodules.

In the majority of patients, the disease starts gradually and insidiously. However in a few it can be acute. The early symptoms include malaise, tiredness, weight loss, fever, vague pain and morning stiffness. These tend to abate after the patient has 'limbered up', but as the disease advances they tend to become progressively more severe and persistent. Swelling of the involved joints is prominent, accompanied by limitation of movement and, consequently, a diffuse wasting of adjacent muscles. This ultimately results in total loss of joint function.

Over 80% of patients follow a characteristic course of remissions and exacerbations, with 20% having a complete remission following the initial episode with neither recurrent nor residual inflammation.

Rheumatoid arthritis is divided into two categories; seropositive and seronegative disease, depending on the presence or absence of RFs respectively, in the patients' serum. These RFs are antibodies of immunoglobulin G (IgG), IgA and IgM classes directed against native IgG as the autoantigen. In the early stages of RA many patients are classified as seronegative but convert to seropositive with time, others convert, either spontaneously or while receiving remissive agents, from positive to negative.

1.1.2 Incidence and prognosis

Rheumatoid Arthritis is relatively common in Western communities. The prevalence of RA in the adult population is approximately 1%, females being affected more frequently than males, in a ratio of 3 to 1. No clear explanation for the apparent female preponderance is available.

An estimated 30% of patients do not consult a physician due to the mild nature of the disease. On the other hand, chronic progressive RA has long been felt to be associated with a reduced life expectancy. Debility and increased susceptibility to infection probably play some role. Those who are female and young at the time of an insidious onset have a less favourable prognosis than an older male with an acute onset. Patients who live within the limitations of their disease and who persist in active range-of-motion exercises along with a positive attitude appear to do better than those who refuse to help themselves.

1.1.3 HLA and disease association

The principal known immunogenetic association with RA is the class II human leucocyte histocompatability antigen, HLA-Dw4, as determined by the mixed lymphocyte reaction or the related HLA-DR4 allotype on lymphocytes (Panayi, Wooley and Batchelor, 1979). HLA-Dw14 is also associated with susceptibility to RA (Nepom et al., 1987). Investigations have shown that 70% of patients with RA possess the HLA-DR4 locus and calculations indicate that an individual with the HLA antigen is six to twelve times more likely to develop the disease than one who is negative (Panayi and Chapel, 1987). The specificity

of this locus for RA is by no means as strong as the HLA-B27 locus for ankylosing spondylitis.

It is possible that this HLA-DR4 antigen is a marker for genes which regulate either the development of autoimmunity to such self components as IgG or IgM, or an abnormal host response to an environmental agent. It is worth noting that although some 25% of Caucasians are HLA-DR4 positive, only some 5% at the most of such populations have RA (Panayi and Chapel, 1987), so clearly other factors are involved in causing the disease.

Moreover, 30% to 40% of patients with RA are negative for DR4, depending on the population studied, and in addition Jewish and Asian patients with RA show an association not with DRw4, but with DR1 (Grennan et al., 1983). In the West of Scotland as much as 35% of the population are HLA-DR4 positive (Dick, personal communication).

1.1.4 Theories of aetiopathogenesis

Despite intensive clinical and experimental research, the aetiology of RA has eluded discovery and remains a challenging mystery. The literature on the subject is extensive and no attempt is made here to cover even the tip of this iceberg. Only a short review is given here; more detailed accounts can be found in a recent review (Phillips, 1986).

From experimental models in animals two main hypotheses have been proposed which may not necessarily be mutually exclusive. The first proposes that RA is

caused by infectious agents such as bacteria, mycoplasma or viruses. The second proposes that RA is an autoimmune disease.

The former theory has led to several studies which implicate various organisms in the pathogenesis of RA (Phillips, 1986). Unfortunately, attempts at isolating live bacteria have not yielded consistent or reproducible results. The emphasis then switched to an attempt to detect bacterial cell wall antigens. The idea that bacterial debris can be phagocytosed by macrophages but not degraded, thus allowing it to persist as a chronic irritant has been proposed by Bennett (1978). Ebringer and his associates in 1985, implicated Proteus mirabilis (P. mirabilis) as a causative agent after detecting antibodies (Abs) to these organisms in RA patient sera.

There has been a long-standing fascination with mycoplasmas as a cause of RA, since these organisms can also produce, in a range of animals, an experimental arthritis which bears a striking resemblance to RA in humans (Cole and Cassell, 1979). Infection with a mycoplasma might initiate an inflammatory response that is enhanced by the mitogenic activity of the organism. Cartilaginous and tissue damage ensues, leaving the joint more susceptible to host attack. The mycoplasma may then incorporate the host's cellular antigens (Ags), thereby stimulating an autoimmune reaction that leads to chronic arthritis. Supporting the notion of their involvement in human arthritis, mycoplasmas have been isolated from human synovial fluid with increasing frequency (Taylor-Robinson et al., 1978), primarily as a result of improved culture techniques. There is also the possibility that these could be contaminants.

The past decade has seen a heightened awareness of a possible viral aetiology because of the interesting association of RA nuclear antigen. This is an Ag

induced in lymphoblastoid cell lines infected with Epstein-Barr virus. Serologic studies, confirmed that Ab to this Ag was found in 70% to 95% of RA patients compared with less than 20% of various control groups (Ferrell et al., 1981). Rubella virus has been a perennial aetiologic candidate in RA, but recent, like earlier studies, have yielded conflicting results (Norval, Hart and Marmion, 1979; Ford et al., 1982). Outbreaks of epidemic polyarthritis following infection with the Ross River virus, an arbovirus, have been well documented (Bennett, 1983). Hurst et al. (1983) described three patients who developed polyarthritis after an acute febrile illness with definite Coxsackie B virus infection. Cytomegalovirus has been detected in cells cultured from rheumatoid synovial membrane (Goodacre and Dick, 1984) and more recently parvovirus has attracted attention (White et al., 1985).

To date, no virus has been proven to cause RA, but because of the capacity of viruses to alter immune responses, to produce experimental arthritis, and in certain known naturally occurring viral infections of man to mimic the clinical and pathologic features found in RA, they continue to be principal candidates for the initiation or propagation of the disease.

The second hypothesis proposes that the disease is the expression of disordered immunity leading to an autoimmune attack on the body's own constituents. One possibility is autoimmunity to IgG, which would explain the production of RF. In 70% of patients RF can be demonstrated by serological means and IgM, IgA and more recently IgE (Zuraw et al., 1981) have been detected in RA patient's sera. Rheumatoid factor and the ICs generated from them, may explain the ongoing inflammatory and erosive disease, but not necessarily its onset; that is, these immune responses could be a secondary phenomenon, the result, rather than the cause, of RA.

In normal individuals the synthesis of RF is tightly regulated. Removal of a stimulus results in decreased synthesis, whereas in RA RF is produced in excessive amounts, which appears to represent a fundamental breakdown in immunoregulation. It is also apparent that IgG can self-associate, forming complexes with other IgG molecules that may become large enough to be phagocytosed and to activate the classical complement pathway (Brown, Nardella and Mannik, 1982) (Fig II).

Polymorphonuclear neutrophils, although abundant in SF, are not found in the synovial membrane: characteristic inflammatory cells in the membrane are T and B lymphocytes, plasma cells and macrophages. Phagocytosis by PMNs generates many inflammatory mediators, such as hydrolytic enzymes (elastase and collagenase) which bind to proteinase inhibitors. Under certain circumstances these inhibitors are probably saturated or inactivated, and as a result the proteinases can act without inhibition on substrates within the joint cavity, including cartilage, bone, ligaments and tendons resulting in the characteristic joint deformities of RA.

Presently, there is much debate concerning an inherent defect of the lymphocyte in RA (Goodacre and Dick, 1984).

An attractive alternative hypothesis has recently come to light to explain the autoimmune features and hence the chronicity of RA - the constant or recurring presence of many heterogeneous common agents as triggers, making the search for a single aetiologic agent unsuccessful.

Studies of various environmental factors as possible aetiologic agents include; nutrition (Ziff, 1983), silica (Klockars et al., 1987), asbestos (Turner-Warwick

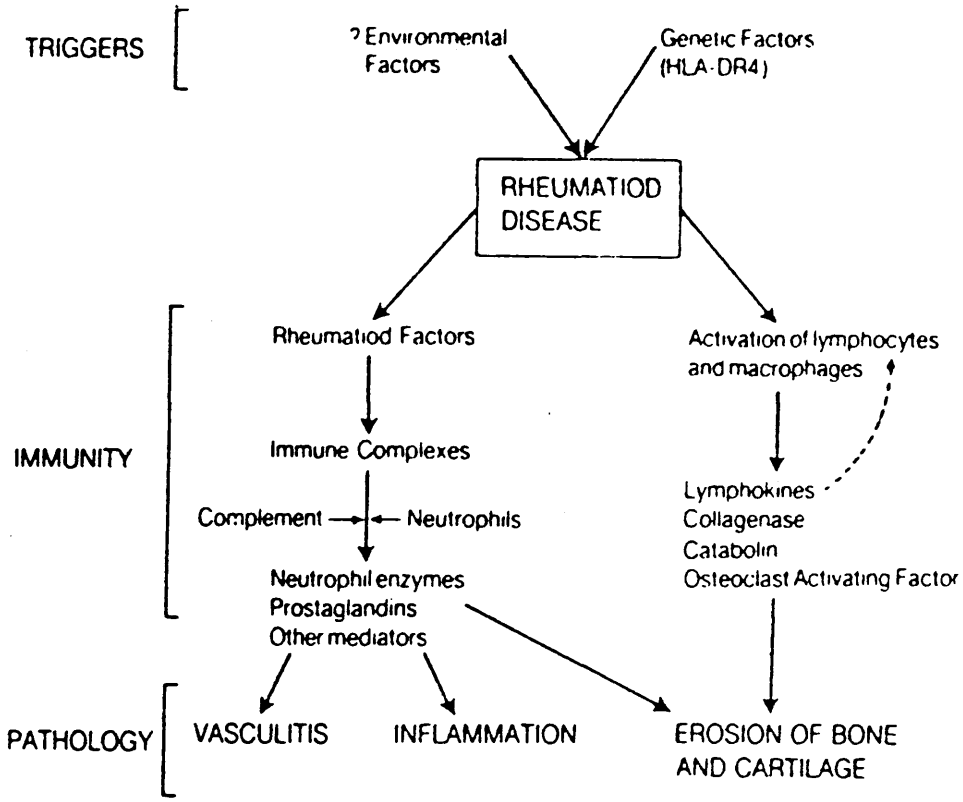


Fig. II A schematic representation of the major events thought to be involved in the immunopathogenesis of rheumatoid arthritis (Panayi and Chapel, 1987).

and Parkes, 1970), smoking (Vessey, Villard-MacKintosh and Yeates, 1987), and climate (Hollander, 1961). There seems to be a high incidence of classic, seropositive erosive RA in male granite workers following silica or asbestos exposure, however the association of RA with nutrition is less definite. As recently as 1987, Vessey and his co-workers reported a strong association between referral to hospital for RA and cigarette smoking; the rate in women non-smokers was 0.27 per 1000 woman-years and in those smoking 15 or more cigarettes per day was 0.64 per 1000 woman-years. Interestingly, surveys have revealed that 2.5% of the adult population in temperate countries suffer from this disease, compared to 1% in the western world. Furthermore, Hollander (1961) observed that arthritis often worsened within a few hours of the onset of a combined rise in humidity and fall in barometric pressure. Such patients appear to be 'weather sensitive'. No pathogenetic mechanism has been proposed to explain this phenomenon.

It was once thought that the anxiety-ridden and depressed person who tends to suppress feelings of hostility and aggression seemed more prone to develop RA than the average, while the psychotic individual seemed less prone. However, the current consensus is that these psychological traits are an understandable reaction to the disease rather than a causative factor. Nevertheless in a given patient, emotional stress is often followed by an exacerbation of rheumatoid activity.

The final trimester of pregnancy is associated with disease remission, which frequently relapses after delivery (Persellin, 1977). This may be explained by the large rise in female sex hormone concentration and the subsequent fall postnatally. Wingrave and Kay (1978) hypothesize that exogenous sex hormones, specifically the oral contraceptive pill, protect against the development of RA.

The association with sex hormones is of interest given that one of the most consistent aspects of the epidemiology of RA is the 2 to 3-fold female predominance. Alternatively, the link between RA and pregnancy may be immunologically mediated by fetal stimulation of the maternal immune system, which might occur progressively with repeated pregnancies and thus result in the production of autoantibodies.

In summary, the causative agent of RA is unknown. However, in the genetically predisposed individual, multiple factors, host and environmental, may have a profound influence on the expression of the disease.

1.1.5 Diagnosis and treatment

There is, as yet, no single clinical or laboratory feature to identify RA; the diagnosis depends upon the presence of a composite of clinical articular and non-articular signs and symptoms, accompanied by radiological, histological and serological abnormalities. Some parameters commonly measured include the erythrocyte sedimentation rate (ESR), which is frequently elevated as are the acute phase reactants, fibronectin and C-reactive protein (CRP). Rheumatoid factor is measured by serological means, such as latex and sheep cell agglutinin test. The RF titre usually reflects the severity of the disease. Analysis of SF shows it to be turbid with excessive numbers of PMNs, low glucose concentration and less viscous than normal.

There is no specific cure for RA , however much can be achieved therapeutically for rheumatoid patients, provided the treatment is tailored to meet the specific needs of each individual. The aims of treatment (Table 1.1) as

1. To help the patient understand the nature of the disease.
2. To provide psychological support.
3. To alleviate pain.
4. To suppress the inflammatory reaction.
5. To maintain joint function and prevent deformity.
6. To correct existing deformity.
7. To improve function.
8. To rehabilitate the individual patient.

Table 1.1 Aims of treatment of RA.

well as the available methods of treatment (Table 1.2) must be considered in planning a program. The goal of therapy in RA is to prevent disability.

Since the cause of the disease remains unknown, therapy has been directed at nonspecific suppression of the inflammatory process in the hope not only of alleviating symptoms, but also of preventing progressive damage to articular structures.

The non-steroidal anti-inflammatory drugs (NSAIDs) provide the 'first-line' of treatment in RA. These give symptomatic relief only, and are thought to have no effect on the underlying disease process. They include the salicylates, for instance aspirin, which is not used very commonly in the United Kingdom because of its association with gastrointestinal irritation and bleeding. Adverse reactions are unfortunate consequences of therapy with most of the anti-rheumatic drugs. One of the most useful and effective anti-inflammatory drugs available is indomethacin.

When the disease process progresses despite NSAID therapy, 'second-line' or disease-modifying agents are implicated. These have minimal nonspecific anti-inflammatory effects, but appear to have the potential to modify the course of the disease in some patients (Wright and Amos, 1980). Their use in RA is based almost entirely on clinical experience rather than knowledge of specific drug action. None of these agents was specifically developed to treat RA, except for sulphasalazine (SASP). The use of gold compounds, for instance, originated with the observation by Koch in 1890 that gold cyanide inhibited the growth of Mycobacterium tuberculosis. Because of the belief that gold compounds possessed nonspecific antiseptic properties, they were use to treat a variety of chronic conditions in which an infectious aetiology was suspected. It was not

1. Psychological considerations.	Relationship between physician and patient developed by sympathetic understanding, free discussion of the nature of the disease, the prognosis and the proposed treatment. Also a need to have complete confidence in the physician.
2. Therapeutic agents.	Non-steroidal anti-inflammatory drugs (NSAIDs) 'First-line' treatment eg. Naproxen Indomethacin Disease-modifying drugs 'Second-line' eg. D-penicillamine Sulphasalazine Corticosteroids, 'third-line' eg. Prednisolone Immunoregulatory agents, eg. Azathioprine
3. Orthopaedic appliances.	Removable splints Remedial shoes Canes and crutches
4. Physical therapy.	
5. Orthopaedic surgical operations.	eg. Synovectomy Prosthetic joint replacement

Table 1.2 Methods of treatment for RA

until 1944 that the results of a properly controlled clinical trial were first published by Fraser in Glasgow (Sneader, 1985) and thereafter the value of sodium aurothiomate ('Myocrisin') in therapy was generally accepted.

A characteristic feature of therapy with these drugs is a delayed onset of clinical effect. Gradual suppression of the signs and symptoms of inflammation may not be apparent until months after initiation of therapy and may persist for weeks after the drug has been discontinued. This is in contrast to clinical responses to NSAIDs which, when observed, are prompt and persist only as long as the drug is continued (Simon and Mills, 1980). Chloroquine and hydroxychloroquine have become the most frequently used antimalarials in RA but they have many minor side-effects such as nausea, headache, dizziness and diarrhoea.

A newer compound, SASP, which consists of sulphapyridine (SP) and 5-aminosalicylic acid (5-ASA) is an antibacterial agent with 'second-line' effects in RA. Svartz (1942) originally formulated SASP because SP alone had been unsatisfactory. It is now the first choice, of some clinicians, when initiating 'second-line' therapy for RA. Although the aforementioned 'second-line' drugs are equally effective in the treatment of RA, this does not diminish the importance of the clinical observation that an individual patient may respond to one, but not another, and that such idiosyncratic responses are unpredictable. There is, however, some evidence that the incidence of toxic reactions differs; antimalarials having a much lower incidence of serious side effects, even after long-term administration (Richter et al., 1980), than D-penicillamine and gold salts. In another study, Situnayake, Grindulis and McConkey (1987) found severe adverse effects were far more common with gold and D-penicillamine than with SASP.

Severe unremitting polyarthritis may be an indication for 'third-line' therapy (cytotoxic agents or corticosteroids), but only after second-line drugs have failed, either due to toxicity or lack of efficacy. This category is used sparingly in view of its greater toxicity, particularly of a cumulative or long-term nature. For patients over 60 years of age and those threatened with immediate disability, the guidelines for with-holding corticosteroid therapy are less stringent. A special situation for systemic therapy is the patient with an active peptic ulcer or gastrointestinal haemorrhage who continues to have active and severe RA. Treatment of these patients always poses a major problem, since most of the medications used to treat RA have the potential for exacerbating the disease.

An additional class of therapeutic agents in RA is the immunoregulatory drugs such as Azathioprine, cyclophosphamide and methotrexate. Most physicians advocate their use late in the course of RA and, like disease-modifying drugs, they have a gradual onset of action. Although they clearly suppress active RA in 60% to 80% of patients in whom they are given, they rarely effect a true clinical remission. If these drugs are discontinued, the arthritis tends to flare again, usually within 1 to 2 months. Initiation of treatment with these agents implies there will be long-term therapy; long-term therapy implies a high risk of toxicity. The major immediate toxic side-effect is severe bone marrow suppression. Fortunately, this problem is not very common with the relatively low doses used in RA. The major long-term complications are carcinogenicity, irreversible sterility and a predisposition to serious infections. The fear of causing a potentially fatal malignancy in a patient whose disease is normally not fatal has severely curtailed the use of immunoregulatory drugs in RA except as a last resort in patients with extremely active, otherwise unresponsive disease. Combinations of immunoregulatory drugs might prove more effective and have

fewer side-effects if lower doses of each could be used. A preliminary study by McCarty and Carrera (1982) suggests that this may be the case.

1.2 SEPTIC ARTHRITIS

The observation that patients with chronic RA who had bacteraemia were susceptible to spontaneous septic arthritis was first reported by Kellgren et al. (1958). Since then more than 100 cases have been reported in the English literature (Gristina, Rovere and Shoji, 1974). Table 1.3 reviews the most frequently encountered micro-organisms reported in the literature.

Although the incidence and causative organisms remain qualitatively unchanged, increasing numbers of cases are being seen among the elderly (Cooper and Cawley, 1986). Staphylococcus aureus is by far the most common causative agent in this country and other European studies (Russell and Ansell, 1972; Lindgren and Lindberg, 1973; Newman, 1976). In a recent 30 year review by Newman (1976) Staphylococcal infections were found to have predominated throughout: but the percentage of penicillin-resistant organisms rose from 29% to 59% during the period reviewed. The American literature suggests a high prevalence of gonococcal and gram negative bacillary infections (Goldberg and Cohen, 1976; Manshady, Thomson and Weiss, 1980). Pneumococcal arthritis was a common complication of pneumonia in the preantibiotic area but it is now rare, representing less than 5% of septic arthritis (Morley, Hull and Hall, 1987).

Suppurative arthritis occurring in rheumatoid disease is relatively rare but dangerous; not only to limb but to life. At particular risk are the elderly with chronic advanced seropositive nodular disease, those on long-term systemic or intra-articular corticosteroid or other immunosuppressive therapy, and those with a primary or secondary infection. Furthermore, patients with RA may be more susceptible because of an inherent phagocytic defect (Mowat and Baum, 1971; Turner, Schumacher and Myers, 1973). The skin and subcutaneous tissues

Study	Year	Organism(s)
Kellgren et al.	1958	S. aureus Escherichia coli
Rosin and Goldberg	1962	S. aureus
Rimoin and Wennberg	1966	S. aureus
Karten	1969	S. aureus Proteus mirabilis
Russell and Ansell	1972	S. aureus
Lindgren and Lindberg	1973	S. aureus Haemophilus influenzae
Gristina et al.	1974	S. aureus Escherichia coli
Goldenberg and Cohen	1976	S. aureus
Mitchell et al.	1976	S. aureus
Newan	1976	S. aureus
Cooper and Cawley	1986	S. aureus Neisseria gonorrhoeae
Morley et al.	1987	Streptococcus pneumoniae

Table 1.3 Micro-organisms associated with septic arthritis

are the most common sites frequently infected by staphylococci. Bacteria may also directly infect a joint from a deep penetrating wound, a concurrent urinary tract infection, a bronchopulmonary infection, prosthetic joint surgery or an intra-articular steroid injection (Rimoin and Wennberg, 1966). Intravenous drug users are prone to septic arthritis; it is often caused by bacteria that do not infect healthy persons, such as Pseudomonas aeruginosa (Roca and Yoshikawa, 1979).

Sudden aggravation of the usual arthritic pain, abrupt onset of swelling, and increased joint temperature are the usual signs of sepsis (Gristina et al., 1974). This often fatal complication may be easily overlooked as the clinical features simulate those of an acute, uncomplicated rheumatoid exacerbation. In some cases malaise and fever may occur. The presence of rigors may be an important differentiating point (Kellgren et al., 1958). The most commonly affected joints are the knee and hip (Cooper and Cawley, 1986) with elbows, shoulders and wrists all being less frequently involved. Monoarticular and polyarticular involvement have been reported (Karten, 1969; Kellgren et al., 1958; Rimoin and Wennberg, 1966). However, in general, the degree of joint involvement in RA patients seems to reflect the severity of the disease. The onset of infection may be acute or insidious.

Early treatment of a septic joint is vital to prevent destruction of cartilage and other joint structures, and therefore prompt diagnosis becomes essential. Diagnosis is suspected in patients showing a deterioration in general health, significant fever (>39°C) sometimes accompanied by rigors, and leucocytosis with more than 90% PMNs, but, as Rosin and Goldberg (1962) pointed out, marked leucocytosis characteristic of a profound bacterial infection may or may not occur in the presence of concurrent RA. Most patients have an elevated

ESR, but this is not specific and therefore of no diagnostic value. Low SF glucose levels (Ward, Cohen and Bauer, 1960; Gristina et al., 1974) and high SF lactate levels (King, 1985) have been suggested as reliable indicators of sepsis, however this remains controversial. Recently, Arthur et al. (1983) were unable to corroborate the findings of Brook et al. (1978) who reported that lactic acid elevations in SF were diagnostic of infectious arthritis. Arthur and coworkers (1983) found no significant difference between RA and septic arthritis. Kellgren et al. (1958) found the presence of chills helpful in diagnosis.

As soon as septic arthritis is suspected, prompt aspiration of the inflamed joint for microscopic examination and culture of SF is imperative. Blood cultures are also important. Aerobic and anaerobic cultures should be routinely grown. Antibiotic therapy should be initiated immediately and then modified when the culture and sensitivity results are available. Since the percentage of penicillin-resistant Staphylococci have significantly increased in the past decade, Penicillinase-resistant agents, such as methicillin, should be included in the initial therapeutic program. Intensive and long-term antibiotic therapy appear essential if recurrence of infection is to be prevented. Before the advent of antibiotics, septic arthritis carried a substantial mortality. One would imagine that death from a pyogenic infection should now be preventable, however, from a review of fatalities in various studies (Table 1.4) this does not seem to be the case. The continuing mortality appears to relate to the rising age incidence and the vulnerability of the elderly, delayed diagnosis, and inadequate local treatment, despite the widespread systemic use of antibiotics. Hip infection, particularly in the elderly, has a poor prognosis.

The principles of management include specific antibiotic therapy and drainage, and joint immobilisation. There is unanimous agreement that systemic antibiotic

Study	Year	Total no. of cases	No. of deaths
Kellgren et al.	1958	13	6
Rosin and Goldberg	1962	5	2
Rimoin and Wennberg	1966	5	3
Karten	1969	5	2
Russell and Ansell	1972	28	2
Gristina et al.	1974	12	1
Mitchell et al.	1976	8	7
Newman	1976	134	1
Cooper and Cawley	1986	74	7

Table 1.4 A review of fatalities from septic arthritis

therapy should be started as soon as a tentative diagnosis of septic arthritis is made, but the use of local antibiotics is still controversial. Mitchell and his associates (1976) showed no good reason for intra-articular injections, as these antibiotics, when administered systemically, pass freely into the joints from the blood stream, easily achieving therapeutic levels. This is the view held by others (Russell and Ansell, 1972; Cooper and Cawley, 1986). However, Kellgren et al. (1958) advised local instillations of antibiotic into infected joints.

Conflicting views also exist as to whether needle aspiration or surgical drainage is the best treatment. Most rheumatologists advocate an initial trial of closed needle aspiration of all joints except hips (Goldenberg and Cohen, 1976). Ward et al. (1960) and Goldenberg et al. (1975) indicate no obvious superiority of surgical drainage over needle aspiration. Gristina and co-workers (1974) showed that needle aspiration was the least effective therapy. They recommend as the treatment of choice: systemic antibiotic therapy and immediate arthrotomy followed by through-and-through irrigation with fluid containing the appropriate antibiotics. Almost all orthopaedic surgeons still advocate surgical drainage (Anon., 1976). Nevertheless, almost all clinicians, medical or surgical, would agree that early aspiration is essential not only for diagnosis but also for treatment. If antibiotic therapy and repeated aspirations prove inadequate surgical drainage is mandatory.

1.3 NORMAL HOST DEFENCE MECHANISMS

Infections predictably occur when an unprotected individual encounters a virulent micro-organism. Normal protective mechanisms include the surface barriers - skin and mucus membranes of respiratory, gastrointestinal, and urinary tracts; leucocytes - PMNs, monocytes, lymphocytes, eosinophils, and basophils; and humoral factors, predominantly Igs and complement (Fig. III). In normal individuals, serious infections occur only rarely because these protective mechanisms are intact. In many diseases, such as leukaemiaand hepatitis, and also as a side effect of many frequently used cytotoxic therapies, host defences are abnormal or depressed and infections occur with increased frequency and severity.

1.3.1 Skin

Normal skin is very resistant to infection by the micro-organisms to which it is constantly exposed. The dryness of the skin, the acid pH of its surface, and certain antibacterial properties of some long-chain fatty acids secreted by the sebaceous glands are thought to be important host defence factors. When the skin is excessively moist, superficial infections often occur. More frequently, infections occur because the horny cutaneous coat is compromised by abrasions, burns, implantation of foreign material, or insertion of needles.

1.3.2 Respiratory and gastrointestinal tracts

Although constantly bathed by micro-organisms, the mucosal surfaces form a very effective barrier against microbial invasion. On the other hand it is

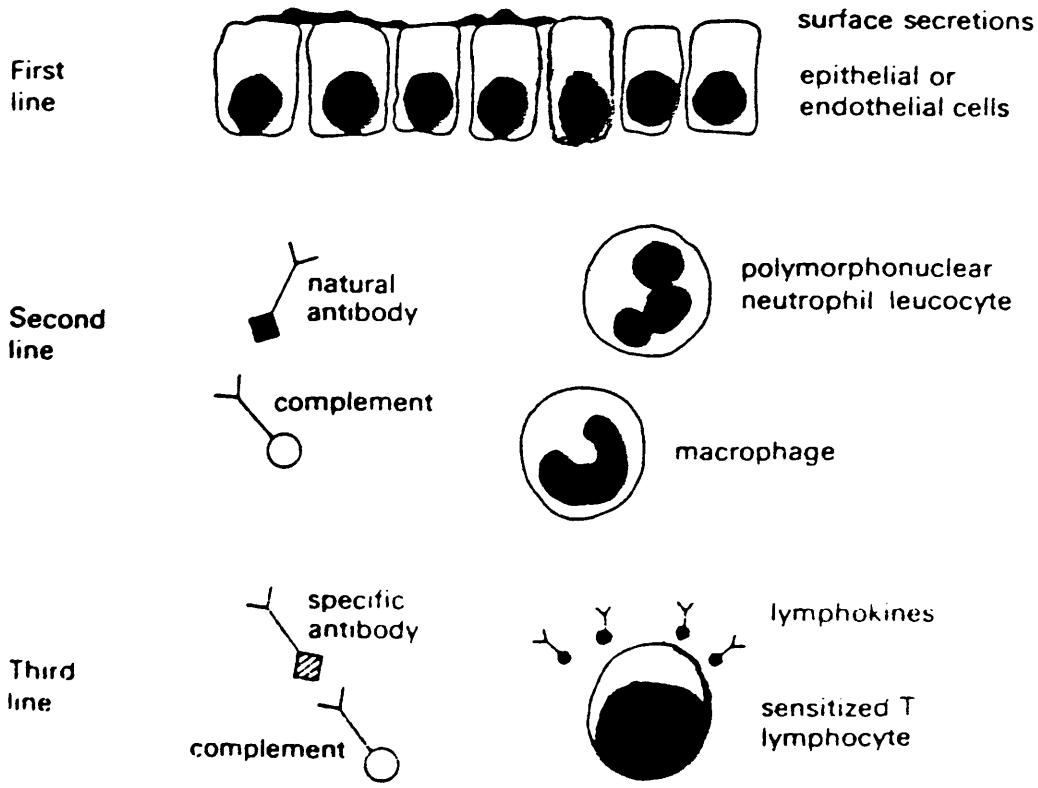


Fig. III Three lines of defence against infection.

through defects in these barriers that systemic infections in compromised hosts most often occur. Non-steroidal anti-rheumatic drugs are associated with increased gastrointestinal permeability (Jenkins et al., 1986) and consequently render it more susceptible to infection.

The two most important factors maintaining the sterility of the upper bowel are gastric acid and gastrointestinal motility. In addition, the host is protected from the bowel flora by the continuity of the epithelial cell layer, mucus, the digestive enzymes, Igs secreted from intestinal-associated lymphoid follicles and the constant exudation of blood PMNs into the intestinal lumen (McGuigan and Leibach, 1978).

The surface of the upper respiratory tract is covered by bacteria, but the lower respiratory tract is sterile. The airway is lined by mucus-covered ciliated epithelium. The mucus blanket is highly impermeable to water and serves to entrap particles reaching the lower levels.

The predominant Ig of the lower respiratory and gastrointestinal tracts is IgA. Its function is somewhat obscure because, in contrast to IgG, it does not opsonise bacteria or fix complement. Immunoglobulin A is thought to facilitate aggregation of foreign particles on the mucosal surfaces and both to prevent their adherence to mucosal membranes and to aid their removal by this aggregation effect (Newhouse, Sanchis and Bienenstock, 1976). The cough reflex is stimulated by inhalation of foreign material and by inflammation in the lungs, trachea or larynx.

1.3.3 Urinary tract

The bladder, ureters and kidneys are sterile in normal individuals. Bacterial colonization in the perineum extends a short distance into the urethra in both the male and female. The principal factor limiting the upward spread of bacteria is thought to be the repeated flushing of this mucosal surface by the downward stream of urine.

The upper urinary system is protected from infection by its anatomical remoteness from surface bacteria.

1.3.4 Polymorphonuclear leucocytes

Polymorphonuclear leucocytes are the first, cellular combatants in the battle that follows microbial invasion. However, these cells would be unable to effectively seek out, recognise, ingest and kill the invaders if it were not for two additional lines of defence provided by specific Ab (section 1.3.6) and the complement system (section 1.3.7). There are approximately 5×10^{10} PMNs, produced and utilised daily under normal circumstances. It is estimated that production increases 10-fold in severe infection (Dale, 1981).

Polymorphonuclear leucocytes, their origin, functions and the process of phagocytosis are discussed in detail in chapter 2.

1.3.5 Monocytes

Monocytes are marrow derived phagocytic cells and share many of their properties with PMNs (Wilkinson, 1983). An important difference between PMNs and monocytes is that monocytes are not end-stage cells; they differentiate in the tissues into macrophages, which are motile and actively phagocytic cells.

Monocytes, like PMNs, have surface receptors for the Fc component of IgG. They also have receptors for IgM, complement and complement-derived chemotactic factors C3a, C5a and $\overline{C5b67}$ (Cline, 1975). They contribute to the immune response, not only as phagocytes, but also as antigen-presenting cells and helper cells in T-cell dependent Ab production. Their capacity to augment their bactericidal mechanism by synthesis of new enzymes is a unique feature of these cells. Furthermore, monocytes can synthesize, upon stimulation, a number of soluble factors that contribute to host defence mechanisms (Dale, 1981). These include IL-1, tumour necrosis factor (TNF), interferon and transferrin. The above features of monocyte function may be responsible for the containment of some organisms such as salmonella and Mycobacterium that can escape PMN bactericidal action.

Interleukin 1 is a cytokine that is produced by numerous mammalian cells in response to various stimuli, including bacteria, bacterial products, viruses, fungi, Ag-Ab complexes and lymphokines (Dinarello, 1984a). Monocytes and macrophages are unquestionably the major source of IL-1. It initiates or augments a number of pro-inflammatory biologic responses including the attraction of PMNs, mononuclear phagocytes and lymphocytes (Hunninghake et al., 1987). Interleukin 1 causes PMNs to degranulate their specific granules to

the exterior and to increase their generation of superoxide. It is also capable of causing B-cells to multiply faster and it potentiates immunological responses which require a clonal expansion of T-cells (Murphy, 1985).

Concerning the therapeutic potential of IL-1, previous studies have shown that administration of recombinant IL-1 (rIL-1) substantially enhanced the antibacterial resistance of mice to Pseudomonas aeruginosa and Klebsiella pneumoniae (Ozaki et al., 1987), and the facultative intracellular pathogen Listeria monocytogenes (Czuprynski et al., 1988). The present consensus is that IL-1 acts indirectly to accelerate the protective response of the infected host. This hypothesis is supported by previous reports that IL-1 initiates or amplifies the in vivo and in vitro production of various biological mediators including interferon, colony stimulating factors, IL-2, TNF, and acute phase serum proteins (Czuprynski et al., 1988). One or more of the above described factors might be required for the IL-1 mediated enhancement of antibacterial resistance.

1.3.6 Lymphocytes

Having evaded the non-specific defence mechanisms, the microbe will then encounter a specific line of defence, the T and B lymphocytes. Approximately 80% of normal blood lymphocytes are T-cells. They emerge from a period of development and differentiation in the thymus as cells capable of a) undergoing blast transformation in response to certain mitogenic substances, b) producing many soluble mediators of immune reactivity, such as IL-2 (formerly T-cell growth factor), B-cell growth factor and IL-3, c) serving as memory cells (Tm) of previous antigenic exposures, d) being helper (Th) and suppressor (Ts) cells

in many B-cell responses, and also e) having the special capacity to kill foreign cells through direct cytotoxic mechanisms (Tc) (David, 1973). Patients with deficient cell-mediated immunity have an increased susceptibility to infection with many viruses, fungi, yeasts, some intracellular bacteria and protozoa. Controversy exists as to the possible association of a T-lymphocyte defect in RA (Goodacre and Carsondick, 1984).

B-lymphocytes are capable of differentiating into plasma cells and of producing Igs. Five classes of Igs are produced; IgG, IgA, IgM, IgD and IgE. Only IgG, IgM and IgA are known to have any specific role in protecting the host from micro-organisms. IgG is particularly important because it facilitates phagocytosis through the process of opsonisation (Huber and Fudenberg, 1968) and fixes complement which would result in bacterial lysis by a direct action on bacterial cell walls. Immunoglobulin M can also fix complement. Immunoglobulin A functions as previously discussed in section 1.3.2. These Abs also neutralize many viruses and prevent their entry into host cells.

A proportion of the B-lymphocyte population differentiate into memory cells, capable of recognising organisms that have previously invaded the host and rapidly secreting specific IgG Abs. B-cell deficiency leads to severe infections by highly encapsulated micro-organisms such as Streptococcus pneumoniae and Haemophilus influenzae.

1.3.7 Complement

The complement system consists of several protein constituents that may be activated by two major pathways; the classical and the alternative. Detailed

accounts of the chemistry of complement proteins and the role of complement in infectious diseases are available in two monographs, respectively (Frank, 1975; Goldstein, 1980).

The complement proteins help protect the host against the overwhelming infections of certain pyrogenic bacteria and fungi. Some microorganisms, even in the absence of specific Ab, call initial attention to themselves by activating the alternative complement pathway. In the presence of Ab, activation of the classical pathway may also occur. Following either or both of these events, biologically active peptides are generated that increase the number of circulating leucocytes, promote adherence of leucocytes to vascular endothelium, and attract PMNs to the site of microbial invasion. Having arrived at the focus of the initial infection, these cells then recognise, ingest and kill the invaders. In some cases, killing may not even require phagocytes because certain microorganisms, especially Neisseria species (i.e. gonococci and meningococci), may be killed by the action of complement alone. These are susceptible to lysis by the terminal membrane attack sequence $\overline{C5b\text{-}9}$ (Table 1.5).

The specific serum requirements of any individual genus or species of microorganism that render it susceptible to phagocytosis and killing cannot be simply summarised. A remarkable diversity exists that depends upon the chemical components of the cell surface of individual organisms. For instance, encapsulated staphylococci require specific IgG Ab as well as complement for optimal ingestion and killing, whereas most strains of S. epidermidis can activate the alternative complement pathway and can be phagocytosed and killed by PMNs in the absence of specific Ab.

INTERACTION WITH PHAGOCYTIC CELLS	
Mobilization of leucocytes	C3e
Adherence to vascular endothelium	C5a
Chemotaxis	C3a,C5a,C$\overline{5b67}$,Ba
Opsonisation (phagocytosis)	C3b
Stimulation of oxidative metabolism (killing)	C3b,C5a
Degranulation (killing and digestion)	C3b,C5a
DIRECT ACTION OF SUSCEPTIBLE BACTERIA (lysis)	C$\overline{56789}$

Table 1.5 Activities of complement that contribute to the maintenance of normal host defences

From the foregoing, one might predict that in certain clinical situations in which specific complement components are either depleted (as a consequence of in vivo complement activation), totally absent (as a consequence of a congenital defect), or depressed (as an effect of immunosuppressive drugs), host defences would be severely compromised. This is indeed the case. C3 and C6 deficiencies render the host susceptible to repeated bacterial infections. Persons lacking C6, C7 or C8 appear to be particularly susceptible to infections with Neisseria (Goldstein, 1980).

An involvement of the complement system in the pathogenesis of RA was suspected when it was observed that the total haemolytic complement activity and C2, C4 and C3 concentrations in joint fluids from RA patients, were depressed as compared to levels measured in SF from patients with degenerative joint disease (Hedberg, 1963). Furthermore, the simultaneous presence of C3 and Ig deposits in synovium and in phagocytic cells of articular effusions was suggestive of the presence of ICs, which were subsequently directly demonstrated (Hannestad, 1968). Many researchers have reported high levels of RF and raised C3 split products, an indication of complement catabolism, in the plasma and SF of RA patients (Berkowicz et al., 1983; Robbins, Feigal and Leek, 1986). These observations may indicate a continuous breakdown of C3 is taking place in the joints in active RA; it is not surprising then that an increased incidence of infection is a feature of this and other autoimmune diseases when compared to control populations.

CHAPTER TWO - PHAGOCYTOSIS

2.1 INTRODUCTION

2.1.1 Polymorphonuclear leucocytes

(i) Origin and fate

It is over 200 years since an English surgeon, William Hewson (Gulliver, 1846) first described the white blood cells. In 1883, Eli Metchnikoff, the famous Russian zoologist, injected foreign particles (starfish larvae) into metazoans. He observed that these were taken up by a population of 'wandering mesodermal cells' that resided in interstitial tissues. He named these wandering cells 'phagocytes'. A little more than 20 years were to pass before Metchnikoff observed and appreciated that the phagocytic white cell is capable of intracellular killing of bacteria (Metchnikoff, 1905). The predominant phagocytic white cells are the circulating PMNs.

Polymorphonuclear leucocytes are produced in the red bone marrow from a stem cell pool (Fig. IV). The stem cell is believed to be pluripotential, the common ancestor, also, of erythrocytes and platelets (Dunn, 1971). The granulocytic line begins as myeloblasts, which divide and differentiate into myelocytes over a 7-day period (Wade and Mandell, 1983). The transition from myelocyte to metamyelocyte marks the end of proliferation but the granulocyte continues to mature in the bone marrow, passing from metamyelocyte to band form to segmented PMN. This postmitotic period lasts approximately 6.5 days (Wade and Mandell, 1983). Thus the evolution from stem cell to mature blood PMN takes approximately 13-14 days.

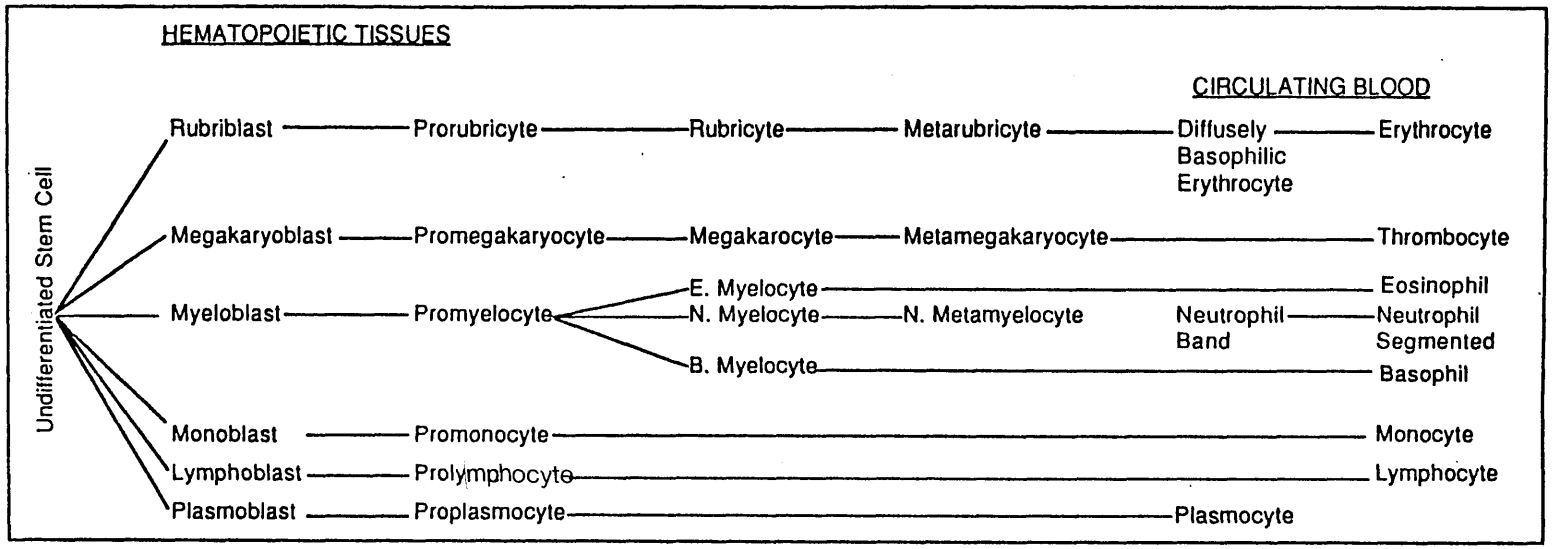


Fig. IV Origin and differentiation of blood cells (Diggs, Storm and Bell, Abbott Laboratories).

Each day 10^{11} mature PMNs enter the blood stream from the bone marrow. At any one time about half the intravascular population are not circulating but are adherent to the endothelium of small vessels, a process called margination. The number of PMNs circulating represent only 5% of the total body content. A large percentage of mature cells are stored in the bone marrow, ready to be called upon as needed to replenish cells in the circulation.

The half-life of PMNs in the blood stream is 6 hours; they are removed at senescence by splenic macrophages. From the bloodstream, PMNs enter the tissues, it is here that they are destined to fulfill their functional role, and it is here that the PMN ultimately dies after 2 to 3 days. Polymorphonuclear leucocytes are end cells; they have no regenerative capacity. Also, having migrated into the tissues, unlike mononuclear phagocytes, PMNs do not return to the circulation. Thus, from birth to senescence, the total life span of a PMN is of the order of 15 days.

(ii) Morphology

The PMN has a characteristic large multilobal (3-5) nucleus (Fig. V) and numerous cytoplasmic granules, which appear pale pink on Leishman stain. These granules are either primary (azurophil) or secondary (specific). The former appear early in development at the promyelocyte stage and are electron dense with a rich content of acid hydrolases (Table 2.1). The specific granules are formed during the maturation period and they are 3 times as numerous as the primary granules. They are called specific since they contain lactoferrin and cobalophilin (Table 2.1) which are regarded as specific cytochemical markers in PMNs. These are smaller, electron dense granules which lack digestive enzymes and peroxidase.

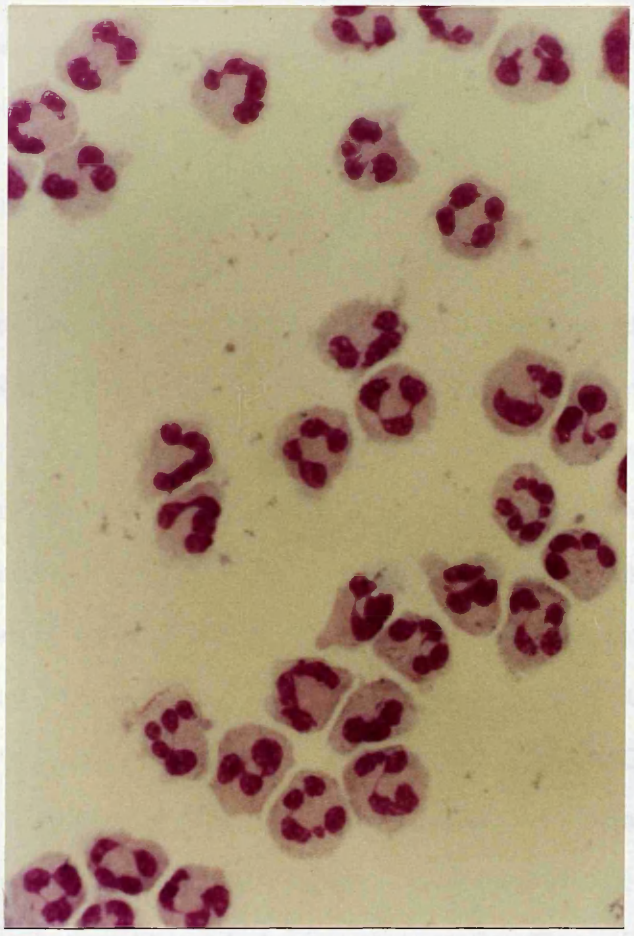


Fig. V The multilobed nucleus of the polymorphonuclear leucocyte.

(iii) Polymorphonuclear leucocyte functions

Adhesion

The PMN has a selective preference for adhering to endothelial cells (Hoover, Briggs and Karnovsky, 1978). The mechanism of adhesion is not known precisely. It involves both net surface negative charges (Hoover et al., 1978) and physical contact, probably by microfilaments (Malech, Root and Gallin, 1977), with the substrate. The cell surface charge may be important in allowing cells to approach one another sufficiently closely so that microfilament attachment can occur. Adherence to a surface is necessary for PMN locomotion. This is probably because microfilaments are involved in both processes.

Locomotion

Three types of locomotion are recognised: random migration, chemokinesis and chemotaxis. Random migration is multidirectional and occurs in the absence of any stimulus to motility. Chemokinesis is the stimulated random movement of PMNs by chemotactically active molecules present in uniform concentration without a gradient (Keller et al., 1977). Chemotaxis is the directional movement of PMNs along a concentration gradient of chemotactic factors (McCutcheon, 1946). The most important known source of chemotactic factors in vivo are generated by complement activation. Complement components C3a and C5a can be generated by activation of either the classical or the alternate pathway. A trimolecular complex of complement proteins acting later in the haemolytic sequence, $C\overline{5b67}$, assembles and also has chemotactic effects (Goldstein, 1980). A fourth fragment, Ba (a fragment of factor B resulting from the action of factor D in the alternate pathway) has chemotactic activity (Spragg and Austen,

1977). Since both complement pathways act on C3 and C5, these proteins are absolutely required for the chemotactic activity of complement.

Phagocytosis

Phagocytosis is the act of internalisation and subsequent sequestration into phagosomes of extracellular particulate matter. The process is accompanied by profound metabolic changes in the cell designed to digest, or render inert, the ingested material. The subject of phagocytosis will be discussed in detail in section 2.1.2.

Secretory function

Over recent years it has been appreciated that exocytosis - that is the fusion of specific granules or lysosomes with the cell membrane and subsequent release of their contents into the surrounding medium - occurs in response to certain triggering factors, eg. bound ICs (Henson, 1971). It is an active process and is accompanied by metabolic sequelae associated with phagocytosis. The PMN may therefore legitimately be considered as a secretory cell. This release may be important for destruction of large pathogens which are not easily ingested.

This aspect of PMN function is of particular relevance from the standpoint of the mediation of tissue injury in the rheumatic diseases. Synovial fluid from patients with RA is characterised by the presence of PMNs, the major effector cells of acute inflammation. Also present, often in high concentration, are the constituents of azurophilic granules (Table 2.1) (Hensen, 1971) and products of oxygen metabolism in the form of cytotoxic free radicals and compounds (eg. superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2)). Among the many lysosomal constituents released to the surrounding

PRIMARY (AZUROPHIL)	SECONDARY (SPECIFIC)
Lysozyme	Lysozyme
Myeloperoxidase	Lactoferrin
Cationic proteins	Gelatinase
Neutral proteases	Collagenase
elastase	Cobalophilin (vitamin B-12 binding protein)
collagenase	
Acid hydrolases	
ß-glucuronidase	
arylsulphatase	
cathepsin	

Table 2.1 The contents of polymorphonuclear leucocyte granules

tissues, particular attention has been devoted to the role of elastases and collagenases, as well as myeloperoxidase (MPO), which are associated with the breakdown of cartilage and connective tissues. The serine proteinase, elastase, is misnamed because elastin is not its only substrate. This proteinase can also degrade proteoglycan, type III collagen, and type IV collagen, found in basement membrane (Harris, 1984). It has been calculated that about 8mg of elastase is released into a typical rheumatoid knee each day. Unneutralised, this would be sufficient to release the total proteoglycan content of the joint together with 10-20% of the collagen content every 24 hours (Barrett, 1978).

Products of arachidonic acid metabolism may also generate and sustain inflammation. Leukotriene B4, produced by the action of lipoxygenase on arachidonic acid is one of the most potent chemotactic factors known. The resulting convergence of PMNs into the SF produces a surplus of extracellular enzymes, which bind to the synovial proteinase inhibitors alpha 2 macroglobulin and alpha 1 macroglobulin. If these inactivators are saturated or inactivated, the proteinases act without inhibition on substances within the joint cavity, including cartilage, bone, ligaments and tendons (Harris, 1984) resulting in joint damage.

(iv) The role of polymorphonuclear leucocytes in immune response.

Metchnikoff, and his contemporaries, argued as to whether the PMN was a 'brave soldier', defending the organism from foreign intruders, or that it might be causing considerable harm, since it is found in the rheumatoid joints. It appears to be involved in both the aforementioned.

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.

The importance of PMNs to host resistance is illustrated by the consequence of their absence, agranulocytosis, or relative lack, neutropenia. Agranulocytosis is generally fatal. Infections with organisms such as S. aureus, Escherichia coli and Pseudomonas are not uncommon. The effects of the absence of PMNs on the overall response to infection shows that fever and malaise can still occur and be the only symptoms for 2 to 3 days, to be followed by necrotic ulcers at sites where a large microflora exists; skin, nose, rectum and anus (Harkness, 1981). Neutropenia results in recurrent infections, especially in the mouth and skin.

The brief life of the PMN is devoted primarily to the task of migrating into the site of an inflammatory lesion. Having arrived, it responds with pronounced adhesion to the substratum, significant release of lysosomal constituents and production of oxygen radicals. Therefore, PMNs have the potential to be responsible for at least some of the joint destruction seen in RA.

2.1.2 Phagocytosis

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

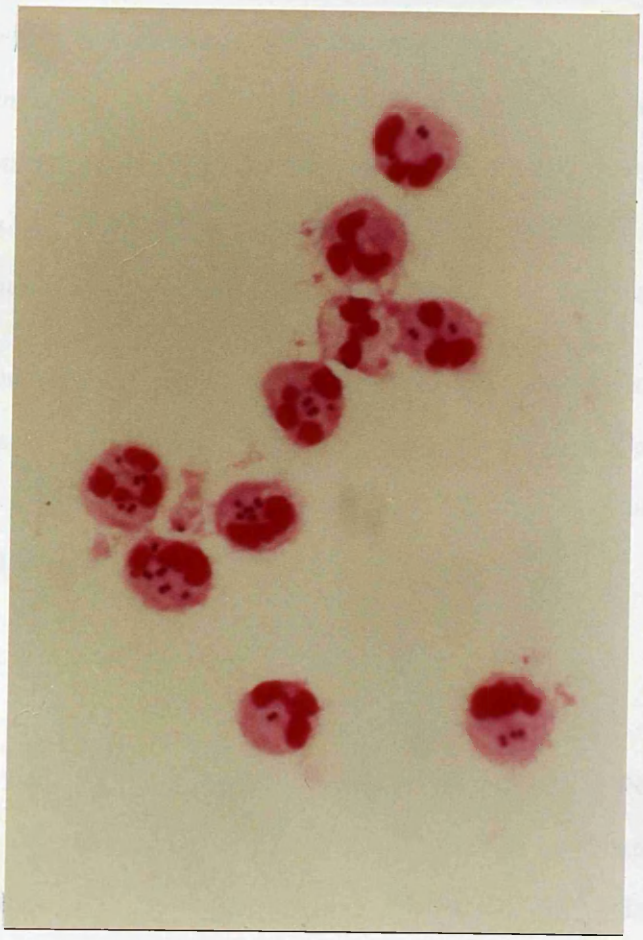


Fig. VI Polymorphonuclear leucocytes with internalised Staphylococcus aureus.

environment. Metchnikoff (1905) championed the cause of phagocytosis as the basis of survival against pyogenic infection.

Controversy arose from the proponents of humoral mechanisms of host protection, but from this dispute came about the realisation that humoral factors and phagocytes cooperate (Wright and Douglas, 1904), a concept popularised by George Bernard Shaw in The Doctor's Dilemma:

> *The phagocytes won't eat the microbes unless the microbes are nicely buttered for them. Well, the patient manufactures the butter for himself all right: but my discovery is that the manufacture of that butter, which I call opsonin, goes on in the system by ups and downs..... There is at the bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes.*

The subsequent passages include an introduction to phagocytosis by dividing it into convenient stages (Fig. VII). This sequence of activities occurs over approximately 6 hours. Death of the PMN in 1-2 days is believed to follow these events (Harkness, 1981).

(i) Production

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 which may represent an adherent Tcell population. Such a leucocytosis is not accompanied by an increase in the total number of PMNs in the body.

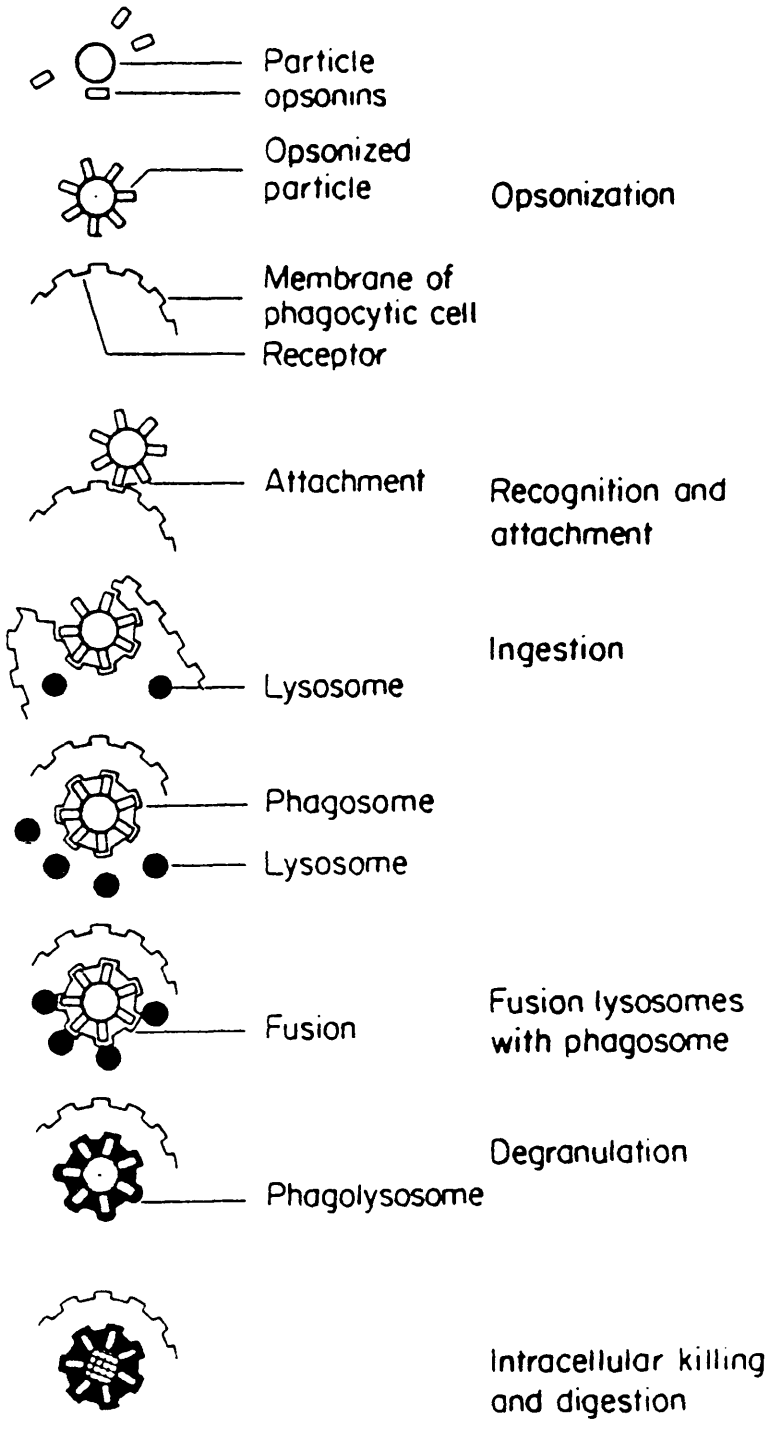


Fig. VII Schematic representation of the various stages of phagocytosis and intracellular killing of micro-organisms (van Furth, Zwet and Leijh, 1978).

(ii) Chemotaxis

A variety of mechanisms for attracting phagocytes into inflammatory sites is presumably advantageous to the host, and the small molecular size of the attractants, namely C3a and C5a, may enhance their access by diffusion to phagocytes at distant locations.

A fundamental observation relevant to the cellular aspect of chemotaxis is that PMNs rapidly enter inflammatory foci whereas monocytes arrive at a slower pace (Metchnikoff, 1905). Greater mobility underlies the prompt response of PMNs to chemotactic stimulation relative to monocytes. Although the early influx of PMNs could be explained by their speed, this hypothesis does not explain why PMNs stop appearing once the mononuclear cell infiltrate is established. Two speculative possibilities could explain this. Firstly, it could be due to the accumulation of inhibitors of PMN motility. Secondly, the PMNs may have a higher response threshold than that of monocytes, for reacting to chemotactic agents. As these chemotaxins are absorbed or inactivated by cells in the exudate, their concentration may fall below the response threshold of the PMNs.

(iii) Recognition and attachment

Having arrived at the invaded site, to attach to a bacterium, phagocytes must recognise it, i.e. phagocytes need specific receptors for something on the bacterial surface. Opsonins are humoral substances that can coat the bacterium to provide this recognition. Wright and Douglas (1903) originated the term opsonise from Greek, meaning 'to prepare for dining'. Immunoglobulin G and C3b are the primary opsonins which bind PMNs to bacteria and promote

ingestion; PMN surfaces have receptors for C3b and the Fc portion of IgG (Horwitz, 1982). It was not until 1968 that Lay and Nussenzweig reported a complement receptor. Three types of C3 receptors have been described, one for C3b, one for C3bi (formed when C3b is cleaved by C3b inactivator and ß1H globulin), and one for C3d (formed when C3bi is degraded further by proteases). C3bi or C3d receptors are, however, apparently lost by PMNs during maturation, C3b therefore being the predominant receptor. Of the IgG Abs, the subclasses IgG_1 and IgG_3 are the 'species' that participate in opsonisation. The presence of specific Ab as opsonin is termed immune opsonisation. The Fc portion of the Ab molecule, as well as the Fab segment that attaches to the particle, must be intact for expression of opsonic activity (Quie, 1972). Since the IgG immunoglobulins are resistant to heating, the heat-stable opsonic power of serum is due to the presence of Abs. Normal serum has little heat-stable opsonic activity. On the other hand, all normal sera have heat-labile opsonic activity, which can be entirely attributed to the complement protein C3b.

Certain organisms do not require Ab or complement for phagocytosis to take place. Examples of these are 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 Ab and/or complement is present, a characteristic that contributes to their pathogenicity.

(iv) Ingestion

Whereas attachment is independent of cell metabolism, ingestion requires the action of microfilament networks and active metabolism in the form of anaerobic glycolysis.

Once particle attachment is complete the PMN puts out cup-like pseudopodia which surround the particle as a result of the sequential and circumferential interaction of receptors on the phagocyte surface with IgG or C3b ligands distributed diffusely over the particle surface. This has been termed the 'zipper mechanism' (Griffin et al., 1975).

The pseudopodia fuse at the distal side of the particle, which thereby becomes encased within a phagocytic vesicle, the phagosome, the lining of which being inverted plasma membrane. The phagosome gradually pinches off and moves centripetally away from the hyaline ectoplasm of the PMN.

(v) Fusion with cytoplasmic organelles

Membrane fusion between the phagosome and one of the specific granules ensues to produce 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. Thus phagolysosomes contain a potent fungicidal, bactericidal and virucidal mixture of lysosomes, proteolytic enzymes, hydrolytic enzymes and lactoferrin. As a result of metabolic activity and the addition of specific granule contents, the pH inside the phagolysosome increases slightly and then falls (Segal et al., 1981). Subsequent to this, the primary granules fuse with the phagosome and release their contents within. Coincident with these events, a series of biochemical changes occur that result in the killing of ingested micro-organisms (Root and Cohen, 1981).

(vi) Microbicidal activity

The microbicidal activities of PMNs can be divided into two major categories: 1) oxygen-dependent and 2) oxygen-independent systems.

The oxygen-dependent process involves a co-ordinated 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. During phagocytosis, an NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase, located in the plasma membrane becomes activated (Babior, 1978), allowing formation of superoxide (O_2^-). Collectively, the stimulation of these metabolic pathways is termed the 'respiratory burst'. This is characterised by 1) an increase in glycolysis, via the hexose monophosphate shunt, which provides the substrate NADPH as electron donor, 2) an elevation in oxygen consumption, 3) generation of O_2^- anion and other oxy radicals and 4) the formation of lactic acid, which is in part responsible for the fall in pH and thus provides conditions favourable for the action of digestive enzymes (Babior, 1978). This respiratory burst is accompanied by the emission of light: chemiluminescence (Klebanoff and Clark, 1978). In addition to ingestion, binding to substances such as ICs, Igs, complement proteins and endotoxin, are capable of activating PMNs and triggering a respiratory burst (Wade and Mandell, 1983) (Fig. VIII).

The reduction of oxygen is a complex process, as outlined in Fig. IX. It results in the production of O_2^- anion, H_2O_2, OH^-, and singlet oxygen ($^1O_2$), a highly toxic bactericidal mixture (Babior, 1978). Almost all oxygen consumed by PMNs is reduced to O_2^- anion. Superoxide is short lived because it

Membrane receptor dependent

Soluble stimuli	Particulate stimuli
Platelet activating factor	opsonised bacteria
C5a, C3b, $C\overline{5b67}$	yeast
IgG-Fc complexes	viruses
Leucotriene B4	

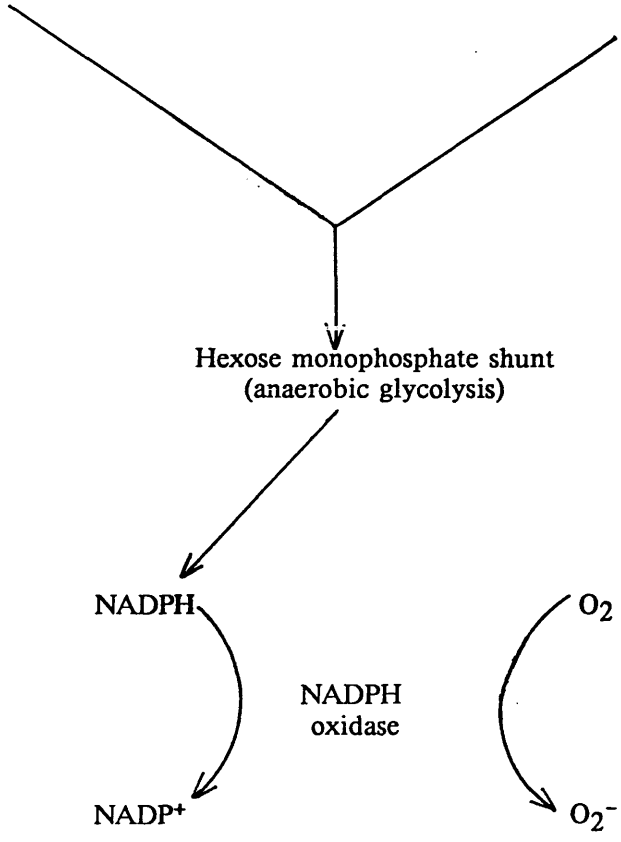
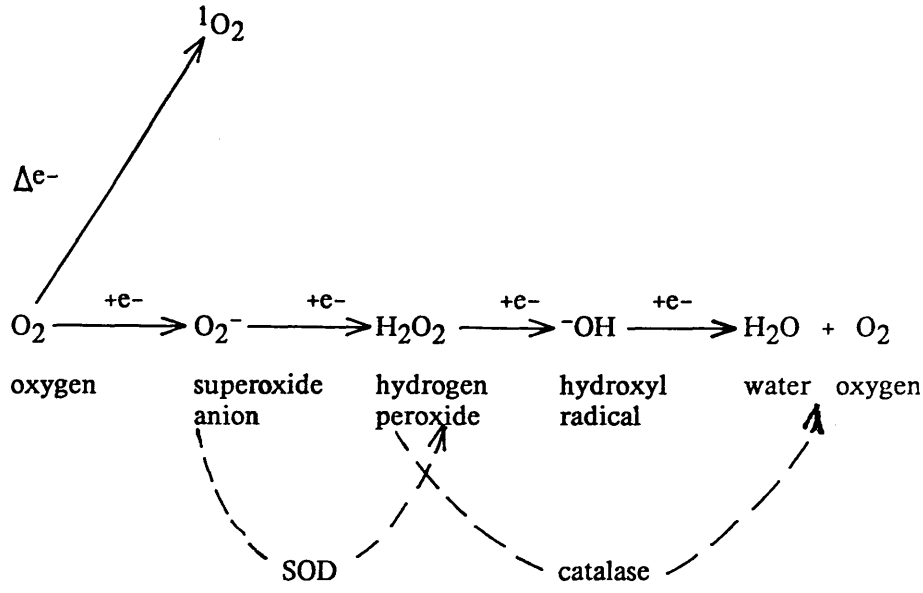


Fig. VIII Activation of the 'respiratory burst'



SOD: superoxide dismutase

Fig. IX The products of oxygen reduction

undergoes rapid spontaneous or superoxide dismutase (SOD)-accelerated dismutation to H_2O_2.

Although H_2O_2 is bactericidal in its own right, its potency is greatly augmented (approx. 50-fold) in the presence of MPO and halide ions (I^-, Cl^-, Br^-) (Klebanoff, 1980). Myeloperoxidase, present within azurophilic granules is excreted into phagolysosomes. This excretion occurs concomitantly with activation of the respiratory burst. This process yields, for example, hypochlorite ion which allows destructive halogenation or oxidation of a wide variety of substances. In the presence of ammonia or amino acids, MPO and H_2O_2 can react to form N-chloroamines, which appear to act as long-lived microbicidal products (Weiss, Lampet and Test, 1983).

The H_2O_2-MPO-halide system is thought to be the major bactericidal peroxidative mechanism in PMNs. This system has an acidic pH optimum; a condition which is met in the phagocytic vacuole.

A system of detoxifying substances within each cell serves to protect against its own oxidants. These include superoxide dismutase which converts O_2^- to oxygen and water; and catalase which converts H_2O_2 to oxygen and water. Staphylococcus aureus is a catalase-positive micro-organism. It contains high catalase activity, which destroys H_2O_2 and consequently reduces the killing efficiency of the PMN. In contrast, bacteria that are catalase-negative, for instance Escherichia coli, are effectively destroyed.

The oxygen-independent mechanisms are responsible for microbial killing in an anaerobic environment or in PMNs in which the oxidative capacity is deficient or absent (eg. chronic granulomatous disease). These factors are the acidic

environment of the phagolysosome (pH 4.0 to 6.0), lactoferrin; an iron binding protein whose extracellular release can destroy microbes by starving them of iron (Root and Cohen, 1981), and the direct action of lysozyme, which attacks the peptidoglycan of some bacterial cell walls resulting in lysis of the micro-organisms (Unanue, 1986). 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 serve 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. The two mechanisms may be synergistic, compatible, or unilaterally or mutually inhibitory. Interactions might include a cooperative attack of oxidants and hydrolytic enzymes. For example, exposing bacteria to a free radical-generating system increases their susceptibility to attack by lysozyme (Thomas, Lehrer and Rest, 1988).

The foregoing stages are convenient for outlining the biology of phagocytosis but are not necessarily discrete in either space or time.

2.1.3 Measurement of phagocytic activity

The process of phagocytosis can be studied in vivo and in vitro. The latter offers several advantages: known homogeneous populations of phagocytic cells can be used, and the effects of serum and other factors on phagocytic function can be controlled. Many in vitro methods have been employed, and though they

may be different in detail their general principles are similar. These are summarised in Table 2.2. The use of subjective techniques, such as microscopic examination of smear preparations and colony counting, have several disadvantages: they are tedious, time consuming, and subject to interobserver error. Also, results cannot be obtained before 24-48 hours. By contrast, the radiometric assay is an objective and sensitive method which eliminates the aforementioned drawbacks. Moreover, the test is easily quantitated so that results can be obtained within several hours. However, in all the above methods, there often lacks distinction between intracellular ingestion and extracellular attachment, therefore care must be taken when interpreting the results.

Polymorphonuclear leucocyte killing can be measured directly by the decrease in total number of live micro-organisms by radioactive probe or microbiological method. Recording 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 be accompanied by intracellular killing and therefore this type of measurement is unreliable.

Different methods have utilised bacteria, notably S. aureus (Bodel and Hollingsworth, 1966; Udén et al., 1983), fungi such as baker's yeast and Candida albicans (Brandt and Hedberg, 1969; Turner et al., 1973; Corberand et al., 1977; Wilton, Gibson and Chuck, 1978; Sheehan, Brown and Durmonde, 1984), and inert particles including polyvinyl toluene latex and colloidal carbon (Hällgren, Häkansson and Venge, 1978; Attia et al., 1982a).

PRINCIPLE

Phagocytosis	Method	Study	Year
Determination of increase in number of intracellular particles	Microscopic examination	Bodel and Hollingsworth	1966
		Brandt and Hedberg	1969
		Turner et al.	1973
		Corberand et al.	1977
		Wilton et al.	1978
		Bültmann et al.	1980
		Attia et al.	1982a
		Breedveld et al.	1985
	Labelling of particles with radioactive probe	Foroonzanfar et al.	1976
		Verhoef et al.	1977a
		Sheehan et al.	1984
Determination of decrease in number of extracellular particles	Direct microbiological methods - colony counting	Breedveld et al.	1985
	Electronic particle counter	Hällgren et al.	1978

Table 2.2 In vitro methods of phagocytosis

2.1.4 Phagocytosis of synovial fluid derived polymorphonuclear leucocytes

There is general agreement in the literature that SF-PMNs from patients with RA have a defective phagocytic capacity as measured by a number of different systems. Bodel and Hollingsworth (1966) using live S. aureus demonstrated that SF-PMNs from 18 patients had depressed phagocytosis and this could be improved, but not normalised, by RA serum. Phagocytosis of baker's yeast particles and Candida albicans was also found to be defective (Brandt and Hedberg, 1969; Turner et al., 1973; Wilton et al., 1978).

The basis for the defect shown is unknown but it has been attributed to the presence of SF (Bodel and Hollingsworth, 1966); ingestion of IgG ICs in vivo (Turner et al., 1973); hyaluronic acid (Brandt, 1974); a functional deficiency in C3b receptors on SF-PMNs (Wilton et al., 1978); binding of soluble ICs and the consequent alteration of cell kinetics (Strakebaum, Jimenez and Arend, 1982); and the decreased expression of Fc receptors (Breedveld et al., 1984).

2.1.5 Phagocytosis of peripheral blood derived polymorphonuclear leucocytes

The phagocytic capacity of PB-PMNs in patients with RA is more contentious than that of SF-PMNs. There are many conflicting reports, some of which are summarised in Table 2.3.

It is interesting that in the study by Wilton et al. (1978), they also noted a positive correlation between the phagocytic capacity of SF-PMNs and PB-PMNs which may suggest that the defective blood PMNs migrate into the SF.

Study	Organism/Particles used	Observation
Bodel and Hollingsworth, 1966	Staphylococcus aureus	no defect
Brandt and Hedberg, 1969	Baker's yeast	no defect
Turner et al., 1973	Baker's yeast	no defect
Goetzl, 1976	Erythrocytes	no defect
Hällgren et al., 1978	Latex particles	no defect
Bültmann et al., 1980	Candida albicans	no defect
Sheehan et al., 1984	Candida albicans	no defect
Breedveld et al., 1985	Staphylococcus aureus	no defect
King, 1985	Staphylococcus aureus and P. mirabilis	no defect
Corberand et al., 1977	Baker's yeast	decreased phagocytosis
Wilton et al., 1978	Candida albicans	decreased phagocytosis
Attia et al., 1982a	Latex particles	decreased phagocytosis

Table 2.3 Phagocytic capacity of blood polymorphonuclear leucocytes from patients with rheumatoid arthritis

In the SF, a further inhibition takes place since the defect of SF-PMNs was significantly greater than that shown by blood PMNs.

These contradictory reports may have arisen through the use of various subjective techniques.

2.1.6 The effect of rheumatoid arthritis serum on phagocytosis

Serum from RA patients has been shown to inhibit phagocytic uptake by normal PMNs (Attia et al., 1982b). It may then be proposed that the defective phagocytosis seen in RA PMNs, tested in a serum-free environment in vitro, is the consequence of these cells not having recovered from the extrinsic inhibitory effect of sera in vivo. Corberand et al. (1977) showed that RA PB-PMNs bathed in autologous sera had defective phagocytosis; this is possibly due to an extrinsic defect arising from the inhibitory action of RA serum.

Others reported that RA serum did not affect the phagocytosis of S. aureus (Breedveld et al., 1985), baker's yeast (Turner et al., 1973), or Candida albicans (Sheehan et al., 1984) by normal PMNs.

There is evidence that ICs and aggregated IgG will inhibit phagocytosis (Turner et al., 1973; Breedveld et al., 1985). Furthermore, the study by Starkebaum et al. (1982) confirms that insoluble ICs are ingested more readily by normal PMNs than are soluble complexes. Also, preincubation of either soluble ICs or soluble aggregates of IgG with PMNs, inhibits subsequent phagocytosis of insoluble ICs in a process that appears to involve metabolic activation of the PMN and may be accompanied by the loss of cell surface Fc receptors. Changes

in the expression of Fc and C3 receptors on PMNs may influence cell functions (Breedveld et al., 1985).

Hällgren et al. (1978) showed impaired PB-PMN phagocytic ability in 5 of his patients, which he correlated with high RF titres in their sera. Interestingly, Turner et al. (1973) also found a significant negative correlation between the RF titre and phagocytic uptake by normal PMNs suspended in RA serum. Attia et al. (1982b) found no correlation between titres of RF, or circulating ICs with the inhibition of PMN phagocytosis by RA serum. This is in agreement with Corberand et al. (1977).

2:1.7 The effect of synovial fluid from patients with rheumatoid arthritis on phagocytosis

The RA joint is the site of an active inflammatory response, resulting in joint destruction. Tissue deposition of circulating ICs, with subsequent complement fixation and release of lysosomal enzymes, highly reactive oxygen radicals and their derivatives from activated SF-PMNs, is thought to be important in the pathogenesis of immune complex diseases (Panayi and Chapel, 1987). Previous exposure of normal PMNs to ICs or IgG aggregates produces a burst of chemiluminescence (Starkebaum et al., 1981). As ICs are present in the SF and SF-PMNs of RA patients, and can be detected in normal PMNs preincubated with SF (Cats, Lafeber and Klein, 1975) it has been proposed that they activate PMNs.

It has been illustrated that SF from RA patients was inhibitory to phagocytosis by normal blood PMNs (Turner et al., 1973; Wilton et al., 1978). Turner et al. (1973) noted that this inhibitory activity was not specific to SF from RA

patients. He compared SF from RA, osteoarthritis and a miscellaneous arthritis group. His results showed phagocytosis to be decreased equally and significantly in all SF. However, RA SF-PMNs showed significantly less phagocytosis than miscellaneous arthritis SF-PMNs, or PB-PMNs from all three groups. This might suggest an additional intrinsic defect in RA SF-PMNs.

This depressed phagocytic activity of SF-PMNs from RA patients has been ascribed to the ingestion of ICs in vivo (Turner et al., 1978). Binding of ICs, in the SF, to normal PMNs, may block surface Fc and C3b receptors; induce interiorisation of unoccupied Fc receptors; or damage Fc receptors (Starkebaum et al., 1982), and thereby render them unrecognizable to opsonised particles, which consequently escape phagocytosis. Breedveld et al. (1984) have shown that PB-PMNs from RA patients also show decreased expression of Fc receptors and increased expression of C3b receptors after ingestion of ICs. In a more recent study (Breedveld et al., 1986), he indicated that phagocytosis of S. aureus was as effective in SF-PMNs with SF as in PB-PMNs with serum, and that the presence of intracellular ICs in SF-PMNs did not influence the phagocytic activity of these cells.

Synovial fluid also contains hyaluronic acid and free iron (Gutteridge, Rowley and Halliwell, 1982). It has been shown that hyaluronate molecules depress the ability of PMNs to dispose of particulate material. Ingestion being inversely related to both the concentration and molecular weight of the synovial hyaluronate (Brandt, 1974). There is evidence that Fe(III) can induce a defect in phagocytosis, however this is dependent on the nature and concentration of the ligand attached to the iron ion. It has been suggested that the noxious effect of iron on PMN function is a result of its ability to catalyse the generation of toxic oxygen species by these cells (Sweder van Asbeck et al., 1984).

Such a defect of phagocytosis, combined with the chemotactic defect described by Mowat and Baum (1971) could jeopardize the inflammatory response and allow infection to become easily established.

2.1.8 Opsonic activity of serum and synovial fluid from patients with rheumatoid arthritis

A lack of opsonins, C3b or IgG, or the presence of inhibitors of opsonisation will impair phagocytosis. Although the level of serum complement in RA is usually normal or slightly elevated, 4% of RA patients are hypocomplementaemic (Hunder and McDuffie, 1973) and, as one might expect, these patients have an abnormally high incidence of bacterial infection. Measurement of C3d, a breakdown product of C3b, provides a useful means of detecting in vivo complement activation. Nydegger et al. (1977) and Mallya et al. (1982) found elevated plasma C3d concentrations in RA patients. This suggests significant breakdown of C3b and consequently fewer opsonically active complement fragments. The low levels of haemolytic complement and the presence of complement fixing materials in hypocomplementaemic serum suggests that activation and utilisation of complement by ICs is responsible (Hunder and McDuffie, 1973). In support of this theory, Mallya et al. (1982) found a significant correlation between C3d concentration and circulating ICs in their patients.

Fluid C3b, a by-product of the activation of large amounts of C3, has been shown to inhibit phagocytosis, possibly by competing with C3 for the C3 convertase on the bacterial cell surface or by blocking the sites where nascent C3b would bind covalently to manifest its opsonic function (Ogle, Ogle and Alexander, 1983).

Several authors have shown titres of whole complement and individual complement proteins to be lower in SF from RA patients than levels present in RA serum, normal serum or serum from patients with other arthritic diseases (Hedberg, 1963; Ruddy and Austen, 1970). A few years later, Pruzanski, Leers and Wardlaw (1974) reported that the bacteriolytic and bactericidal activity of RA SF was less than that of autologous serum, or SF and serum from osteoarthritic patients.

The presence of elevated levels of C3 breakdown products in serum or SF indicates that the lack of complement components is due to the continuous activation of the complement pathway by both the classical and alternate routes (Hunder, McDuffie and Clark, 1979).

2.1.9 The effect of drugs on polymorphonuclear phagocytosis

It seems reasonable to suggest that the aforementioned discrepancies of PMN phagocytosis, may be due to differences in drug regimens. Drugs used in the treatment of RA are discussed in chapter 1. The NSAIDs have been shown to depress phagocytosis and intracellular killing in vitro (Whittaker, Hughes and Khurshid, 1975; Robinson, 1978) and in vivo (Whittaker et al., 1975) though high concentrations of drug are frequently required. Inhibition may also occur with therapeutic concentrations of corticosteroids (Jones, Morris and Jayson, 1983). There is a widespread clinical experience that corticosteroid treatment increases the incidence of bacterial infection. There are several reports that phagocytosis is increased following treatment with penicillamine (Hällgren et al., 1978). The use of D-penicillamine, a reducing and chelating agent, in the treatment of RA is based upon the observation that this drug acts by

dissociating the disulphide bonds of RF (Jaffe, 1962). This may lead to decreased IC formation and consequently reduced blocking of C3b and Fc receptors on the PMN cell surface. These receptors are then free to attach and ingest opsonised particles. Some antirheumatic drugs have been reported to decrease PMN adherence (MacGregor, Spagnudo and Lentnek, 1974). However, the significance of this to phagocytic function is unclear since Youinou and Le Goff (1987) showed that inhibition of bactericidal ability of the PMN is not the consequence of weakened adherence or impaired bacterial engulfment, but an induced defect in the respiratory burst. Impairment of lysosomal enzyme release by PMNs treated with indomethacin, as demonstrated by Northover (1977), is another possibility for the depressed killing ability of PMNs. Despite these theories, Wilton et al. (1978) found no correlation between drug therapy and defective phagocytosis by either SF-PMNs or PB-PMNs. It may be necessary to differentiate between the effects of drugs on RA and normal PMNs, since Spisani and colleagues (1982) reported that rifamycin sv inhibited phagocytosis by RA but not normal PMNs.

2.1.10 Aims of the study

In the light of conflicting reports of PMN phagocytic function, this study set out to investigate 1) a possible intrinsic cellular defect in RA PMNs compared to PMNs from healthy controls, 2) possible extrinsic inhibitory activity inherent to RA serum or SF and 3) if present, could this inhibitory activity be transferred in vitro to normal PMNs? Furthermore, the opsonic activity of serum and SF from normals and patients with RA was investigated, to determine whether the phagocytic dysfunction could be the consequence of an opsonic defect rather than a PMN abnormality.

The fact that patients with RA are susceptible to infection, together with the high incidence of isolation of S. aureus as the causative agent, suggests a possible defect in PMN phagocytosis.

2.2 MATERIALS AND METHODS

2.2.1 Patients

All RA patients studied in this and subsequent chapters had clinical or definite RA according to the American Rheumatism Association (Appendix A). They were either in-patients or attending clinics at the Centre for Rheumatic Diseases, Glasgow Royal Infirmary. Controls free from any clinically detectable disease and non-users of drugs, were similarly tested in parallel for each test. Some patients and controls were tested on more than one occasion.

2.2.2 Collection of samples

All centrifugation througout this study was performed at 37°C unless otherwise specified.

To aid puncture of an antecubital vein, and consequent blood flow, a rubber tourniquet applied gentle pressure. Using either a 19 or 21 gauge, sterile, disposable butterfly needle (Abbot Irland Ltd. U.K.), blood was slowly withdrawn into a sterile plastic syringe (Becton, Dickinson & Co. Ltd., U.K.). The whole blood samples were collected into sterile universal containers (Laboratory Sales Ltd., U.K.) containing preservative free sodium heparin to a final concentration of 10 Units/ml of blood (1000 Units/ml, Leo Laboratories, U.K.), as anticoagulant. The universals were securely sealed and the contents gently mixed.

For separation of serum, blood was collected into glass tubes containing no anticoagulant (Labco, U.K.). These were clotted at ambient temperature for 80 minutes. The tubes were then centrifuged (Mistral 4L, MSE, U.K.) at 2000g for 10 minutes, the serum removed and stored at -70°C in 1ml aliquots.

All blood samples were collected between 9.00 and 10.00 am to minimise the effect of diurnal variation. Samples were processed as soon as possible after collection.

Synovial fluid was aspirated from the joints of RA and non-RA patients, and collected into universals containing no anticoagulant. Synovial fluid was centrifuged at 2000g for 10 minutes to pellet all the cells present and the supernatant removed and stored at -70°C in 1ml aliquots.

Serum and SF samples, separated for the measurement of complement components, were centrifuged at 2000g for 10 minutes, within 30 minutes of collection.

2.2.3 General media

(i) Dextran 150

A 5% (w/v) dextran 150 (Fisons, U.K.) solution was prepared in saline (0.9% (w/v) sodium chloride (NaCl); BDH Chemicals Ltd., U.K.). Once totally dissolved, it was filtered through a 0.2μ sterile, disposable, filter assembly (Gelman Sciences, U.S.A.) into a sterile glass bottle and stored at 4°C.

(ii) Lymphoprep

Lymphoprep (Nyegaard, U.K.) is a commercially available sterile solution of sodium metrizoate and Ficoll in concentrations of 9.6% (w/v) and 5.6% (w/v) respectively. It has a density of 1.077 g/ml and an osmolarity of 300 mOsm/kg. This solution is stored at or below 20°C protected from light for optimum stability.

(iii) Ammonium chloride

Ammonium chloride (NH_4Cl: Sigma, U.K.) was prepared as a 0.87% (w/v) solution in distilled water and stored at 4°C.

(iv) Gel-Hanks

Gel-Hanks (GH) was prepared by adding 10ml of Hanks' Balanced Salt Solution (HBSS: 10x, Gibco Ltd., U.K.), without calcium and magnesium, to 80ml of sterile distilled water. Gelatin (1% (w/v), BDH Chemicals Ltd., U.K.) (10ml), 20mM N-2-hydroxyethylpiperazine-N2-ethansulphonic acid (HEPES buffer) (1M solution, Northumbria Biologicals Ltd., U.K.) and sodium bicarbonate solution (7.5% (w/v); Northumbria Biologicals Ltd., U.K.) (1.1ml) were added to stabilize the solution. The pH was adjusted to 7.2 with 1M sodium hydroxide (BDH Chemicals Ltd., U.K.) and 1M Hydrochloric acid (HCl). Gel-Hanks was used as a washing medium and stored at 4°C.

(v) Tris (hydroxymethyl)aminomethane (Tris) buffer

Stock solutions

A: 0.2M solution of Tris (Sigma, U.K.).

B: 0.2M HCl (specific gravity 1.18; BDH Chemicals Ltd., U.K.).

50ml of A + xml of B diluted to a total of 200ml with distilled water.

x	pH
5.0	9.0
8.1	8.8
12.3	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.6	7.8
38.1	7.6
41.4	7.4
41.2	7.2

2.2.4 Isolation of polymorphonuclear leucocytes from whole blood

Polymorphonuclear leucocytes were prepared from heparinised peripheral blood using a modification of the method described by Böyum (1968). Approximately 7ml of blood was mixed with a 5% (w/v) solution of dextran 150 (3ml) in 15ml plastic conical centrifuge tubes (diameter 12mm: Elkay products Inc., U.S.A.),

Fig. X Dextran sedimentation of whole blood.

Fig. XI Lymphoprep separation of leucocyte-rich plasma.

and allowed to gravity-sediment for 30 minutes at ambient temperature. Erythrocytes settle at the bottom of the tube (Fig. X). The PMN-rich plasma was layered onto lymphoprep (4ml) and centrifuged at 290g for 25 minutes. Polymorphonuclear leucocytes pellet while the monocytes settled at the interphase (Fig. XI). The supernatant was decanted and the pellet resuspended in cold 0.87% NH_4Cl (10ml), to lyse any contaminating erythrocytes. After 20 minutes the PMNs were centrifuged at 290g for 5 minutes, resuspended and washed once in GH for 5 minutes. The PMNs were finally resuspended in GH (1 ml). A small volume of PMN suspension was diluted 10-fold in white cell diluting fluid (2% glacial acetic acid (May and Baker, U.K.), and a few grains of crystal violet (BDH Chemicals Ltd., U.K.) in distilled water), to immobilize the cells, and 10µl was added to an improved Neubauer haemocytometer (Gallenkamp, U.K.). The cells were counted under a light microscope using x10 magnification. (Watson microsystem 70, U.K.). The white cell count was determined by taking the mean number of cells (Y) in the four outermost corners (each of 16 squares) of the grid within the chamber. Total white cell count = Y x dilution (i.e 1/10) x 10^4 giving number of cell per ml, i.e. Y x 10^5 cells per ml. The PMNs were adjusted to a concentration of 1 x 10^7 colony forming units (c.f.u.)/ml for the phagocytic assays.

Use of this method for counting cells had the advantage that purity of individual fractions could be assessed along with cell counts, as the staining allowed differentiation between the nuclei of mononuclear leucocytes and PMNs.

The viability of the PMNs was determined at the beginning and end of the phagocytosis experiments, by trypan blue exclusion (0.5% solution in 0.85% saline: Flow laboratories, U.K.). Equal volumes (10µl) of PMN preparation and

	RA	Controls
Number	38	38
Mean age (years) (range)	53 (28-76)	29 (23-43)
Sex*	M=14 F=24	M=17 F=21
Mean duration of disease (years) (range)	10 (1-27)	NA NA
Drug therapy	NSAID only = 15 2nd line only = 13 3rd line only = 5 2nd line + NSAID = 5	NA

* M: male; F: female
RA: rheumatoid arthritis
NSAID: Nonsteroidal anti-inflammatory drug
2nd line: gold, penicillamine, auranofin or sulphasalazine
3rd line: cyclophosphamide, methotrexate or azathioprine
NA: not applicable

Table 2.4 Clinical data of rheumatoid arthritis and controls

trypan blue were mixed together and the cells examined within 2 minutes. Non-viable cells stained deep blue whereas viable cells remained unstained. Viability of more than 95% was found in all samples examined, with greater than 94% purity.

2.2.5 Radiometric phagocytic assays

A modification of the method of Verhoef, Peterson and Quie (1977a) was used, and is described in the following text.

(i) Patients and controls

For the study of phagocytic uptake of S. aureus and P. mirabilis, PB-PMNs from 38 patients with RA were compared to PB-PMNs from 38 sex-matched, healthy laboratory staff. The clinical data of patients and controls are shown in Table 2.4.

(ii) Bacterial strains and radioactive labelling.

Mueller-Hinton broth (10ml) (Oxoid Ltd., U.K.) containing [^{3}H]-adenine (0.1ml) (TRK.343, specific activity 851 Gigabequerels/mmol; Amersham International, U.K.) was inoculated with one colony of S. aureus cowan 1 or P. mirabilis (laboratory strain). The broths were incubated overnight at 37°C, in which time the growing bacteria incorporated the [^{3}H]-adenine into their own DNA sequence. The bacteria were washed thrice in sterile phosphate-buffered saline (PBS: Oxoid Ltd., U.K.), pH 7.4, at 2000g for 10 minutes (MSE, Super Minor, U.K.). The concentration of the bacterial suspension was adjusted to 1 x 10^7 cfu/ml using a Cecil spectrophotometer (Unicam, S.P. 1700, U.K.), set at a

wavelength of 620nm and slit width of 0.1mm. An optical density (O.D.) of 0.025 corresponded to 1 x 10^7 cfu/ml in the case of both organisms. This was confirmed by the Miles and Misera technique (1938). For standardisation of bacterial concentrations see Appendix B.

(iii) Bacterial opsonisation

Bacteria were opsonised with an equal volume of pooled human serum diluted in GH. Opsonisation of S. aureus and P. mirabilis with 10% and 50% serum respectively, was found to give optimum phagocytosis (Appendix B). The bacteria and serum mixtures were incubated at 37°C on an orbital shaker (150rpm). After 15 minutes, they were removed and centrifuged at 2000g for a further 15 minutes. The supernatant was discarded and the bacteria resuspended in the original volume of GH.

(iv) Uptake of bacteria

To measure phagocytic uptake (Fig. XII), duplicate polypropylene scintillation vials (Pony vials: Canberra Packard, U.K.) containing PMNs (0.1ml) and opsonised bacteria (0.1ml) were incubated at 37°C, on a shaking incubator (150rpm). A 1:1 ratio of PMN:bacteria was found to give the optimum phagocytosis (Appendix B). After 15 minutes the vials were removed and phagocytosis was terminated in one of the duplicate vial sets (Fig. XII) by the addition of scintillation fluid (3ml) (299: Canberra Packard, U.K.). Ice cold PBS (3ml) was added to the other set of vials, which were washed thrice with PBS at 160g for 5 minutes. After the final wash, the pellet was resuspended in scintillation fluid (3ml). The purpose of the PBS washes was to remove any extracellular, non-phagocytosed bacteria which were not pelleted during the differential centrifugation. Thus, only those organisms which were leucocyte-

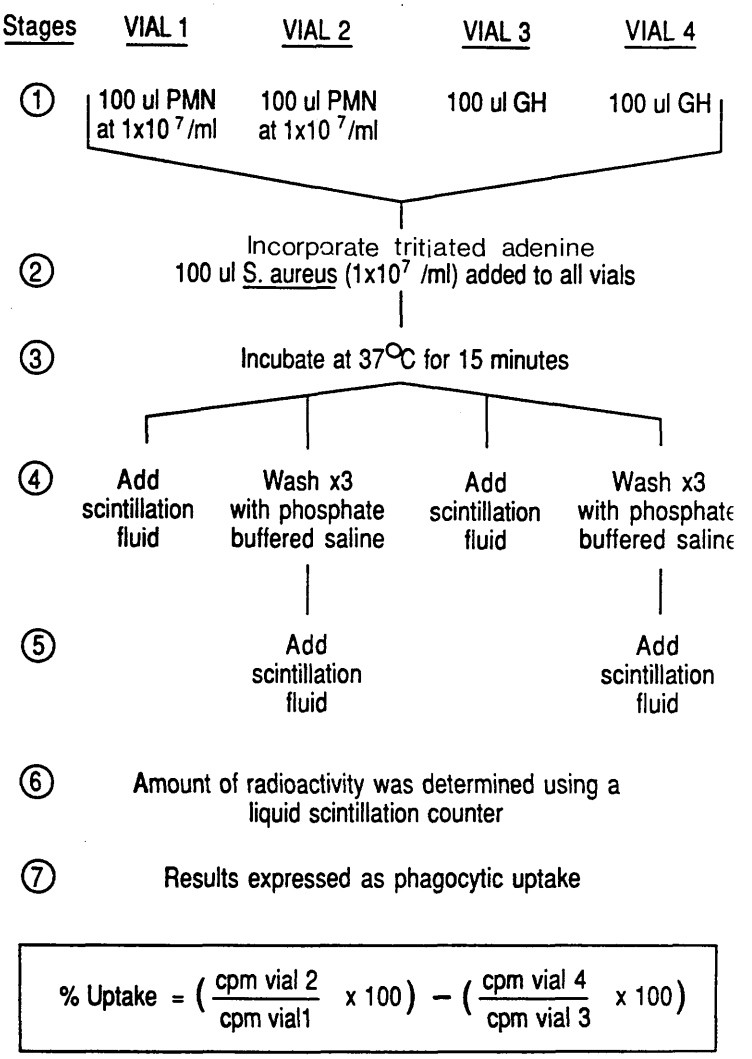


Fig. XII Outline of the phagocytic assay.

associated would remain. The amount of radioactivity was determined using a liquid scintillation ß counter (1216 Rackbeta II, LKB, U.K.). Each sample was counted for 60 seconds. All experiments contained duplicate control vials in which PMNs were replaced by GH. These were treated as described above. They were included to control for non-specific bacterial aggregation and adherence to the vials. Phagocytic uptake was calculated as the percentage of radioactivity associated with the PMN (Fig. XII).

(v) The effect of drugs on phagocytic uptake of S. aureus by normal polymorphonuclear leucocytes.

The drugs studied were Tenidap (CP-66,248; Pfizer, U.K.), SASP (Pharmacia Ltd., U.K.), Hydrocortisone sodium phosphate (Efcortesol, 100 mg/ml solution, Glaxo, U.K.), and Indomethacin (Sigma, U.K.). Sulphapyridine and 5-ASA, the two components that constitute SASP, were also studied individually. Tenidap, SASP, SP and 5-ASA were dissolved in Tris buffer pH 9.0, neutralised with 0.2M HCl, as far as possible without precipitation, at concentrations of 10mg/ml for all except Tenidap which was at 1mg/ml. Indomethacin was dissolved in 95% ethanol (James Burroughs (F.A.D.) Ltd., U.K.) at 10mg/ml and was diluted just before use to 1µg/ml, the final concentration of ethanol being approximately 0.01%. All drugs were filter sterilised (0.2µ) before use. Tenidap, SASP, SP and 5-ASA were stored at -70°C in 0.5ml and 0.1ml aliquots. Indomethacin and Hydrocortisone were stored at 4°C. The drugs were diluted with GH to the appropriate working concentrations (Table 2.5), prior to use in the assay. The final concentrations of drugs used in in vitro experiments are as detailed in Table 2.5.

Polymorphonuclear leucocytes (0.1ml, 1 x 10^7/ml) were incubated with each drug (0.1ml) at 37°C. As a control, PMNs were incubated with GH alone. After

Drugs	Working concentrations (μg/ml)	Final concentrations (μg/ml)
Tenidap	2 4 10 20	1 2 5 10
Sulphasalazine	20 40 200 1000	10 20 100 500
Sulphapyridine	20 40 200 1000	10 20 100 500
5-Aminosalicylic acid	20 40 200 1000	10 20 100 500
Hydrocortisone sodium phosphate	20 40 60	10 20 30
Indomethacin	2	1

Table 2.5 Drugs and concentrations studied

60 minutes, samples were removed and washed with GH (2ml) at 160g for 5 minutes. The cells were resuspended in GH (0.1ml) to restore them to their original concentration. The ability of PMNs to phagocytose S. aureus was measured as described in section 2.2.5(iv). The effect of drugs on the phagocytic uptake of S. aureus by PMNs was calculated from the phagocytic differential (PD).

PD (uptake) = % uptake (PMN-drug) - % uptake (PMN-GH)

where % uptake is calculated as described in Fig. XII.

(vi) The effect of rheumatoid factor on phagocytosis of S. aureus by normal polymorphonuclear leucocytes.

Commercially available RF (American Hospital Supply company, U.S.A.) at a concentration of 250 International Units (IU)/ml was purchased. Rheumatoid factor (0.1ml) was added to PMNs (0.1ml) at 37°C in an orbital shaking incubator (150rpm). The concentration of PMNs was 1 x 10^7 cfu/ml and final assay concentrations of RF were 125, 100, 75, 50, 25, and 0.5 IU/ml in GH (PMN-RF). As a control, PMNs were incubated with GH alone (PMN-GH). After 30 and 60 minutes, samples were removed and washed with GH (2ml) at 160g for 5 minutes. Cells were resuspended in GH at 1 x10^7 cfu/ml. The ability of normal PMNs to phagocytose S. aureus was measured using the radiometric assay described in section 2.2.5(iv). The effect of RF on phagocytosis by normal PMN was calculated from the phagocytic differential (PD).

PD (uptake) = % uptake (PMN-RF) - % uptake (PMN-GH)

where % uptake is calculated as described in Fig. XII.

(vii) The effect of serum on phagocytosis of S. aureus by polymorphonuclear leucocytes

In this study serum was obtained from 33 RA patients and 16 healthy controls. The clinical data are shown in Table 2.6.

Polymorphonuclear leucocytes (0.1ml) were incubated at 37°C, with sera (0.1ml) from RA patients (Groups I, II and III) or controls (PMN - serum). Serum was present at a final concentration of 50% and PMNs at 1 x 10^7 cfu/ml in GH. As a control, PMNs were incubated with GH alone (PMN-GH). After 60 minutes, samples were removed and washed with GH (2ml) at 160g for 5 minutes. Cells were resuspended in GH (0.1ml) to restore them to their original concentration. The ability of PMNs to phagocytose S. aureus was measured as described previously.

Group I : serum from RA patients with high RF titre (>1/1024) (n=11).

Group II : serum from RA patients with low RF titer (<1/64) (n=12).

Group III : serum from RA patients with no detectable RF (seronegative) (n=10).

Group IV : serum from healthy controls (n=16).

The effect of serum on the phagocytic uptake of S. aureus by PMNs was calculated from the phagocytic differential (PD).

	RA	Controls
Number	33	16
Mean age (years) (range)	54 (28-78)	26 (23-32)
Sex*	M=11 F=22	M=9 F=7
Mean duration of disease (years) (range)	7 (1-20)	NA NA
Drug therapy	NSAID only = 15 2nd line only = 17 2nd line + NSAID = 3	NA

* M: male; F: female
RA: rheumatoid arthritis
NSAID: Nonsteroidal anti-inflammatory drug
2nd line: gold, penicillamine, auranofin or sulphasalazine
NA: not applicable

Table 2.6 Clinical data of patients and controls, from whom serum was collected

PD(uptake) = %uptake (PMN-serum) - %uptake (PMN-GH)

Where % uptake is calculated as described in Fig. XII.

(viii) The effect of synovial fluid on the phagocytosis of S. aureus by polymorphonuclear leucocytes.

In this study, the effect of SF from 30 RA patients, on PB-PMN phagocytosis was compared with SF from 15 non-RA patients. This group consisted of 1 adult Still's disease, 1 systemic lupus erythematosus, 2 psoriatic arthritis, 3 juvenile RA, 3 reactive arthritis and 5 ankylosing spondylitis patients. The clinical data of RA and non-RA patients is presented in Table 2.7.

Polymorphonuclear leucocytes (0.1ml) at a final concentration of 1×10^7 cfu/ml were incubated at 37°C with SF (0.1ml) from RA and non-RA patients (PMN-SF). Synovial fluid was present in the assay at final concentrations of 25%, 50% and 75%. As a control, PMNs were incubated with GH alone (PMN-GH). After 60 minutes, the samples were removed and washed with GH (2ml) at 160g for 5 minutes. Cells were resuspended in GH (0.1ml). The phagocytic uptake assay was measured as previously described.

The effect of SF on phagocytosis was calculated by the phagocytic index (PI).

$$\text{PI (uptake)} = \frac{\text{\% uptake (PMN-SF)}}{\text{\% uptake (PMN-GH)}} \times 100$$

Where % uptake is calculated as described in Fig XII.

	RA	Non-RA
Number	30	15
Mean age (years) (range)	57 (36-77)	29 (16-46)
Sex*	M=20 F=10	M=11 F=4
Mean duration of disease (years) (range)	9 (2-20)	4 (1-10)
Drug therapy	NSAID only = 9 2nd line only = 14 3rd line only = 1 2nd line + NSAID = 6	NA

* M: male; F: female
RA: rheumatoid arthritis
NSAID: Nonsteroidal anti-inflammatory drug
2nd line: gold, penicillamine or sulphasalazine
3rd line: azathioprine

Table 2.7 Clinical data of patients with and without rheumatoid arthritis

(ix) Opsonic activity of serum and synovial fluid

Serum and SF were collected as detailed in section 2.2.2. Opsonic activity was examined in the serum samples described in 2.2.5(vii), and the SF samples described in section 2.2.5(viii).

Several dilutions (10%, 20%, 25%, 50% and 75%) of SF in GH were tested for optimal opsonic activity. No significant differences were found between any two dilutions. However, 50% SF gave the lowest percentage precision value (Appendix B). As a consequence of these preliminary experiments, 50% SF was used as opsonin in subsequent opsonisation experiments. Serum, as an opsonin, was used at a concentration of 10% in GH. Radioactively labelled ([^{3}H]-adenine) S. aureus was opsonised as previously specified. Serum or SF, both inactivated and untreated were used as opsonins. Inactivation was performed by incubating the serum or SF for 30 minutes at 56°C, before use.

Phagocytic uptake was measured as described in section 2.2.5(iv).

2.2.6 Immune complex assay

A commercially available kit (M201 CIC screening kit: Mercia diagnostics, U.K.) was used to measure circulating ICs (IgG and IgM) in serum and SF. The assay procedure was based on precipitation of ICs by polyethylene glycol (PEG). It was a two stage procedure; an initial precipitation by PEG followed by the subsequent quantitation and characterisation of the complexes by single radial immunodiffusion. The assay was performed according to the instructions supplied with the kit.

Immune complex study groups.

The same groups of sera and SF were used in this study as in section 2.2.5(vii) and (viii) respectively. Sera or SF that were known to be positive and negative for ICs were included as quality control samples in each assay. This enabled the precipitation to be qualitatively validated. In addition to this the kit included plate controls, which allowed both quantitative and qualitative validation.

2.2.7 Evaluation of immunological parameters in serum and synovial fluid

Complement and RF titre were quantitated by the Department of Clinical Immunology, Glasgow Royal Infirmary. Complement components, C3 and C4, were quantitated using commercially available immunodiffusion plates. Rheumatoid factor titre was determined by the Rose-Waaler agglutination technique (Rose et al., 1948).

Immunoglobulin and CRP were evaluated by the Department of Clinical Biochemistry, Glasgow Royal Infirmary. Immunoglobulin (IgG, IgM and IgA) levels were obtained by immunoturbidimetry using an 'Encore' centrifugal analyser (Baker Instruments, U.K.). C-reactive protein was estimated by fluorescence polarization immunoassay technology using an Abbott TDx system (Abbott laboratories, U.K.).

2.2.8 Statistical analysis

Statistical analyses of all results were performed by computer analysis using the Statgraphics software package (version 2.6) (STSC Inc., U.S.A.). All correlations

were performed using Spearman's rank correlation procedure. Statistical significance was sought using the non-parametric Mann Whitney U test for unpaired data, or the Wilcoxon rank test for paired data. All significance was assessed at the 5% probability level. Methods are detailed in Appendix C.

2.3 RESULTS

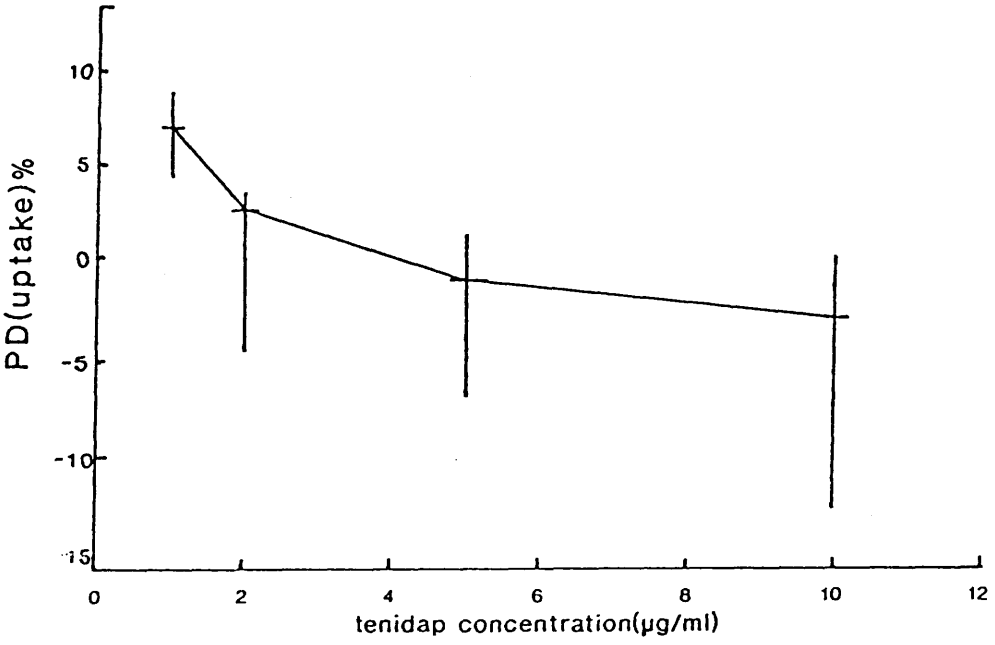
2.3.1 Effect of drugs on phagocytic uptake by normal polymorphonuclear leucocytes

As shown in Fig. XIII phagocytic capacity was depressed in a dose dependent fashion, in normal PMNs treated, in vitro, with Tenidap. The concentrations, 10, 5, and 2μg/ml significantly inhibited PMN uptake compared to 1μg/ml ($p=0.014$, $p=0.002$, $p=0.005$ respectively), but not when compared with untreated PMNs ($p>0.05$). Interestingly, the lowest concentration of 1μg/ml significantly increased phagocytic uptake when compared with untreated PMNs ($p<0.001$).

Sulphasalazine weakly depressed phagocytic uptake (Fig. XIV), as did the two individual components that constitute SASP; SP (Fig. XV (A)) and 5-ASA (Fig. XV (B)), however, this difference was not significant. A fall in uptake was observed when the concentration of SP was increased from 10μg/ml to 20μg/ml. However, at higher concentrations, uptake remained relatively constant, at a level comparable to that at 20μg/ml. A similar response was found with 5-ASA, but not with SASP. No dose dependent response was observed in PMNs pretreated with SASP. Also, SASP did not induce significantly greater inhibition than either SP or 5-ASA alone ($p>0.05$).

No significant impairment of uptake could be detected with hydrocortisone sodium phosphate although PMN uptake showed a definite downward trend with increasing drug concentration from 10μg/ml to 20μg/ml to 30μg/ml (Fig. XVI).

Fig. XIII Effect of tenidap on polymorphonuclear phagocytosis.

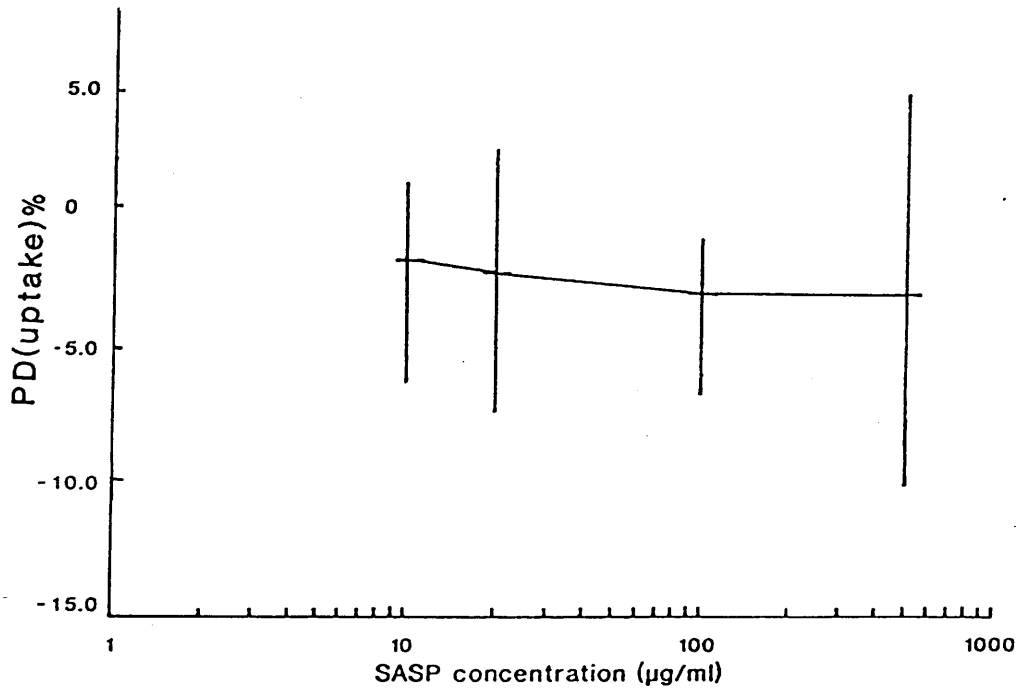


┼ median plus interquartile range (8 experiments)

PD : phagocytic differential
PD (uptake) = % uptake (PMN - tenidap) - % uptake (PMN-GH)
PMN : polymorphonuclear leucocyte
GH : gel-hanks

Significant difference between concentrations 1µg/ml and 2µg/ml: p=0.005, 1µg/ml and 5µg/ml: p=0.002, and 1µg/ml and 10µg/ml: p=0.014
Wilcoxon rank test.

Fig. XIV Effect of sulphasalazine on polymorphonuclear phagocytosis.



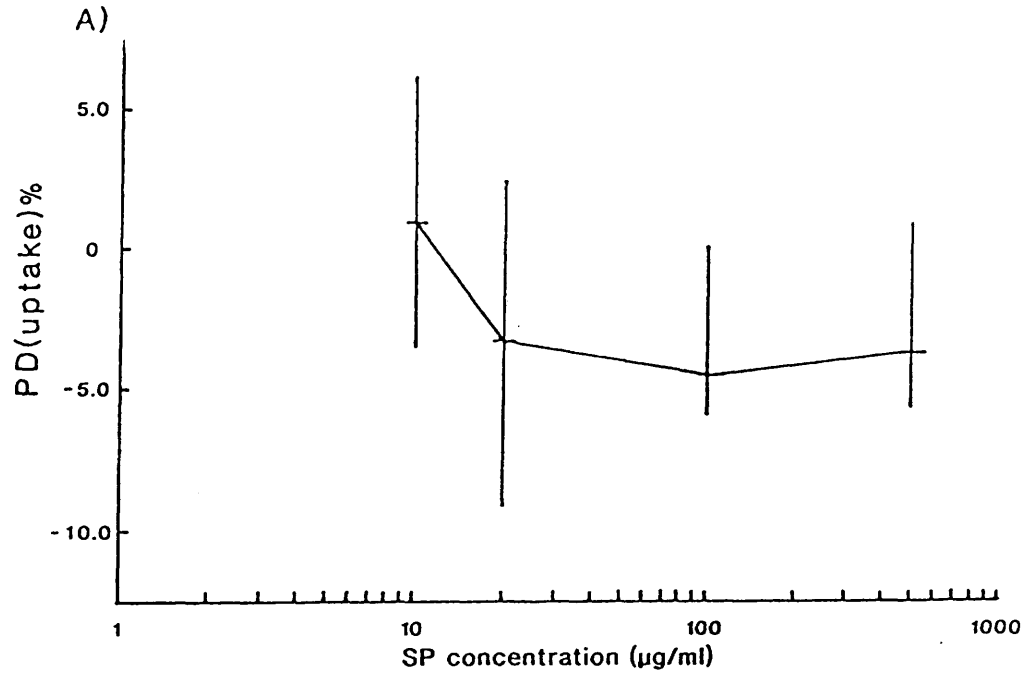
median plus interquartile range (8 experiments)

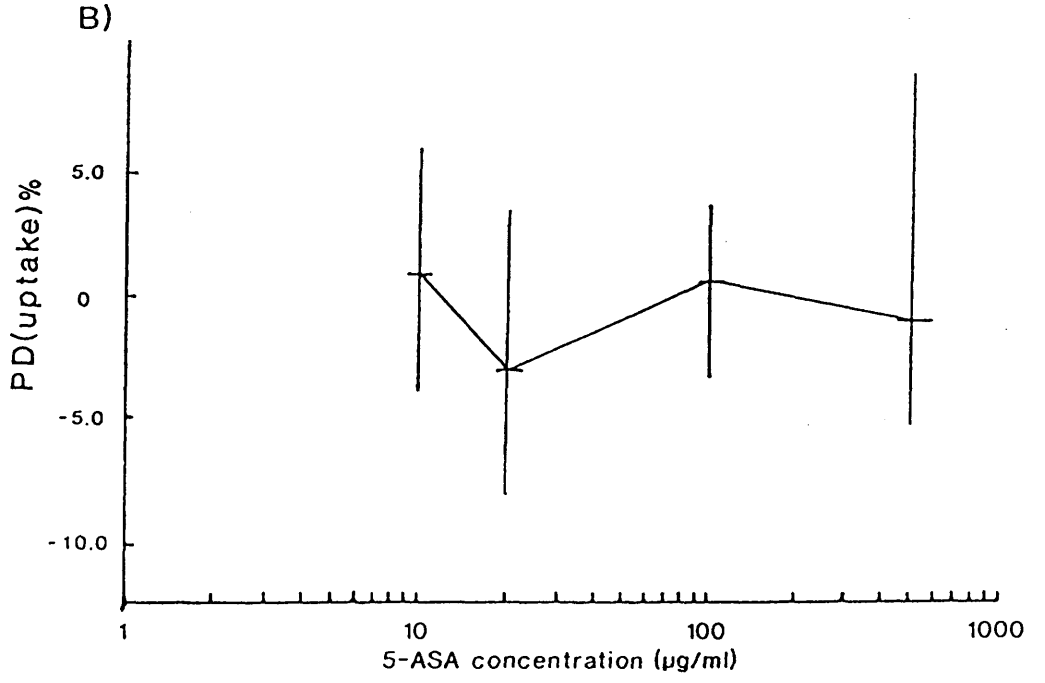
PD : phagocytic differential
PD (uptake) = % uptake (PMN-SASP) - % uptake (PMN-GH)

PMN : polymorphonuclear leucocyte
SASP : sulphasalazine
GH : gel-hanks

No significant difference between concentrations. Wilcoxon rank test.

Fig. XV Effect of A) sulphapyridine and B) 5-aminosalicylic acid on polymorphonuclear phagocytosis.

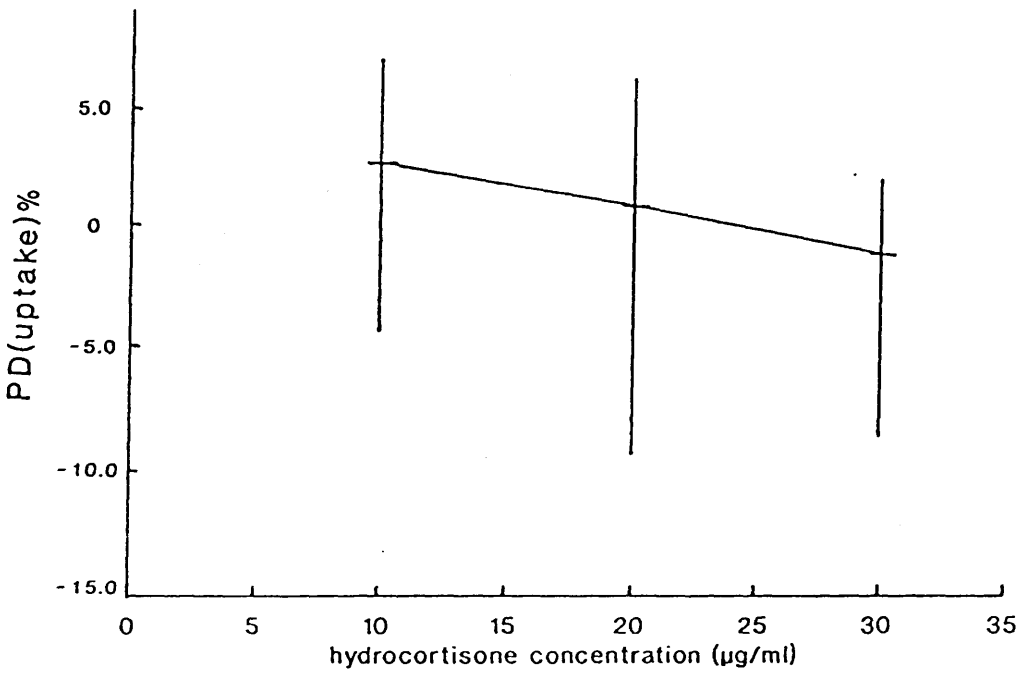




median plus interquartile range (8 experiments)

PD : phagocytic differential
PD (uptake) = % uptake (PMN-drug) - % uptake (PMN-GH)
PMN : polymorphonuclear leucocyte
GH : gel-hanks
SP : sulphapyridine
5-ASA : 5-aminosalicylic acid
No significant difference between concentrations. Wilcoxon rank test.

Fig. XVI Effect of hydrocortisone on polymorphonuclear phagocytosis.



┼ median plus interquartile range (8 experiments)

PD : phagocytic differential
PD (uptake) = % uptake (PMN-drug) - % uptake (PMN-GH)

PMN : polymorphonuclear leucocyte
GH : gel-hanks

No significant difference between concentrations. Wilcoxon rank test.

Indomethacin, at a concentration of 1μg/ml, had no effect on normal PMN phagocytosis.

2.3.2 Comparison of the phagocytic function of peripheral blood polymorphonuclear cells from rheumatoid arthritis patients and controls

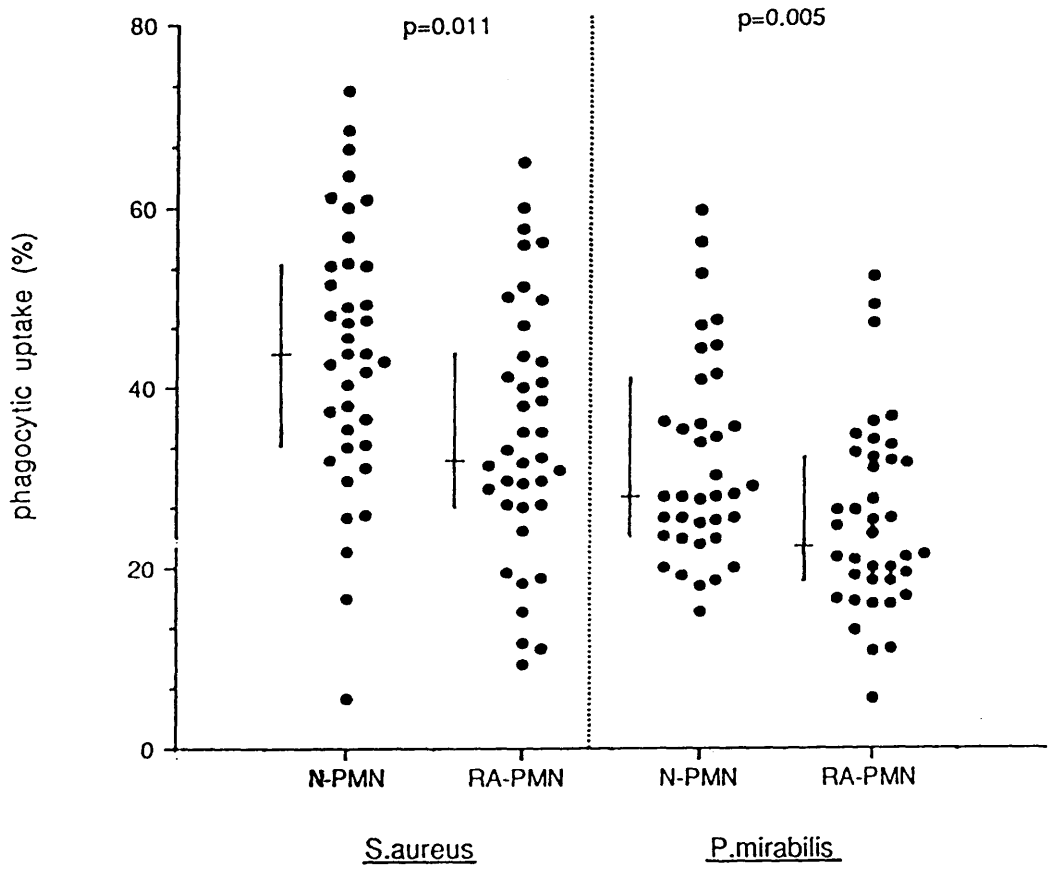
As shown in Fig. XVII, the phagocytic capacity of PB-PMNs from RA patients was significantly impaired, in the case of S. aureus ($p=0.011$) and P. mirabilis ($p=0.005$). No significant correlation could be found between the duration of disease, age, or drug therapy of the patients, and defective phagocytosis by their PB-PMNs.

A similar study using PMNs from controls showed a poor correlation between age and PMN uptake (Fig. XVIII) ($rs=0.416$). However, this study showed an upward trend in uptake up to approximately 50 years of age, thereafter uptake dropped slightly and remained relatively constant. Phagocytosis by PMNs from other age groups was by no means impaired when compared with PMNs from the younger age groups.

2.3.3 Effect of rheumatoid factor on phagocytosis by normal polymorphonuclear leucocytes

There was no significant difference in the phagocytic uptake of S. aureus by normal PMNs pretreated with various concentrations of RF for 30 minutes or 60 minutes (Table 2.8: $p>0.05$). No dose-dependent response was detected.

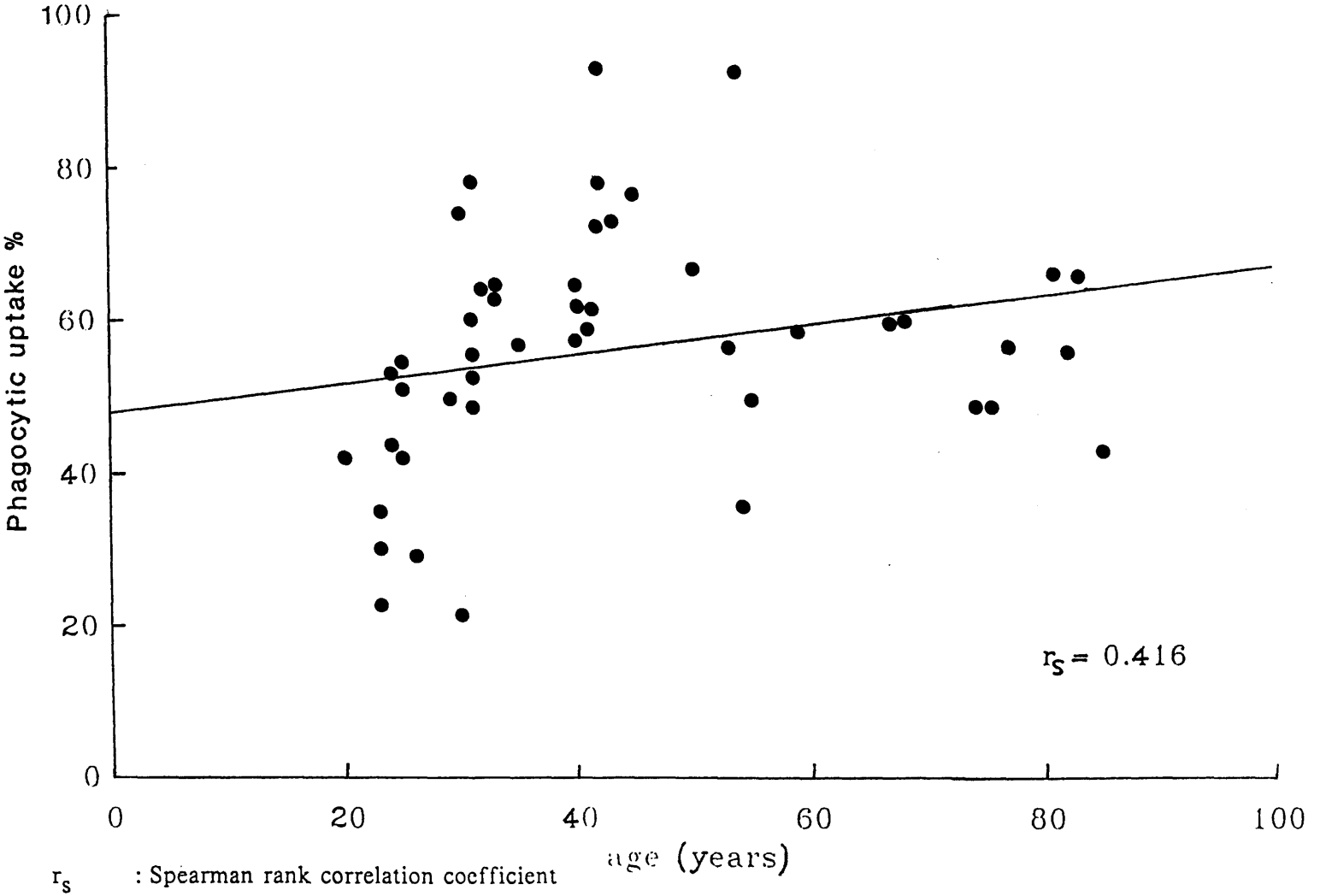
Fig. XVII Phagocytic uptake of S. aureus and P. mirabilis by polymorphonuclear leucocytes from normals and patients with rheumatoid arthritis.



median plus interquartile range

N : normal
RA : rheumatoid arthritis
PMN : polymorphonuclear leucocyte
S. aureus : Staphylococcus aureus
P. mirabilis : Proteus mirabilis
P : significance level. Mann Whitney U test

Fig. XVIII Correlation between age and phagocytic uptake of S. aureus.



r_s : Spearman rank correlation coefficient

RF (IU/ml)		Incubation time (minutes) 30	60
		PD uptake (%)	
25	*	-2.4	4.3
	**	(-6.5,8.9)	(-4.11,11.0)
50		-3.8	1.7
		(-6.2,13.8)	(-1.7,8.2)
75		4.1	2.9
		(-2.3,15.2)	(-4.1,8.8)
100		1.0	1.1
		(-2.1,11.9)	(-6.3,6.5)
125		-1.1	2.7
		(-3.7,12.4)	(-2.0,6.3)

* Median.
** Range.
RF: rheumatoid factor.
PD: phagocytic differential.

PD (uptake) = % $\text{uptake}_{(\text{PMN-RF})}$ - % $\text{uptake}_{(\text{PMN-GH})}$

PMN: polymorphonuclear leucocytes.
GH: gel-hanks.
IU: international units.

No significant difference between 30 and 60 minute incubations, $p>0.05$.
No significant difference between any two concentrations, $p>0.05$.
Mann Whitney U test.

Table 2.8 Effect of rheumatoid factor on phagocytic uptake of Staphylocuccus. aureus by normal polymorphonuclear leucocytes

2.3.4 Effect of serum on phagocytosis by polymorphonuclear cells from rheumatoid arthritis patients and normals

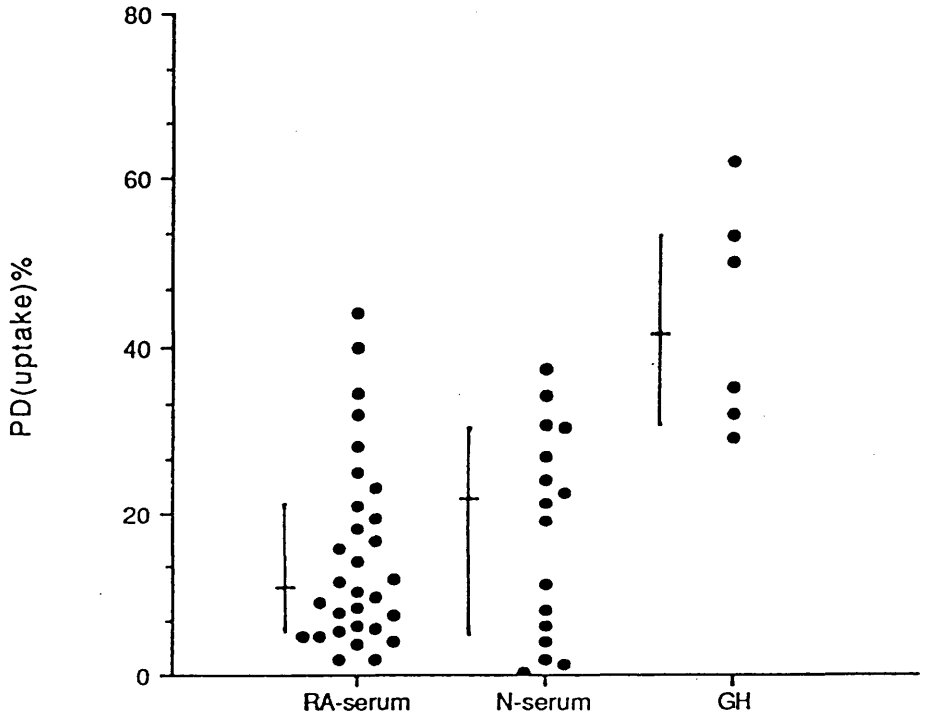
As shown in Fig. XIX, the phagocytic capacity of normal PB-PMNs, pre-incubated for 60 minutes in 50% serum from 36 RA patients, did not differ significantly from PMNs pretreated with 50% normal serum ($p>0.05$). However, both groups showed significantly depressed phagocytosis of S. aureus when compared to PMNs incubated with GH alone (patient serum, $p=0.001$; normal serum, $p=0.003$).

No significant difference of normal PMN or RA PMN function was observed between RF positive, RF negative or normal serum (Fig. XX: $p>0.05$; Fig. XXI: $p>0.05$).

Significantly higher IC levels were detected in the RA serum (IgG: median 34.5μg/ml, range 0-357μg/ml, $p<0.001$; IgM: median 69μg/ml, range 0-221μg/ml, $p<0.001$) than in normal serum (IgG: median 0μg/ml, range 0-58μg/ml; IgM: median 0μg/ml, range 0-41μg/ml). In an extended study, 20 out of 48 (IgG: 42%) and 15 out of 48 (IgM: 31%) were outwith the normal range. No significant correlation was found between RF titre and either IgG ($rs=0.192$, $p>0.05$) or IgM ($rs=0.311$, $p>0.05$) IC concentrations. Comparison of uptake of bacteria by normal PMN or RA-PMN with serum IC levels revealed no significant correlation.

The results of quantitative Ig, complement and CRP determination in RA serum revealed a wide range of values (Fig. XXII). Immunoglobulin A, C3c, C4 and CRP concentrations were significantly higher than normal serum values (IgA: $0.01<p<0.05$; C3c: $0.001<p<0.01$; C4: $0.001<p<0.01$; CRP: $p<0.001$). Serum

Fig. XIX Effect of serum on phagocytic uptake of S. aureus by normal polymorphonuclear leucocytes.

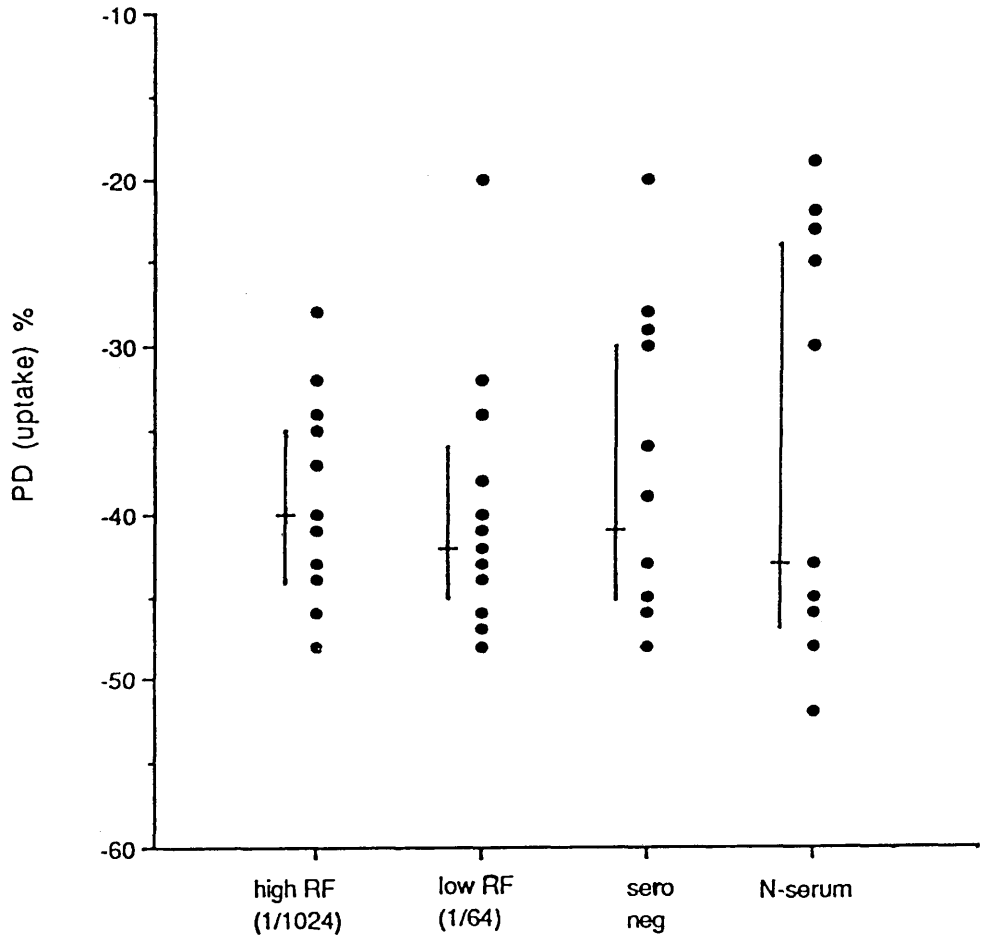


median plus interquartile range

RA : rheumatoid arthritis
N : normal
GH : gel-hanks
S. aureus : Staphylococcus aureus

Significant difference between RA-serum and GH: p=0.001, and N-serum and GH: p=0.003, but no significant difference between RA-serum and N-serum: p=0.05. Mann Whitney U test.

Fig. XX Effect of seropositive and seronegative serum on phagocytic uptake of S. aureus by normal pholymorphonuclear leucocytes.

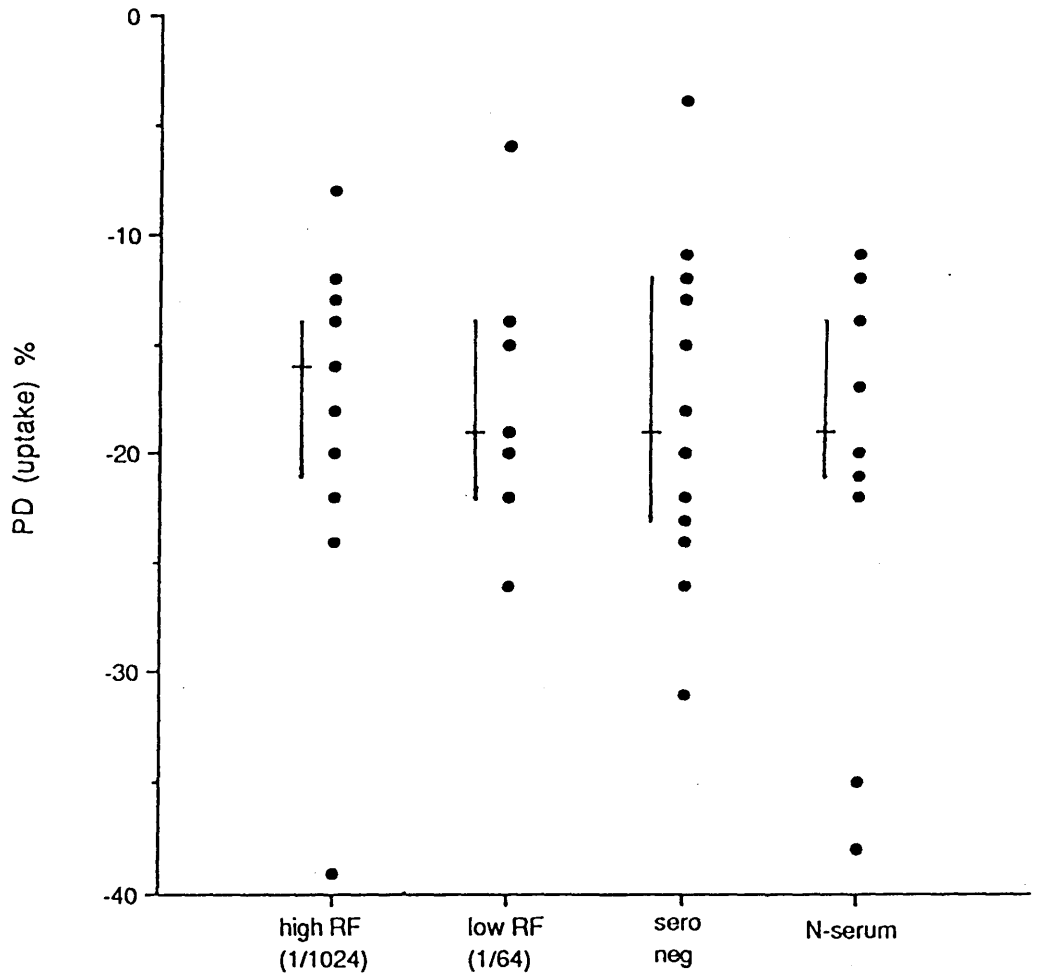


median plus interquartile range

RF : rheumatoid factor
Sero neg : serum with no detectable rheumatoid factor
N : normal
PD : phagocytic differential
PD (uptake) : % uptake (PMN-serum) - % uptake (PMN-GH)
PMN : polymorphonuclear leucocytes
GH : gel-hanks

No significant difference between groups: $p>0.05$.
Mann Whitney U test.

Fig. XXI Effect of seropositive and seronegative serum on phagocytic uptake of S. aureus by polymorphonuclear leucocytes from patients with rheumatoid arthritis.



median plus interquartile range

RF : rheumatoid factor
Sero neg : serum with no detectable rheumatoid factor
N : normal
PD : phagocytic differential
PD (uptake) : % uptake (PMN-serum) - % uptake (PMN-GH)
PMN : polymorphonuclear leucocytes
GH : gel-hanks

No significant difference between groups: $p>0.05$.
Mann Whitney U test.

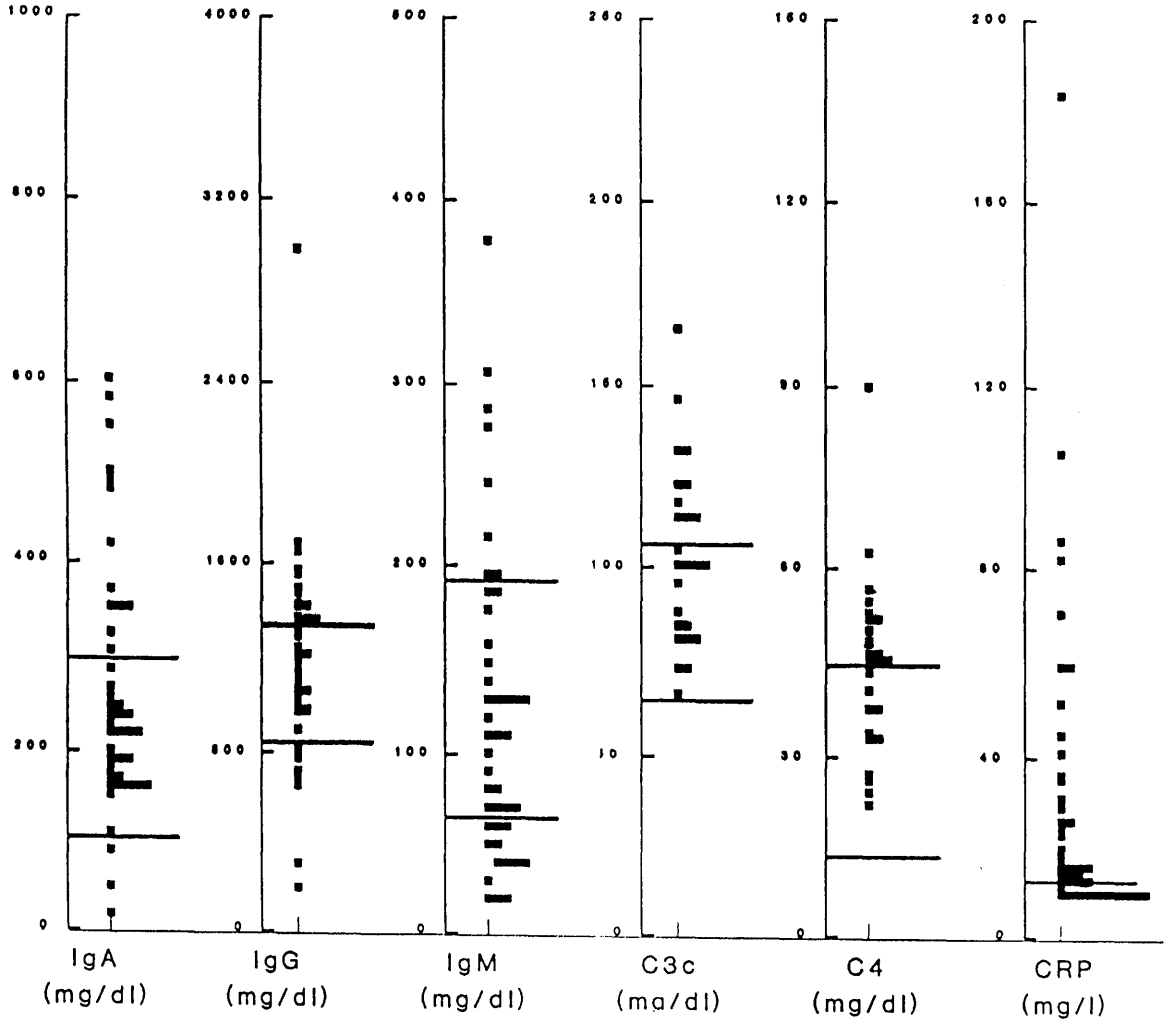


Fig. XXII Immunoglobulin, complement, and C-reactive protein concentrations in serum from patients with rheumatoid arthritis.
Horizontal bars represent normal serum range. CRP: C-reactive protein

IgG and IgM from patients with RA, were comparable to levels detected in normal serum. Overall, no parameter was found to be significantly lower in the RA group compared to the normal group studied, although 4, 5 and 10 individual patients showed reduced IgA, IgG and IgM respectively.

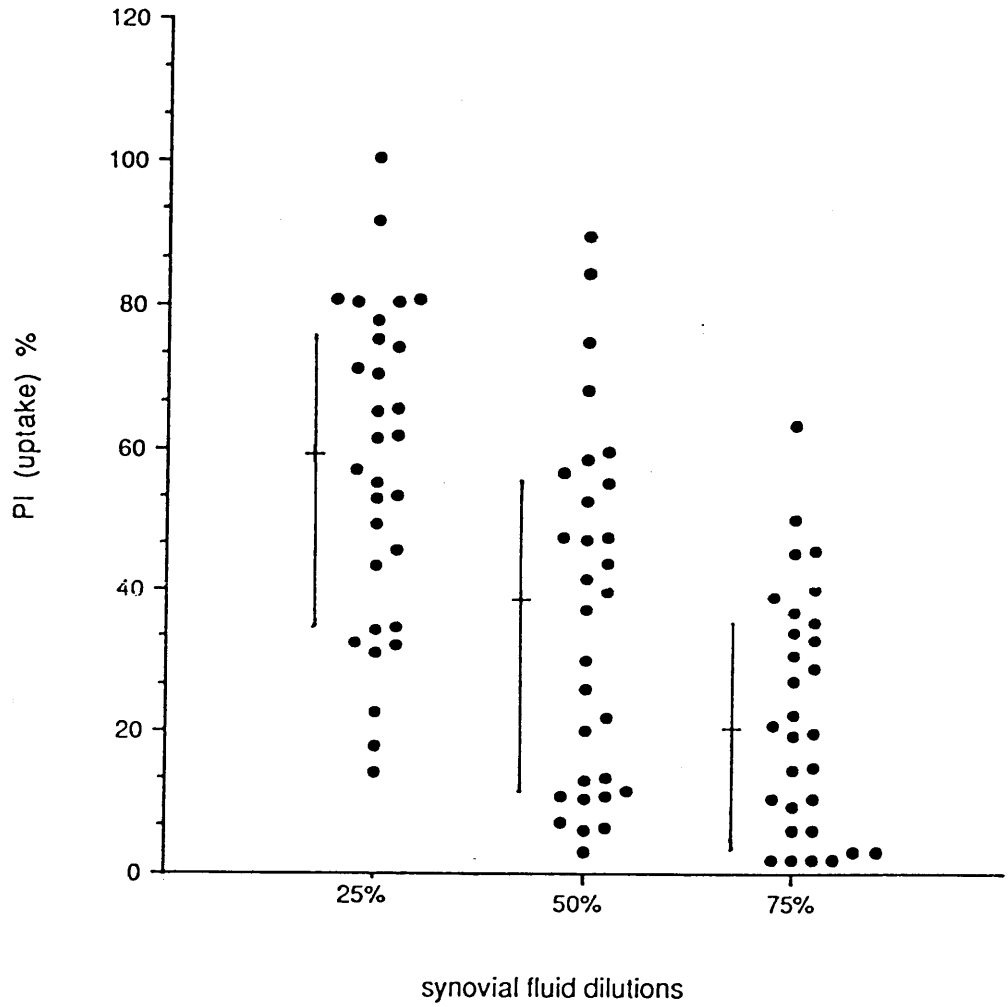
Comparison of the serum mediated effect on phagocytosis by normal or RA PMNs with immunological parameters measured in the serum revealed no correlation.

2.3.5 Effect of synovial fluid on the phagocytosis of Staphylococcus aureus

Synovial fluid from 30 RA patients inhibited the uptake of S. aureus by normal PB-PMNs in a dose-dependent fashion (Fig. XXIII). It was observed that this inhibitory activity was not specific to RA SF. A similar but less striking inhibition was recorded with SF from 15 patients with other arthritic diseases (Table 2.9). However, there was significantly greater inhibition of uptake induced by 75% SF from RA patients compared with 75% SF from other arthritides (Fig. XXIV, $p=0.0001$). Synovial fluid from patients with RA had a similar dose-dependent inhibitory effect on RA PB-PMNs as on normal PB-PMNs. The inhibition induced by 75% SF was significantly greater in normal PMNs compared to RA-PMNs (Fig. XXV, $p<0.001$).

Immunological parameters measured in RA SF revealed a wide range of values (Fig. XXVI). As illustrated, IgG and IgM were found to be significantly lower in SF compared to normal serum concentrations (IgG, $p=0.027$; IgM, $p=0.003$), whereas IgA did not differ significantly from the normal range ($p>0.05$). The values of CRP were significantly higher in 30 out of 37 (81%) SF tested

Fig. XXIII Effect of synovial fluid from patients with rheumatoid arthritis on phagocytic uptake of S. aureus by normal polymorphonuclear leucocytes.



┼ median plus interquartile range

S. aureus : Staphylococcus aureus
PI : phagocytic index
PI (uptake) = $\frac{\%\ \text{uptake (PMN-SF)}}{\%\ \text{uptake (PMN-GH)}} \times 100$

PMN : polymorphonuclear leucocyte
SF : synovial fluid
GH : gel-hanks

Significant difference between 25% and 50% SF: p=0.002, 25% and 75% SF: p<0.001, and 50% and 75% SF: p=0.014.
Wilcoxon rank test.

SF concentration		PI (Uptake) %
25%	*	56.65
	**	(9.45 - 97.66)
50%		57.76
		(13.30 - 79.43)
75%		47.43
		(21.36 - 93.00)

* median
** range

SF: Synovial fluid,
PI: phagocytic index

$$\text{PI (uptake)} = \frac{\text{\% uptake (PMN-SF)}}{\text{\% uptake (PMN-GH)}} \times 100$$

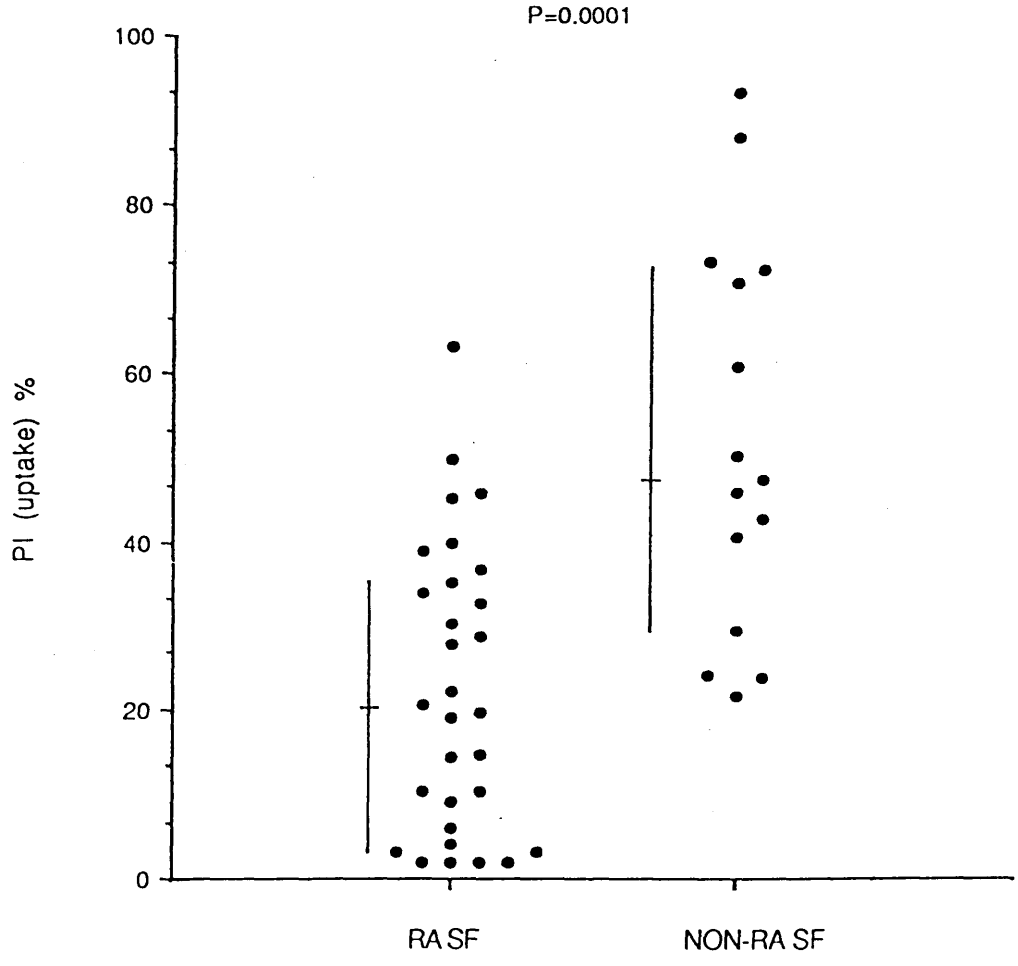
PMN: polymorphonuclear leucocyte

GH: gel-hanks

No significant difference between 25% and 50% SF ($p>0.05$), 50% and 75% SF ($p>0.05$) or 25% and 75% SF ($p>0.05$).
Mann Whitney U test.

Table 2.9 Effect of synovial fluid from patients with other arthritic diseases on phagocytic uptake by normal polymorphonuclear leucocytes.

Fig. XXIV Synovial fluid induced inhibition of phagocytic uptake of S. aureus by normal polymorphonuclear leucocytes.

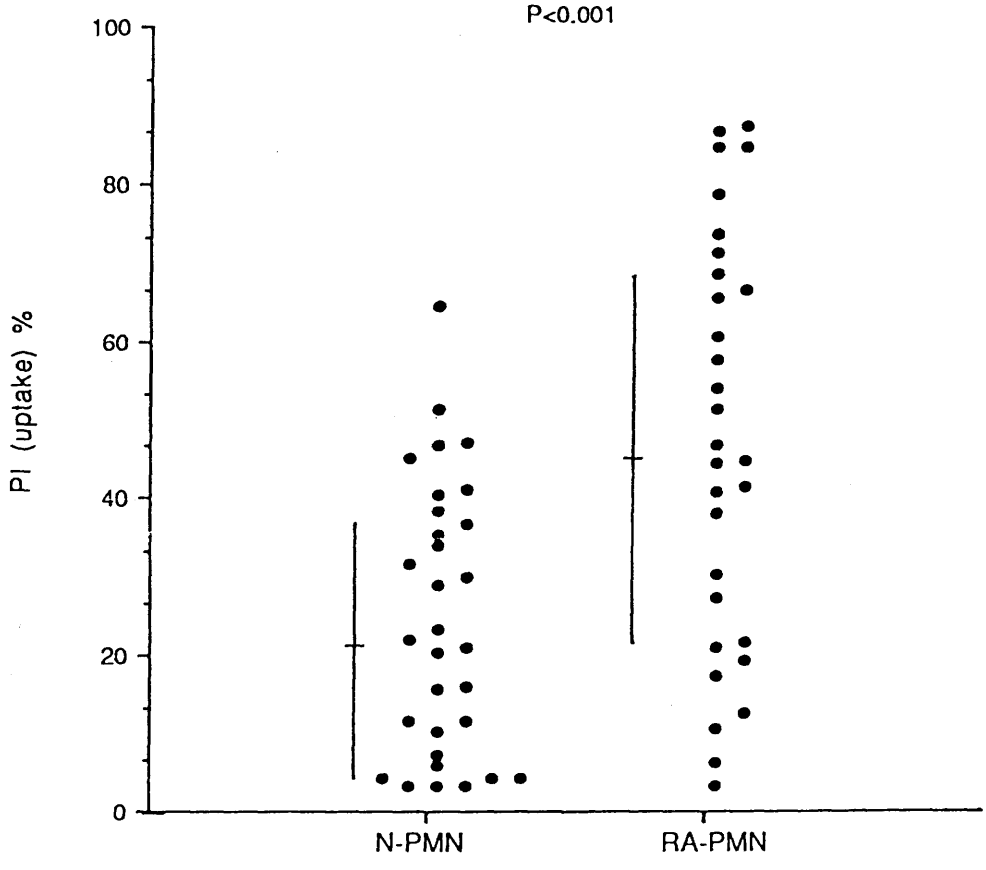


┼ median plus interquartile range

S. aureus : Staphylococcus aureus
RA : 75% rheumatoid arthritis synovial fluid
NON-RA : 75% synovial fluid from patients with other arthritic diseases
PI : phagocytic index
PI (uptake) = $\frac{\text{\% uptake (PMN-SF)}}{\text{\% uptake (PMN-GH)}}$ x100

PMN : polymorphonuclear leucocyte
SF : synovial fluid
GH : gel-hanks
P : significance level determined by Mann Whitney U test

Fig. XXV Synovial fluid induced inhibition of phagocytic uptake of S. aureus: normal PMNs versus rheumatoid arthritis PMNs.



median plus interquartile range

S. aureus : Staphylococcus aureus
N : normal
PMN : polymorphonuclear leucocyte
RA : rheumatoid arthritis
PI : phagocytic index
PI (uptake) = $\frac{\text{\% uptake (PMN-SF)}}{\text{\% uptake (PMN-GH)}} \times 100$

SF : 75% synovial fluid from patients with RA
GH : gel-hanks
P : significance level determined by Mann Whitney U test

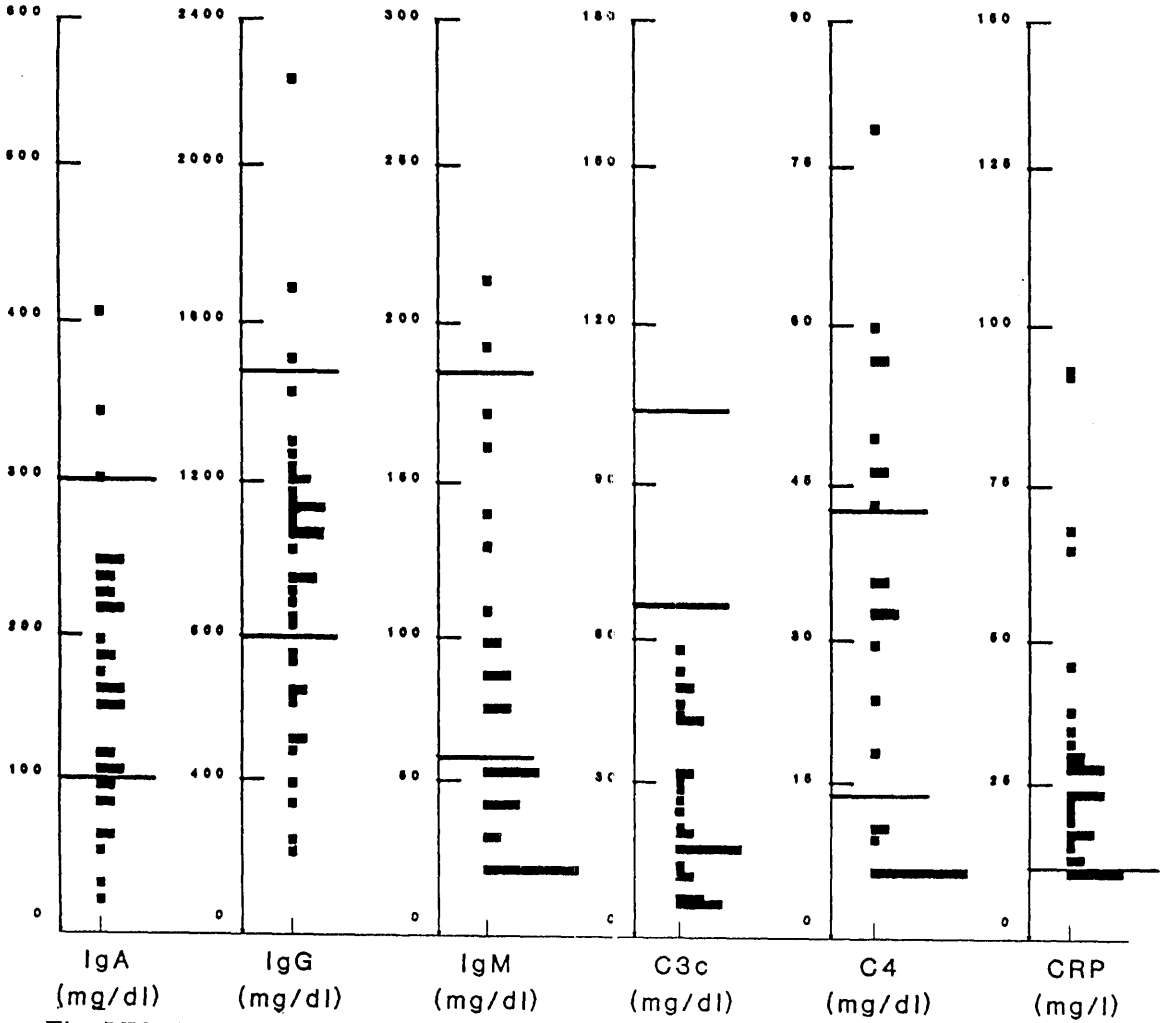


Fig. XXVI Immunoglobulin, complement, and C-reactive protein in synovial fluid from patients with rheumatoid arthritis. Horizontal bars represent normal serum range. CRP: C-reactive protein

($p<0.0001$). C3c was found to be significantly lower than the normal range in all of the samples tested ($p<0.0001$). Although 25 out of 43 (58%) of C4 results were lower than the normal range, this did not reach significance ($p>0.05$).

No correlation was found between inhibition of uptake by normal or RA PMNs and the pH of SF, titres of RF, IgG or IgM ICs, C3c, C4, IgG, IgA, IgM or CRP in the SF.

2.3.6 Opsonic activity of serum

The concentration of C3c and C4 was not significantly different in serum from RA patients compared to control serum (Fig. XXII, $p>0.05$). Also, there was no significant difference between the uptake of S. aureus opsonised with patient serum or normal serum (Table 2.10: $p>0.05$). Bacteria opsonised with heat-inactivated normal or RA serum were significantly less phagocytosed than those opsonised with the corresponding untreated sera (normal serum: $p<0.001$; RA serum: $p<0.001$). There was no significant difference between phagocytic uptake of S. aureus opsonised with heat-inactivated normal or RA serum ($p>0.05$).

2.3.7 Opsonic activity of synovial fluid

The C3c and C4 concentrations were below the normal serum range in the majority of SF tested (Fig. XXVI). The concentration of C3c in SF from RA and non-RA patients was significantly lower than normal serum values ($p<0.001$ and $p=0.001$ respectively). The median value of complement levels in fluids from patients with RA was lower than that in a control group of patients with

OPSONIC ACTIVITY

Uptake of Staphylococcus aureus (%)

		Untreated	Inactivated	p
RA serum	*	29.51	5.03	<0.001
	**	(23.23 - 35.22)	(3.48 - 8.85)	
Normal serum		24.44	3.34	<0.001
		(20.46 - 29.96)	(0.61 - 8.04)	
RA SF		12.80	10.30	<0.001
		(5.64 - 32.06)	(4.87 - 15.99)	
Non-RA SF		18.84	8.68	>0.05
		(13.40 - 32.28)	(3.65 - 20.36)	

* median
** interquartile range
RA: rheumatoid arthritis
SF: synovial fluid
p: significance value determined by Mann Whitney U test for unpaired and Wilcoxon test for paired data
RA SF: RA serum; p = 0.003
Non-RA SF: RA serum: p = 0.002
Inactivated RA SF: Inactivated normal serum; p = 0.004
Inactivated Non-RA SF: Inactivated normal serum; p = 0.007
Inactivated RA SF: Inactivated RA serum; p = 0.03
Inactivated Non-RA SF: Inactivated RA serum; p = 0.04

Table 2.10 Opsonic activity of serum and SF from patients with and without RA and controls

other forms of arthritis, although this difference did not reach significance (Table 2.11: C3c, $p>0.05$; C4, $p>0.05$). There was no significant difference between the opsonic activity of untreated or heat-inactivated synovial fluids from patients with and without RA (Table 2.10: $p>0.05$). Also, there was no significant difference in opsonic activity of RA SF or non-RA SF when compared to normal serum ($p>0.05$). However, this opsonic activity was significantly less than that of RA serum (RA SF: $p=0.003$; non-RA SF: $p=0.002$). There was a significant increase in the phagocytic uptake of S. aureus opsonised with inactivated SF compared with inactivated normal serum (Table 2.10: RA SF, $p=0.004$; non-RA SF, $p=0.007$) and inactivated patient serum (RA SF, $p=0.03$; non-RA SF, $p=0.04$).

Opsonic differentials (OpD) were calculated for RA and non-RA fluids and compared with those for normal serum.

$$\text{OpD (uptake)} = \begin{array}{c}\text{\% uptake of S. aureus}\\ \text{opsonised}\\ \text{with untreated}\\ \text{serum or SF}\end{array} - \begin{array}{c}\text{\% uptake of S. aureus}\\ \text{opsonised}\\ \text{with inactivated}\\ \text{serum or SF}\end{array}$$

The OpD calculated for RA SF were significantly lower than those calculated for normal serum (Table 2.12; $p=0.03$), but not those calculated for non-RA SF ($p>0.05$). There was no significant difference between OpD calculated for non-RA SF and normal serum ($p>0.05$).

Synovial fluid		C4 (mg/dl)	C3c (mg/dl)
	*	22.0 - 55.0	55.0 - 120.0
RA	**	7.5	19.0
	***	(6.0 - 30.0)	(12.5 - 35.0)
Non-RA		11.0 (7.0 - 37.0)	34.5 (33.0 - 43.0)

* normal serum range
** median
*** range
RA: rheumatoid arthritis
Non-RA: patients suffering from other arthritic diseases.
SF: synovial fluid
RA SF and normal serum: $p<0.001$; Non-RA SF and normal serum: $p=0.001$

No significant difference between the concentrations of C4 ($p>0.05$) or C3c ($p>0.05$) in synovial fluids from RA and non-RA patients. Mann Whitney U test.

Table 2.11 Concentration of complement components in synovial fluid

opsonin	OpD (uptake) %	
RA SF	*	10.99
	**	(4.75 - 25.03)
Non-RA SF		17.62 (14.23 - 29.06)
normal serum		18.87 (16.17 - 26.34)

* median
** interquartile range
OpD: opsonic differentials
OpD(uptake) = % uptake of S. aureus opsonised with untreated serum or SF - % uptake of S. aureus opsonised with inactivated serum or SF
SF: synovial fluid
RA: rheumatoid arthritis

Significant difference between RA SF and normal serum ($p=0.03$)
No significant difference between RA SF and Non-RA SF ($p>0.05$)
or Non-RA SF and normal serum ($p>0.05$). Mann Whitney U test.

Table 2.12 Opsonic differentials of rheumatoid arthritis and non-rheumatoid arthritis synovial fluid and normal serum

2.4 CONCLUSIONS

In vitro cell assay systems were utilised throughout, as these have several advantages over in vivo methods: some of the clinical and immunochemical variables inherent in patient-orientated studies can be eliminated, known homogenous granulocyte cell populations can be used, the effect of serum and SF on PMN phagocytosis can be controlled, and the process of phagocytosis and intracellular killing can be assessed independently of each other, if desired.

2.4.1 Effect of drugs on PMN phagocytosis

This study was performed to assess whether any relationship exists between the reactivity of PMNs and drug therapy. Since RA is characterised by chronic inflammation with a vast accumulation of PMNs at sites of joint inflammation it is possible that, by changing PMN function with drugs know to inhibit or stimulate PMNs, the activity of the disease may be modified. However, clinicians must be careful not to prescribe such high doses which would suppress PMN phagocytic function to such an extent that would render the patient more susceptible to bacterial infection.

Tenidap [(z)-5-chloro-3, 2-dihydro-3-(hydroxy-2-thienyl methylene)-2-oxo-1H-indole-1-carboxamide], a novel NSAID, possesses good anti-inflammatory-analgesic activity in laboratory models, possibly by impairing chemotaxis of PMNs into the inflammatory site and it has been found to inhibit arachidonic acid-induced PMN degranulation (Otterman, Personal communication). In the present study, a dose-dependent inhibition of uptake of S. aureus was observed, compared to untreated PMNs. Although this impairment did not reach

significance, it is possible that higher concentrations of tenidap will be detrimental to PMN phagocytosis. To the best of my knowledge, no other studies concerning this aspect of tenidap activity have been published.

In in vitro studies of PMNs from healthy subjects, SASP inhibited random and chemotactic migration but had no effect on phagocytosis (Molin and Stendahl, 1979). Furthermore, long term treatment with SASP has shown depressed PMN chemotaxis but no consistent change in PMN phagocytic function (Hermanowicz et al., 1985). The present study confirms that over a wide concentration range (10μg/ml-500μg/ml), SASP, SP or 5-ASA had no significant inhibitory effect on normal PMN phagocytic activity. The impaired chemotactic response of PMNs pre-incubated with SASP, will attribute to the anti-inflammatory property of this drug. The efficacy of the antibacterial component of SASP permits speculation about the role of a bacterial pathogen in the aetiopathogenesis of rheumatoid disease.

Indomethacin has proved, at least, in vitro to be a very potent anti-inflammatory drug. The present in vitro study used indomethacin at a concentration of 1μg/ml, such as that which occurs in the blood plasma during normal drug therapy, and no impairment of PMN phagocytosis was observed. This is in agreement with the findings of Youinou and Le Goff (1987). In contrast, Hannelore, Stelzner and Kunze (1984), found that indomethacin dissolved in ethanol clearly inhibited the phagocytosis of both S. aureus and Escherichia coli. This inhibition was significant at concentrations of $1x10^{-4}$ and $2x10^{-4}$M/l (equivalent to 35.78 and 71.56μg/ml respectively). In clinical practice indomethacin is often given in multiple divided doses at 6 or 12 hour intervals (50-200mg maximum). Emori et al.(1973) found a mean peak serum level of 2.88μg/ml, for SF it was lower, 0.69μg/ml (1 and 2 hours respectively

following a single 50mg dose). In a later study, Emori et al. (1976) showed that with multiple dose schedules plateau levels were reached with maximum peak levels not exceeding 2μg/ml. Therefore, it is unlikely that a patient on the maximum dose of 200mg (4x50mg) is going to develop serum or SF concentration of 35μg/ml due to the short half-life of indomethacin (approx. 5 hours). The results of Hannelore et al. (1984) are therefore not surprising; indomethacin at such high concentrations are perchance toxic to the PMN, resulting in cell death. The use of indomethacin in children was curtailed in 1967 on the basis of a report that high doses might impair responses to infection, with serious consequences (Jacobs, 1967). Such findings have not been reported since.

Non-steroidal anti-inflammatory drugs certainly influence the behaviour of PMN cells. The impairment of lysosomal enzyme release, demonstrated by Northover (1977) in the case of indomethacin, and the inhibition of neutral proteases (Kruze et al., 1976) are no doubt important anti-inflammatory mechanisms. The general consensus however, is that indomethacin does not impair PMN phagocytosis.

It is possible that the vast majority of drugs that interfere with host defence mechanisms do so not by influencing phagocytosis or bacterial killing but by injuring haematopoietic stem cells in the bone marrow or adversely affecting the function of differentiated lymphoid cells. Since phagocytosis is augmented by bacterial opsonisation, interference by drugs with Ab production is ultimately reflected in diminished phagocytic function.

It must be emphasised that results obtained in vitro do not always reflect what happens in vivo. This reinforces the need to study the effect of drugs in vivo

and thus to focus attention on patient's PMNs, taking into account their current drug treatment. Patients with chronic RA are often treated with multiple-drug regimens which complicates the interpretation of in vivo studies. Other factors must also be taken into consideration; such as frequency of administration, dosage and food intake which may have important modifying effects on serum drug concentrations and consequently PMN function. It is probable that some of the conflicting results in the literature, may be explained by varying drug regimens.

The majority of patients in the following studies were either treated with NSAIDs or '2nd line' drugs. Since SASP and indomethacin did not significantly impair PMN phagocytosis, drug interference by some of the commonly used anti-rheumatic drugs does not seem to be responsible for the impaired phagocytosis observed with PMNs from patients with RA, in this study.

2.4.2 Peripheral blood polymorphonuclear phagocytosis

It has previously been suggested that the defective capacity of PMNs in RA is due to intrinsic cellular defects (Attia et al., 1982a). Using a radiometric assay, the present study provides presumptive support for the theory of an intrinsic defect in PMN phagocytosis. This study further demonstrates that the defect is not specific for gram positive organisms but also encompasses gram negative bacteria.

This PMN phagocytic impairment is in agreement with the findings of Corberand et al. (1977) and Wilton et al. (1978). However, other investigators found no anomaly of PB-PMN phagocytosis (Brandt and Hedberg, 1969;

Sheehan et al., 1984; Breedveld et al., 1985). Previous contradictory reports can be ascribed to differences in techniques employed and also to the use of different micro-organisms (baker's yeast, Candida albicans), since all micro-organisms have specific opsonic requirements, (van Furth, Leijh, and Klein, 1984). It has been suggested that the failure to remove autologous sera in some studies (Brandt and Hedberg, 1969; Corberand et al., 1977; Bültmann et al., 1980) makes interpretation of their results difficult and thus, the intrinsic phagocytic ability of PMNs cannot be distinguished from the effect of serum upon the PMNs. Furthermore, some researchers failed to separate the processes of opsonisation and phagocytosis (Brandt and Hedberg, 1969; Turner et al., 1973; Sheehan et al., 1984). In this study S. aureus and P. mirabilis were pre-opsonised, PMNs were suspended in GH, and the assay performed in a serum free milieu. The results therefore suggest an intrinsic PMN defect. Another possible explanation could be that the PB-PMNs from RA patients have migrated from the SF and have not had sufficient time to fully recover from the inhibitory activity of the SF (section 2.4.4). Alternatively, the reduced uptake may indicate either a reduced flow of young PMNs from the bone marrow into the circulation, a rapid elimination of recently formed PMNs or a high proportion of older PMNs at the time of bleeding. The impaired phagocytosis in RA-PMNs is unlikely to be attributed to the treatment per se, in light of the results of the previous study. Of all patients studied only 5 were receiving third line therapy, none were receiving salicylates, hydrocortisone or tenidap, however, they were on NSAIDs. One other possible explanation could be the advancing age of the patients in the study, compared to the control group. Although there was no significant correlation between impaired uptake and drug therapy, disease duration or age, a slightly downward trend in uptake with increasing age was observed. This was not confirmed in a larger

study, which revealed that PMNs from older normal subjects were no less efficient phagocytes than PMNs from young normal subjects.

Vaughan and others (1968) whilst examining blood leucocytes of patients with RA, found Ig inclusions, and a considerable number of them. This would result in interiorisation of Fc receptors, leaving fewer membrane receptors for subsequent attachment of opsonised bacteria. This could be partly responsible for the reduced PMN uptake observed.

The majority of investigators used subjective techniques for measuring phagocytosis (Bodel and Hollingsworth, 1966; Brandt and Hedberg, 1969; Turner et al., 1973; Bültmann et al., 1980; Breedveld et al., 1985), such as microscopic assays which have several limitations: the small number of PMNs examined may be unrepresentative of the behaviour of the PMN population as a whole, it is impossible to determine whether organisms or particles associated with PMNs have been ingested or are merely adherent to the cell membrane, and microscopic determination is open to interobserver error. By contrast, the radiometric assay described herein is both objective and sensitive. Furthermore, it has been shown, by means of Giemsa-stained preparations and electron-microscopic observations that after being washed twice, PMNs no longer carry any bacteria on their surface (van Furth et al., 1984). In this investigation the PMNs were washed thrice. It is therefore highly likely that the assay system is measuring ingestion and not merely bacterial attachment.

2.4.3 Effect of serum on phagocytosis by polymorphonuclear leucocytes

Results of the present study revealed significant impairment of phagocytosis in normal PMNs pre-incubated in serum from normals and patients with RA, compared to untreated PMNs. However, there was no significant difference in PMN uptake whether in the presence of patient serum or normal serum. This is in agreement with the recent observation made by Breedveld et al. (1985). Nor was there any increase of phagocytic activity of patient PMNs when suspended in normal serum compared to patient serum. Furthermore, no significant difference was observed between the effect of seropositive or seronegative serum on phagocytosis.

Disparate results have been reported concerning the effect of serum on phagocytosis (Turner et al., 1973; Attia et al., 1982b; Breedveld et al., 1985) which may be the consequence of different test procedures. In these previous studies PMNs were not pre-incubated with serum, and organisms were opsonised in test serum, before or during the measurement of phagocytosis. By separating opsonisation, pre-treatment of PMNs with test serum and phagocytosis, and washing PMNs and bacteria after each process, as was done in this study, the phagocytic uptake can be attributed to the effect of the serum on the PMNs, and not differences in the opsonic activities of the serum.

It has been suggested that the presence of RF can activate the complement pathway, producing ICs, which can bind to, or be phagocytosed by PMNs, via their C3b receptor. This may interfere with subsequent phagocytosis of complement-coated bacteria. Turner et al. (1973), discovered a significant relationship between the increase in RF titre and the phagocytic capacity decrease of normal PMNs, as was also observed by Hällgren (1978), but not

other investigators (Corberand et al., 1977; Attia et al., 1982b). The findings of the present investigation do not support the theory that RF interferes with phagocytic uptake as no correlation between the two could be detected. Also, normal sera was described as seronegative and the majority contained no detectable ICs of either the IgG or IgM class, yet both normal sera and patient sera inhibited phagocytosis to the same extent. No correlation was found between RF and ICs illustrating that there was no equilibrium between complexed and free RF in the sera tested, nor was there any relationship found between ICs and phagocytic uptake. Furthermore, PMNs in this study were washed after treatment with serum. This process is likely to dislodge any surface bound complexes and consequently reveal free C3b receptors.

The concentration of serum complement in RA has been reported as normal or elevated (Brandt, 1967; Mallya et al., 1982). Measurement of C3c or C3d is a useful means of detecting in vivo complement activation. Increased levels are in agreement with Nydegger et al. (1977) who showed that complement activation is detectable in the circulation of the majority of patients with RA and is not confined to the joint space as previously reported (Versey, Hobbs and Holt, 1973). Others found lower complement values (Hunder and McDuffie, 1973) which accompanied the high incidence of recurrent bacterial infections. Comparable levels of C3c and C4 were detected in serum from RA patients and normals, but like previous studies, no relationship was found between phagocytosis and the complement level, if the latter was normal or increased (Brandt, 1967). Mallya et al. (1982) found a significant correlation between C3d concentration and circulating ICs in their patients, supporting the hypothesis that activation of the complement system may be due to the presence of ICs. This study failed to confirm this.

As non-immune serum was used as opsonin in this study, the majority of bacteria will be coated with C3b, however, approximately 5% of normal uptake occurs when opsonisation is performed using heat-inactivated serum (section 2.4.5). This may represent Fc-mediated phagocytosis arising from the presence of non-specific Abs. Loss of Fc-mediated phagocytosis by PMNs which have fewer/no Fc receptors as a consequence of ingestion of ICs cannot explain such a large decrease in phagocytic uptake as detected in the present study. Depressed phagocytosis observed in this system is most possibly due to masking of C3b receptors by RA or normal serum C3.

2.4.4 Effect of synovial fluid on phagocytosis by polymorphonuclear leucocytes

The S. aureus-PMN system employed here also detected a humoral defect in the SF of patients with RA, significantly greater than in SF of patients with other arthritides. This does not agree with Turner et al. (1973) who reported that phagocytosis of yeast by normal PMNs was depressed equally and significantly in SF from patients with RA, osteoarthritis and miscellaneous arthritides. However, they used the test synovial fluid for opsonisation and failed to separate the processes of opsonisation and phagocytosis. In RA, elevated serum complement levels and depressed joint fluid complement levels, have been reported (Pekin and Zvaifler, 1964), and are in agreement with the results of this study. Since complement enhances phagocytosis, through opsonisation, this difference in complement activity may be responsible for the decreased uptake of Baker's yeast seen in Turner's study, rather than the direct action of SF on the PMNs. Bodel and Hollingsworth (1966) also reported that plasma-containing medium permitted more phagocytosis by both PB-PMNs and SF-PMNs than did the SF-containing medium. Wilton et al. (1978) reported

similar phagocytic inhibition by normal PMNs in SF from RA patients. In the present study, trypan blue staining demonstrated no increased incidence of cell death in the test PMNs as a result of incubation with SF.

Several factors may be implicated to explain this finding. Immune complexes and fluid C3b may be responsible for the inhibitory effect of SF, in a similar fashion, to that described for RA serum. Like RA serum, it is unlikely that the inhibitors responsible in RA SF act by blocking surface receptors as PMNs were washed after incubation with SF. Turner et al. (1973) showed that ingestion of ICs would inhibit subsequent phagocytosis. This would imply that the C3b, or Fc receptors are absent due to internalisation of the PMN membrane, rather than blocked. This is highly feasible since both IgM and IgG ICs were detected in SF from all patients, however no correlation between these and the phagocytic index was detected in this study.

Synovial fluid from patients with RA contains free iron (Gutteridge et al., 1982). Both free iron and ICs stimulate the production of oxygen radicals by PMNs, which prevent phagocytosis, possibly by damaging surface receptors (Starkebaum et al., 1982). Furthermore, joint fluid components such as hyaluronic acid, might impede phagocytosis by interfering with cell-particle association, possibly due to the increased viscosity of the fluid surrounding the PMNs (Brandt, 1984).

The present studies demonstrate that cellular dysfunctions are in part transferable by SF to PMNs of healthy controls, explaining a SF-dependent granulocyte function disturbance in patients with RA, although the inhibitory factor(s) as yet, remain unidentified.

2.4.5 Opsonic activity of serum

Opsonisation of S. aureus was equally effective with patient or donor serum. The concentration of complement and Ig opsonins in the majority of patient sera was within the normal range and the absence of inhibitors of opsonisation was evident by the tantamount ability of RA and normal serum to support bacterial phagocytosis.

2.4.6 Opsonic activity of synovial fluid

This study illustrated significantly less C3c in RA SF than in normal serum. Berkowicz et al. (1983) disagree with this observation; they reported that C3c levels were higher in SF from patients with RA. However, Molenaar et al. (1974) have shown that antisera raised against C3c also binds to antigenic determinants on native C3, thus the C3c levels reported in this study represent the overall concentration of C3, which other investigators have found to be depressed in RA SF (Ruddy and Austen, 1970). Indigenous C3 was removed from the SF by Berkowicz and his colleagues (1983), and therefore they only recorded the concentration of breakdown products of C3. Thus, it appears that SF from patients with RA contain lower total levels of C3, which consist of a higher proportion of C3c than other breakdown products, compared with SF from other forms of arthritis.

The heat-labile opsonic activity of RA SF as measured by the OpD (uptake) was significantly lower than that of normal serum. However, the overall opsonisation of S. aureus was equally effective with normal serum and SF from patients with and without RA. This is in agreement with the findings of

Breedveld et al. (1986). In the present study this was the consequence of an increase in the opsonic activity of inactivated SF compared with inactivated serum, that is, Fc-mediated opsonisation. These Ab opsonins are unlikely to be specific Abs, as no patients had had a recent bacterial infection and no anti-staphylococcal Abs were found in the SF.

Verhoef, Peterson and Quie (1977b) presented evidence that suggested that bacteria opsonised in the presence of complement and Igs are primarily phagocytosed via the complement receptor. The receptor for bacteria opsonised with heat-inactivated serum is specific for the Fc fragment of Ig and appears to play a minor role in the phagocytosis of bacteria opsonised in normal serum. The SF used in this study had a very low concentration of complement and therefore Fc-mediated phagocytosis seems to predominate in this situation.

The in vivo significance of these findings is unclear; the greater uptake of S. aureus opsonised with inactivated SF compared to inactivated serum, suggests a higher IgG content in the SF. It is conceivable that in the rheumatoid joint the Igs could bind to and block the Fc receptors on the PMN membrane, thereby preventing subsequent Fc-mediated phagocytosis. This phenomenon is unlikely to be wholly responsible for the susceptibility to infection seen in RA patients. Nevertheless, in conjunction with a depressed C3b opsonic activity of SF and a possible intrinsic PMN defect, this could result in a detrimental impairment of phagocytosis. Furthermore, the SF tested were observed to be of high viscosity, which may also be detrimental in vivo, causing clumping of PMNs, and preventing sufficient contact between PMNs and bacteria.

In this assay system, opsonisation with SF may have caused aggregation of S. aureus. By clumping together, more bacteria can be phagocytosed via a single

receptor, which could compensate for the decrease in C3b production. Therefore, the fact that there was no significant difference between the opsonic activity of normal serum and SF from RA and non-RA patients may be due to the increased viscosity of SF compared to serum.

In summary, the results of this study suggest, 1) the possibility of an intrinsic cellular phagocytic defect, 2) SF has an inhibitory activity on PMN phagocytosis in the rheumatoid joint and 3) a significantly lower concentration of C3c detected in SF as compared to serum may consequently result in less C3b-mediated phagocytosis in the joint. Some S. aureus strains (eg. Cowan) possess an outer layer called protein A, which renders the bacteria more resistant to phagocytosis unless adequately opsonised. The foregoing may explain, at least in part, the susceptibility of patients with RA to intraarticular infections (most commonly S. aureus) as first described by Kellgren et al. (1958).

CHAPTER THREE - STANDARDISATION OF AN INTERLEUKIN 1 BIOASSAY

3.1 INTRODUCTION

Mononuclear phagocytes are important contributors to the immune response as previously discussed in Chapter 1. A variety of stimulants including bacteria, viruses, immune complexes, and lymphokines can induce monocytes to synthesise a number of soluble factors that contribute to host defence mechanisms (Dinarello, 1984a) including TNF, colony stimulating factor, interferon and IL-1 (Dale, 1981). The most potent stimulator for IL-1 production is bacterial endotoxin, which has been shown to be active down to a concentration of 50pg/ml (Duff and Atkins, 1982). Whole bacteria such as Staphylococcus albus (now designated S. epidermidis) also have been shown to stimulate in low numbers; as few as 10 bacteria per monocyte will stimulate IL-1 production (Gearing, Johnstone and Thorpe, 1985).

Interleukin 1, which is thought to be identical to endogenous pyrogen (EP), is a cytokine produced predominantly by mononuclear phagocytes (Gearing et al., 1985) although it is produced by several mammalian cell types. Two forms of human IL-1 (alpha and beta) are encoded by separate genes with inducible expression in many cell types. The initial product of each is a propeptide of 31 kilodaltons from which a mature peptide of 17 kilodaltons is excised (Dinarello, 1986). Interleukin 1 alpha and beta have only 26% amino acid homology but have similar biological activities (Dinarello, 1986). In resting blood monocytes IL-1 messenger RNA is undetectable but after cellular activation it accumulates rapidly and IL-1 beta is usually the predominant form

(Dinarello, 1986). It is a moderately stable polypeptide surviving temperatures from -70°C to +56°C and pHs ranging from 3 to 11.

Interleukin 1 has a wide range of biological activities (Fig. XXVII) both in vivo and in vitro, many of which are considered essential for the induction of immune responses against infection and perhaps against malignancies (for review, see Dinarello, 1984a). Probably the most significant of these is the capacity to stimulate the production of IL-2 by T-cells (Smith et al., 1980) but it also causes an increase in body temperature in vivo (Rosenwasser and Dinarello, 1981) which may augment the immune response to bacteria and viruses by enhancing the effects of IL-1 on T-cell proliferation (Hanson et al., 1983) and potentiating production and secretion of Ab by B-cells (Lipsky et al., 1983).

There is also evidence that injected IL-1 rapidly induces neutrophilia due to increased release of PMNs from the bone marrow (Oppenheim, 1986). Moreover, in vitro studies indicating that IL-1 is a chemotaxin for PMNs and monocytes (Luger et al., 1983), and lymphocytes (Hunninghake et al., 1987). Virtually all chemotaxins also activate the cells they mobilize. Likewise IL-1 induces PMNs to increase glucose metabolism through the hexose monophosphate shunt and to generate superoxide and intralysosomal enzymes as measured by the capacity to reduce intracellular nitroblue tetrazolium salt (NBT) (Hunninghake et al., 1987). Interleukin 1 also induces PMNs to release specific granules and to selectively release lysozyme and lactoferrin, which in turn sequester iron. In vivo, IL-1 also causes elevation of acute phase proteins (APPs) (Dinarello, 1984a) and some of these can act as opsonins to promote ingestion of invading organisms by phagocytes (Pepys and Baltz, 1983). Furthermore, a combination of IL-1 and LPS or serum factors can increase the

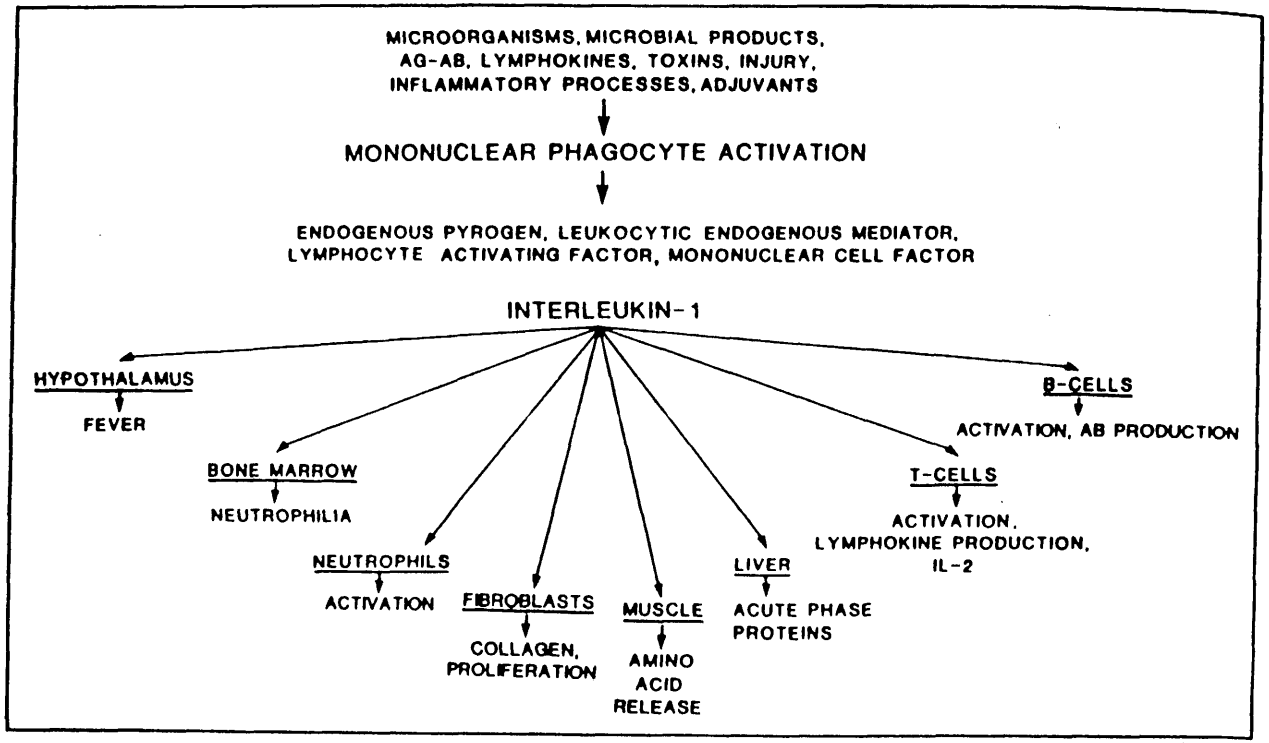


Fig. XXVII The multiple biologic activities of Interleukin I AG denotes antigen, AB antibody, and IL-2 Interleukin 2 (Dinarello, 1984b).

natural killer activity of large granular lymphocytes. Because these cells have natural killer activity and play a significant role in eliminating blood-borne metastasising tumour cells, IL-1 may amplify nonspecific host defence mechanisms against tumours and virus-infected cells.

Together these findings suggest that IL-1 is indeed produced to facilitate the eradication of invading organisms as soon as possible. However, IL-1 does have a number of deleterious effects, such as cartilage destruction, muscle wasting, high fever, anorexia and inflammation. Indeed IL-1 activity has been detected in the SF of patients with RA (Wood et al., 1983); a disease that is characterised by chronic inflammation and cartilage destruction.

The majority of IL-1 assays utilise in vitro cell or tissue culture techniques. Originally, IL-1 was described by Gery and Waksman (1972) in a mouse thymocyte co-mitogenesis assay in which IL-1 augmented the proliferation of thymocytes and production of IL-2 in response to suboptimal concentrations of T-cell mitogens such as phytohaemagglutinin (PHA). Although this assay was easy to perform and relatively sensitive, it required primary cultures of thymus which were non-specific, for instance they responded to IL-2 (Gearing et al., 1985). Therefore, the assay lacked specificity. Furthermore, it required 72 hours for maximum proliferation which is a potential disadvantage.

Because of these problems, several alternative IL-1 assays have been developed. These use T-cell lines such as LBRM 33 or EL-4 which produce IL-2 in response to IL-1 when co-stimulated with lectin or ionophore (Gillis and Mizel, 1981; Simon, Laydon and Lee, 1985). The IL-2 is measured by its effects on IL-2 dependent cell lines eg. continuous T-lymphocyte line. Such two stage assays have the disadvantages that they take 72 hours for completion and that

background IL-2 production is high. Conlon (1983) has improved this system by incorporating the IL-2 dependent continuous T-lymphocyte line in the same assay wells as mitomycin C-inactivated LBRM 33 cells co-stimulated with PHA. In this one stage assay, IL-1 caused increases in IL-2 production, and hence T-cell line proliferation within 24 hours.

The murine T-cell line D10 proliferates in response to IL-1 and has been used to measure IL-1 concentrations (Kaye et al., 1984). However, the D10 cell line is difficult to maintain, very time consuming and relatively expensive. Also, the assay takes 72 hours to complete.

Additional assays, employing non-lymphoid cells, are available. However, many of these are also stimulated by TNF which is probably present in a large number of crude IL-1 containing supernatants Therefore the specificity of these assays is poor unless advantage is taken of neutralising Abs specific for individual cytokines, which are increasingly becoming available.

In the present study, a 24 hour, highly sensitive bioassay for the detection of IL-1 was chosen, which was first described by Larrick, Brindley and Doyle (1985). A 6-thioguanine-resistant mutant of the murine lymphoma cell line LBRM 33 was selected (LBRM TG-6). When this cell line was incubated with a suboptimal concentration of PHA and IL-1 it produced IL-2. Interleukin 2 dependent HT2 cells were co-cultured with the LBRM TG-6 cells to measure the release of IL-2. The principle of the assay relies on the fact that LBRM TG-6 cells do not incorporate thymidine when cultured in the presence of hypoxanthine/azaserine supplemented medium, which metabolically blocks DNA synthesis by LBRM TG-6 cells but not by HT2 cells. This selection medium replaces the conventional hypoxanthine, aminopterin, thymidine

medium. This resulted in an assay with a minimal amount of technical modifications.

These bioassay systems are, however, vulnerable to interference by IL-2 that directly stimulates T-cell proliferation (Lisi et al., 1987).

The measurement of IL-1 present in the supernates from cultured cells in vitro can be affected by the transfer of substances used either to stimulate or suppress IL-1 production. For example, endotoxins and other bacterial products often mimic IL-1 activities in vivo. Several pharmacological agents which inhibit IL-1 production also interfere with biological assays for IL-1, while PHA can affect T-cell assays by the induction of IL-2 production (Lisi et al., 1987) which consequently results in falsely high levels of IL-1 as measured in the bioassay. Furthermore, we cannot rule out the possibility that bacterial stimulants affect the release of both IL-1 and IL-1 inhibitors, which might cause variability in assaying the level of IL-1 activity. Consequently, the determination of IL-1 activity by bioassay is not a straightforward process and great caution must be taken when interpreting the results.

3.1.1 Aims of the study

The present study seeks to define the optimal conditions for in vitro IL-1 synthesis by human monocytes, to be used routinely in the laboratory. In addition the relative amount of biologically active IL-1 produced by monocytes in response to different stimuli (Lipopolysaccharide (LPS) and S. aureus) is compared.

3.2 MATERIAL AND METHODS

3.2.1 Collection of peripheral blood

Peripheral blood was withdrawn from normal healthy volunteers as previously described in 2.2.2. It was collected into sterile universal containers containing ethylenediaminetetra-acetic acid di-potassium salt (K/EDTA:2mg/ml. BDH Chemicals Ltd., U.K.).

Normal blood donors were free from any clinically detectable disease and were non-users of drugs.

3.2.2 Separation media

(i) Dextran 500

A 6% (w/v) dextran 500 (Fisons, U.K.) solution was prepared in saline (0.9% (w/v) NaCl). Once totally dissolved, it was filtered through a 0.2μ sterile disposable filter assembly into a sterile glass bottle and stored at 4°C.

(ii) Nycodenz Monocytes

Nycodenz Monocytes (Nyegaard, U.K.) is a commercially available, slightly hypertonic, sterile solution for the isolation of monocytes from leucocyte-rich plasma. The solution contains Nycodenz, a non-ionic derivative of tri-iodobenzoic acid with a molecular weight of 821, dissolved in: KCL (1.2mmol/l), $CaNa_2EDTA$ (0.1mmol/l), NaCl (0.1mol/l) and Tris buffer (pH 7.5; 0.2mmol/l). It has a density of 1.068g/ml and an osmolarity of 337

mOsm/kg. It is stored at or below 20°C protected from light for optimum stability.

(iii) Washing fluid

After separation of the leucocyte-rich plasma on Nycodenz Monocytes, the plasma was retained and made up to a 5% solution in saline (0.9% (w/v) NaCl). This constituted the 'washing fluid' and was prepared fresh for each monocyte population.

(iv) RPMI 1640

RPMI 1640 (Gibco, UK) was used with the following supplements: 10mM HEPES buffer, 2mM L-glutamine (200mM; Gibco, U.K.), 50 IU/ml penicillin/50 IU/ml streptomycin (5000IU/5000IU; Gibco, U.K.), and $2x10^{-5}$M 2ß-mercaptoethanol (20mM; Sigma, U.K.). This complete medium will be referred to as RPMI 1640 through this text, unless otherwise specified.

This medium was prepared under sterile conditions and stored at 4°C for not more than 3-4 days prior to use. Heat-inactivated fetal calf serum (FCS; Gibco, U.K.) was added to a final concentration of 10%. Heat-inactivation was performed by heating at 56°C for 30 minutes. All FCS used was heat-inactivated unless otherwise stated.

All separation media were checked for contamination with endotoxin by Dr. C. McCartney, Department of Bacteriology, Glasgow Royal Infirmary, using a sensitive chromogenic Limulus Amoebocyte Lysate Micro-assay (Piotrowicz, Edlin and McCartney, 1985).

Nycodenz Monocytes, physiological saline and RPMI 1640 culture medium were found to be free of endotoxin. However, heat-inactivated FCS and 6% Dextran solution were found to contain approximately 0.04ng/ml and 0.08ng/ml respectively, of endotoxin. Consequently, adequate controls were included in the assay system to eliminate any endotoxin effect on monocytes.

3.2.3 Separation of monocytes from whole blood

All centrifugation was performed using a Mistral 4L centrifuge (MSE, U.K.) at 4°C unless otherwise specified.

Monocytes were separated from K/EDTA blood using a modification of the method described by Böyum (1968). Peripheral blood (10 parts) was mixed with 6% (w/v) Dextran 500 (1 part), in a 15ml sterile conical centrifuge tube (12x17mm diameter, Sarstedt, UK). The plasma layer, containing the leucocytes was removed when the erythrocytes had sedimented (30-60 minutes at ambient temperature). The leucocyte-rich plasma (5-6ml) was layered over Nycodenz Monocytes solution (3ml) and centrifuged at 600g for 15 minutes.

The recommended method suggests centrifugation at ambient temperature. However, I found that this caused considerable irreversible clumping of monocytes. For this reason all centrifugation was performed at 4°C which eliminated the problem.

Immediately after spinning, clear plasma was removed down to 3-4mm above the interphase and retained. Thereafter, the remaining plasma together with the interphase and slightly more than half the volume of the separation fluid

was collected into a centrifuge tube. The cells from one tube were diluted to 6-7ml with physiological saline and centrifuged at 600g for 10 minutes. The supernatant fraction was discarded and the cell pellet resuspended in calcium-free and magnesium-free HBSS (1 ml) and layered over platelet-free autologous K/EDTA plasma (3ml). The tube was centrifuged at 50g for 10 minutes and the supernatant carefully removed by pipetting. The monocytes were resuspended in 'washing fluid' (7-8ml) and washed twice at 600g for 10 minutes. The monocytes were suspended in RPMI 1640 supplemented with 10% FCS (1ml). A 1/10 dilution of the monocyte suspension was prepared in white cell diluting fluid and the cells counted in an improved Neubauer counting chamber as before (2.2.4). Cells were adjusted in RPMI 1640 containing 10% FCS to give the desired concentration.

To determine purity, cells were stained for esterase activity, an enzyme specific to monocytes (alpha-Naphthyl Acetate Esterase Kit, Sigma, U.K.). Monocytes separated with Nycodenz Monocytes solution were of high purity (>90%) and greater than 99% viability.

3.2.4 Preparation of standard IL-1

An internal laboratory IL-1 standard was prepared from cultures of human monocytes obtained from one donor, as described in 3.2.3. Cells were adjusted to $1x10^6$/ml. Sufficient autologous plasma and LPS from Escherichia coli 055:B5 (Sigma, U.K.) was added to give final concentrations of 5% and 100ng/ml respectively and incubated in $75cm^2$ tissue culture flasks (Bibby Scientific Products Ltd., U.K.) for 18 hours at 37°C, in a humidified atmosphere of 5% carbon dioxide (CO2)/95% air. The solution was pipetted into sterile universals, centrifuged at 600g for 10 minutes to remove cells and

the supernatant filtered through a 0.2μ sterile, disposable filter assembly, diluted to 1/100 concentration and stored at -20°C in 1ml aliquots. The standard was used at a 1/100 concentration with 6 serial dilutions.

3.2.5 Preparation of IL-1 supernatant

Culture conditions, for the generation of IL-1 from monocytes, were optimised in these preliminary experiments comparing:

a) monocytes at final concentrations of $1.25x10^5$, $2.5x10^5$, $5x10^5$ and $1.0x10^6$ cells/ml; and

b) LPS stimuli at 0.1, 1.0 and 10μg/ml final concentrations or S. aureus (Pansorbin Brand preparation, Behring Diagnostics, U.K.) at final concentrations of $1x10^7$, $5x10^7$ and $1x10^8$ c.f.u./ml. All dilutions were made in RPMI 1640 supplemented with 10% FCS. Monocytes and stimuli were cultured in triplicate, in a final volume of 200μl, in flat bottomed 96-well microtitre plates (Northumbria Biologicals Ltd., U.K.) for 18 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air.

To assay for spontaneous production of IL-1, cell suspensions were incubated without LPS or S. aureus for the same period of time. In addition, control medium was prepared by incubating RPMI 1640 plus 10% FCS with LPS or S. aureus (without monocytes). To determine whether FCS was capable of activating the cells, a control well containing monocytes in RPMI only without FCS was set-up.

After 18 hours, cell-free IL-1-containing supernatants were harvested by centrifugation at 600g for 15 minutes at ambient temperature, triplicate cultures pooled, filter sterilized (0.2μ), and frozen at -20°C until assayed for IL-1 content. Interleukin 2 was not detected in any crude IL-1 supernatants prepared in this manner when they were assayed using the HT2 cell line (Cetus Corporation, U.S.A.) in a microtitre assay as described by Watson (1979).

3.2.6 IL-1 assay

The 24 hour IL-1 assay, described by Larrick et al. (1985) was performed using a slightly modified protocol. The assay makes use of two cell lines, LBRM TG-6 (Cetus Corporation, U.S.A.) and HT2. The LBRM TG-6 cells were subcultured at $1x10^5$/ml in Iscove's modified Dulbecco's medium (Gibco, U.K.) supplemented with 50IU penicillin/50IU streptomycin and 10% FCS, every 3-4 days. The HT2 cells were subcultured to a concentration of $4x10^4$/ml in RPMI 1640 containing 10% FCS and fed with 200 units/ml human recombinant IL-2 (BCL., U.K.) every 48 hours.

The cells were counted and the viability assessed using trypan blue exclusion. Sufficient cells were pelleted, at 600g for 10 minutes, such that in the final suspension there were $1.5x10^5$/ml HT2 cells and $1x10^6$/ml LBRM TG-6 cells. The cells were washed twice in RPMI 1640 supplemented with 10% FCS (10ml), before being made up to the final volume to give the required concentrations of cells. The standard used in the assay was obtained as previously described in 3.2.4. Standard IL-1 was diluted in RPMI 1640 plus 10% FCS to give working concentrations of 1/100, 1/1000, 1/2000, 1/5000, 1/10000, 1/25000 and 1/50000. Interleukin 1-containing supernatants to be

assayed also required to be diluted to give working concentrations of 1/100, 1/1000, 1/5000 and 1/10000 for LPS stimulated cultures, and 1/100, 1/500, 1/1000 and 1/2500 for S. aureus stimulated cultures. The LBRM TG-6 (1x105/well) and HT2 (1.5x104/well) cells were distributed into 96-well (u-shaped) microtitre plates (100ul) in RPMI 1640 plus 10% FCS, further supplemented with PHA (Wellcome, U.K.) to give a final concentration of 1μg/ml. An equal volume (100ul) of IL-1 standard dilutions and experimental sample dilutions was added, in triplicate, to the plates.

A control was required where no lectin was added, to measure spontaneous IL-1 production. Therefore, unstimulated LBRM TG-6/HT2 cells (100ul) were added to triplicate wells containing culture medium only (100ul). An additional control was included which consisted of culture medium and PHA-stimulated cells only, no IL-1-containing supernatant.

The cultures were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air. After the incubation period, hypoxanthine (10^{-4}M; final concentration: Sigma, U.K.) and azaserine (10μg/ml; final concentration: Sigma, U.K.) were added to the cultures and incubated in the same conditions as before. After 4 hours, each well was pulsed with 37 Megabequerels of [^{3}H] thymidine (TRK 418, specific activity $1.67x10^3$ Gigabequerels/mmol; Amersham International, U.K.) and incubated for a further 4 hours at 37°C in a 5% CO_2 humidified incubator. The cultures were then harvested onto glass fibre filter discs (Skatron, U.K.) using a Titertek Cell Harvester (Skatron, U.K.). The discs were left overnight to dry and then transferred to polypropylene scintillation vials, to which was added scintillant (PPO/toluene (4g/l); Canberra Packard, U.K./ May and Baker, U.K.) (3ml). The

incorporated radioactivity was assessed by liquid scintillation using a LKB 1216 Rackbeta II counter.

3.3 RESULTS

3.3.1 Standard preparation of IL-1

The standard IL-1 preparation was diluted and assayed using the bioassay system. As shown in Figure XXVIII, the preparation contains IL-1 activity. The X-axis dilutions are on a logarithmic scale which illustrates the results as a sinusoidal curve. The Y-axis shows the percentage of maximum activity. The assay system is unable to distinguish between the activity due to the alpha and beta form of IL-1. However, in this study such a distinction is not required.

3.3.2 In vitro production of IL-1 by human monocytes

The response to supernatants from $1.25x10^5$/ml monocytes incubated with increasing concentrations of LPS are shown in Figure XXIX. The supernatants from these incubations were diluted 4-fold and assayed for IL-1. The supernatants from cultures of monocytes at $1.25x10^5$/ml and different concentrations of heat-killed S. aureus (Cowan 1 strain) were similarly assayed. The results are shown in Figure XXX. The concentration of LPS and S. aureus which illustrated the greatest range of activity was 1μg/ml and $1x10^7$ /ml respectively.

3.3.3 IL-1 production by varying numbers of monocytes

The minimum number of monocytes which produced detectable IL-1 activity when incubated with 1μg/ml LPS or $1x10^7$ /ml S. aureus was $1.25x10^5$ /ml (Fig.

Fig. XXVIII Standard interleukin 1 curve.

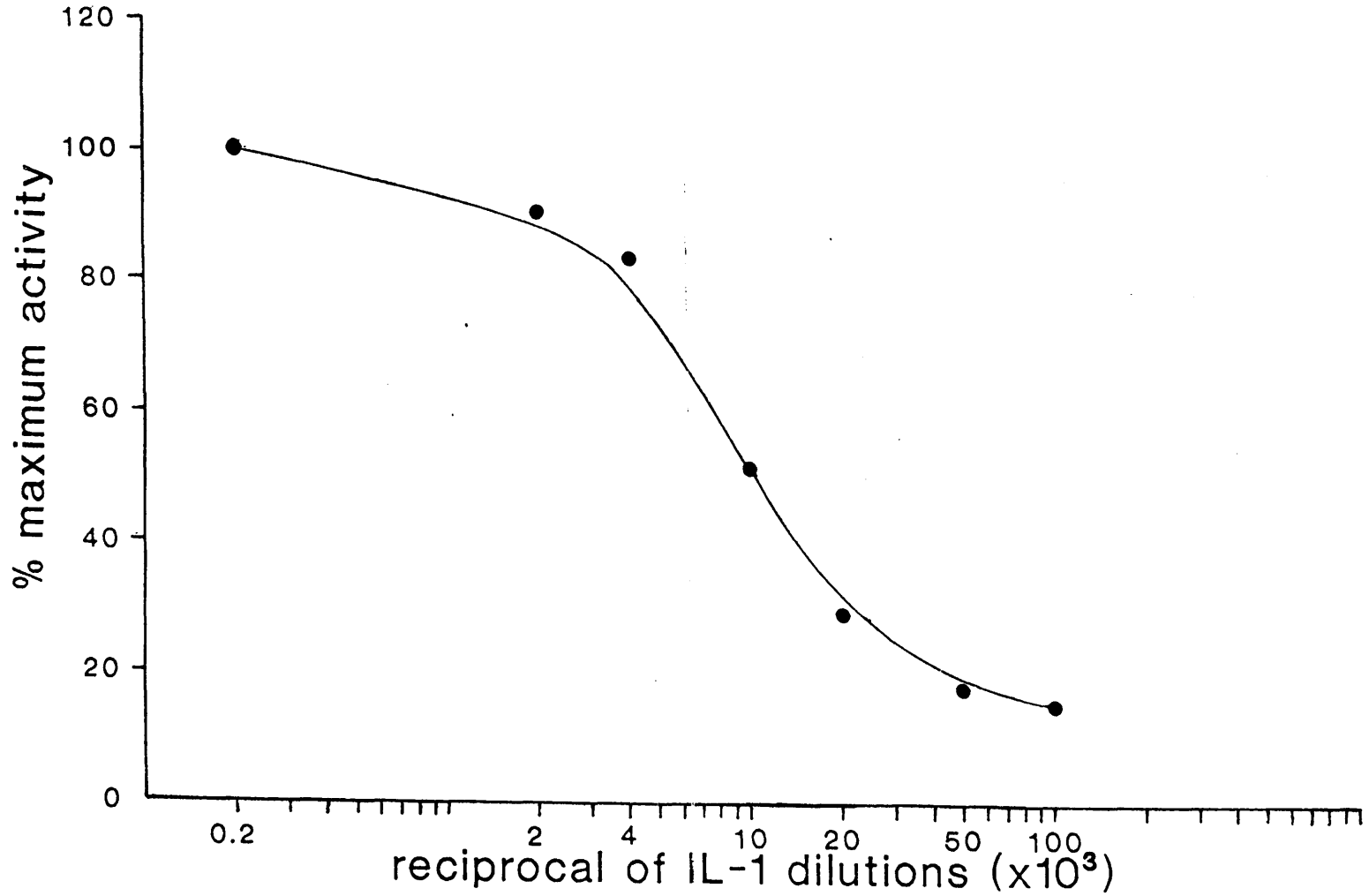


Fig. XXIX Interleukin 1 production by LPS stimulated monocytes at a concentration of 1.25×10^5/ml.

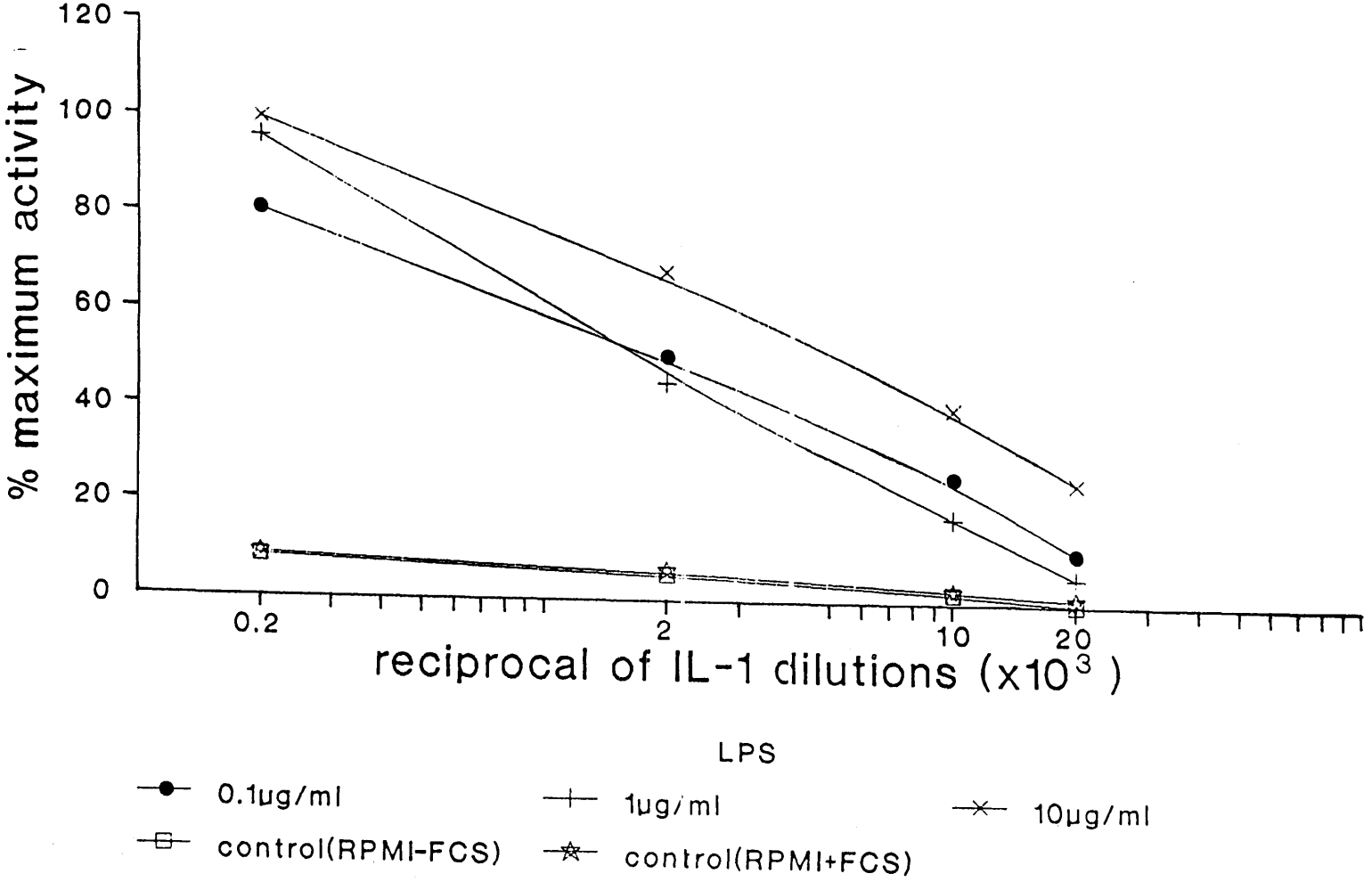
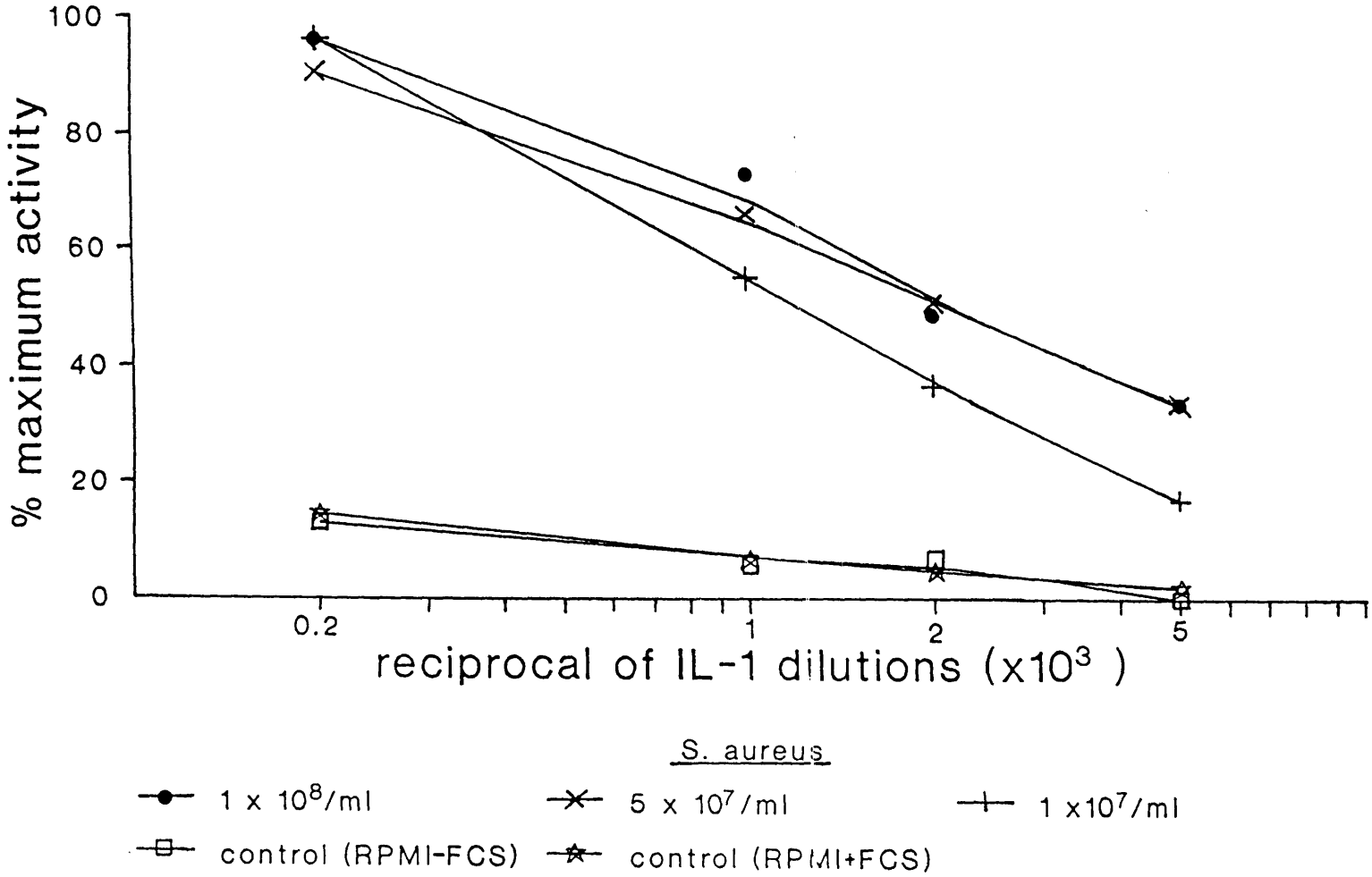


Fig. XXX Interleukin 1 production by S. aureus stimulated monocytes at a concentration of 1.25×10^5/ml.



XXXI and XXXII). When the number of monocytes per incubation was increased but the same concentration of LPS or S. aureus, progressively more IL-1 was produced but the drop off from the lowest dilution to the highest dilution was diminished.

All other possible combinations of monocytes and LPS or S. aureus concentrations were tested. The figures shown represent the optimum monocyte and stimulant concentrations for the purposes of this study.

As a control, monocytes were incubated with RPMI supplemented with 10% FCS. Background levels of IL-1 were produced but this was negligible when compared to test conditions. As an additional control, monocytes were diluted and incubated with RPMI only, in the absence of FCS. From all figures in this chapter it can be observed that the FCS used in these assays did not significantly activate the monocytes in vitro.

As a consequence of these preliminary studies, monocytes at 1.25×10^5/ml, LPS at 1μg/ml and S. aureus at 1×10^7 /ml were chosen as the optimum conditions for future assays.

Fig. XXXI Interleukin 1 production by monocytes stimulated with 1µg/ml of LPS.

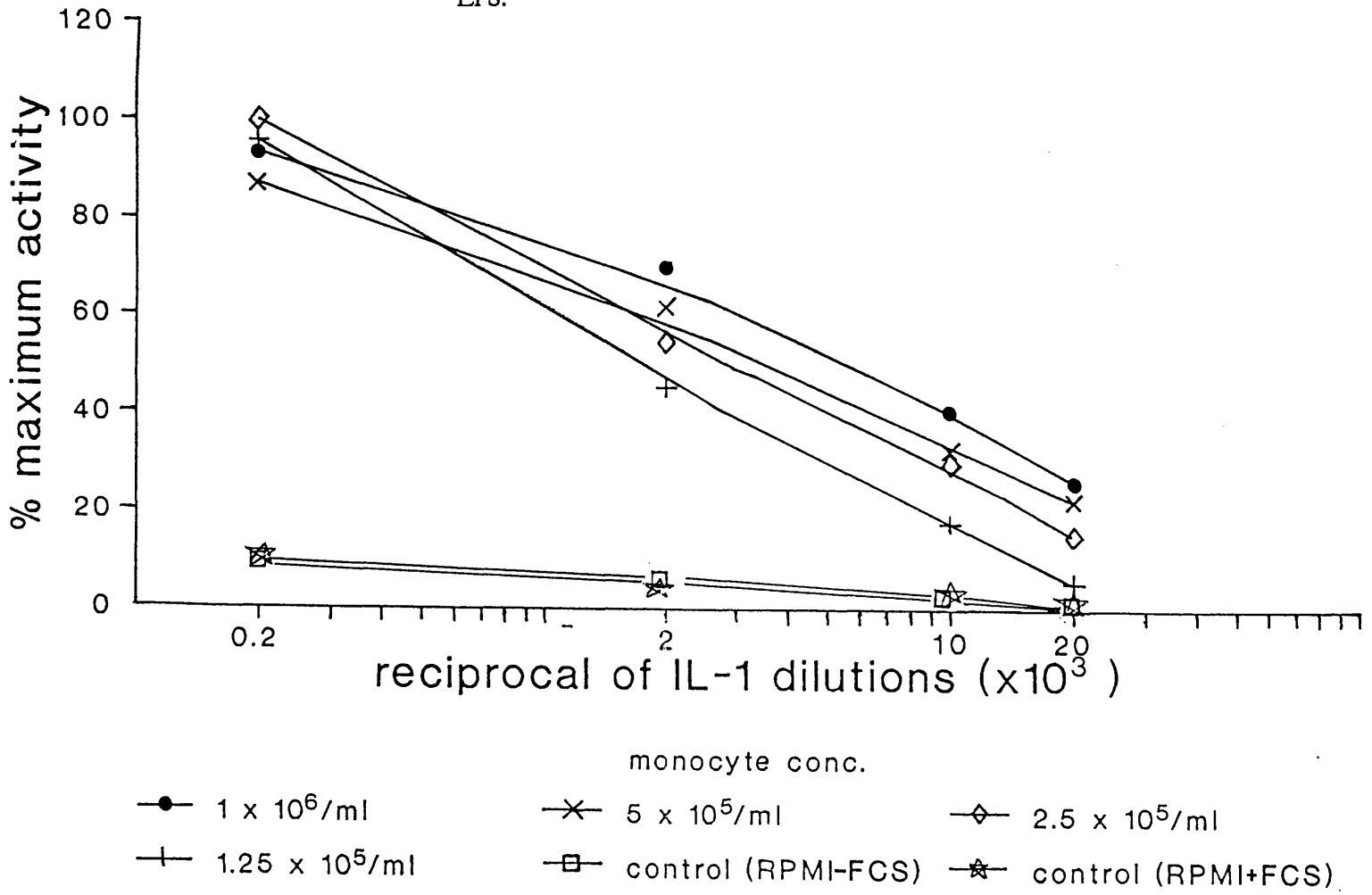
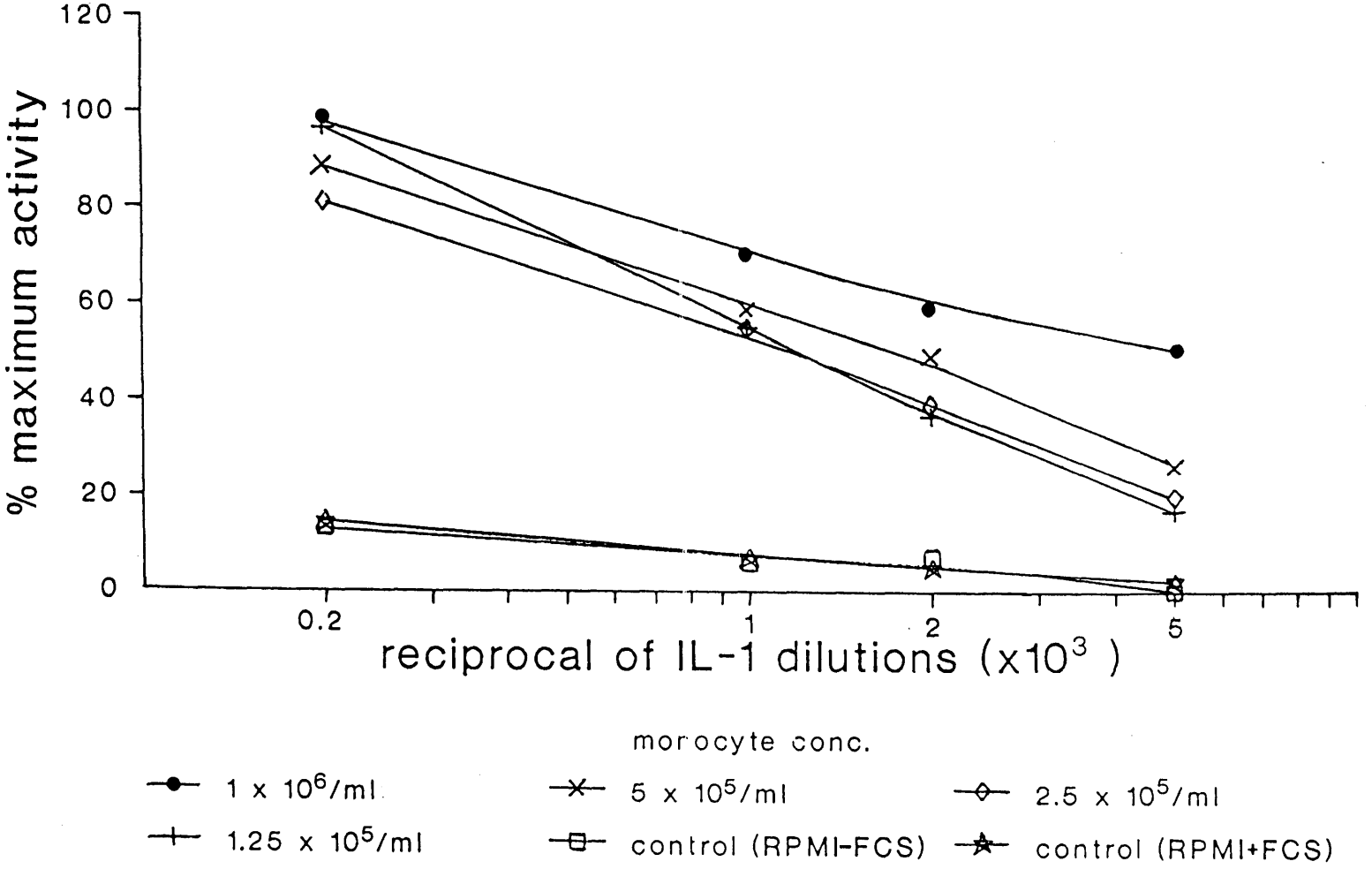


Fig. XXXII Interleukin 1 production by monocytes stimulated with $1x10^7$/ml of S. aureus.



3.4 CONCLUSIONS

The main goal of the present study was to define the experimental conditions for routine in vitro IL-1 production. A prerequisite for defined stimulation of monocytes is endotoxin-free culture conditions. The difficulty was knowing whether cells that release IL-1 in vitro without deliberate stimulation had, in fact, been activated in vivo or inadvertently stimulated during handling. Particular care must be taken to exclude accidental contamination with endotoxin during cell separation or from the culture media. Commercially prepared density gradient media are not necessarily endotoxin free. For this reason all media were tested before use and adequate controls incorporated into the assays. It is also possible that FCS contains factors other than endotoxin which may promote IL-1 release by monocytes (Shore et al., 1986). Therefore, I used filtered, heat-inactivated FCS and included an appropriate control comprising monocytes with RPMI alone (no FCS present), which showed that FCS was not significantly activating the cells. It is relevant that the same batch, not only of FCS, but of all media was used throughout individual studies. These measures enabled me to culture monocytes without significant activation of the cells.

Polymyxin B is a very effective inhibitor of endotoxin activity in vitro (Duff and Atkins, 1982) but the amount of polymyxin B which can be safely added to cell cultures may limit its use in the face of heavy endotoxin contamination. We were measuring the effect of LPS and for this reason could not add polymyxin B to our cultures, which would eliminate the desired response.

While we observed little spontaneous in vitro IL-1 production by monocytes from healthy individuals we were able to induce high levels of IL-1 by 1μg/ml LPS and S. aureus at a concentration of $1x10^7$ /ml.

Most protocols for IL-1 production suggest that cells should be cultured at 1-$2x10^6$/ml, and that their supernatant should be harvested after 18-48 hours of culture to give optimal IL-1 titres. It has been suggested that conditions outside these limits may result in low yields of IL-1 (Gearing et al., 1985). From the preliminary studies herein, a monocyte concentration of $1.25x10^5$/ml was chosen as the standard concentration to be used in subsequent experiments. This allows a large number of samples to be separated daily due to the smaller volume of blood needed to yield the desired monocyte concentration. We also chose 18 hours for culture, since supernatants tested showed demonstrable IL-1 but no IL-2 production within this time period (Smith, Personal communication). It is also important to use fresh monocytes, as cultured monocytes differentiate to macrophages which produce little IL-1 (Gearing et al., 1985).

Interleukin 1 activity in tissue fluids and also from culture supernatants can be obscured by inhibitory material. In culture the most common inhibitor is prostaglandin E_2, release of which can be inhibited by indomethacin (Gearing et al., 1985).

The LBRM TG-6/HT2 bioassay was the assay of choice because it was quick (24 hours), relatively easy to perform and one of the most sensitive available at the initiation of these studies. Furthermore, the cell lines were readily available within the laboratory.

Other potential problems which accompany all systems that utilise cell lines maintained for continuous culture, include possible phenotypic and genotypic changes in the cell, as well as contamination of the cell line with mycoplasma. Larrick et al. (1985) have found the LBRM TG-6 cell line to show no evidence of reversion in over 8 months of continuous culture. Both problems, however, are easily controlled by returning to a frozen stock of cells.

More recently radioimmunoassays (RIA) have become commercially available from Cistron Biotechnology. Radioimmunoassays have several advantages over bioassays: 1) it is possible to measure concentrations in samples containing interfering factors that would otherwise mask IL-1 bio-activities; 2) it is possible to measure one form of IL-1 (ie. either alpha or beta gene product) a specificity that is, at present, not possible with any bioassay. Nevertheless, they are not without disadvantages: 1) the sensitivity of RIA are significantly reduced (250pg/ml) in comparison with bioassay systems (50pg/ml), however, Endres et al. (1988) recently reported IL-1 beta levels as low as 115pg/ml in an RIA system; 2) possibly the Ab may recognise epitopes of the molecule that are not necessarily relevant to biological activity (Symons et al., 1987). This problem may be overcome in the future by the use of monoclonal Abs directed at the active site of IL-1. Another potential disadvantage is the high cost which reduces the feasibility of RIA, especially when measuring large numbers of samples and working within financial constraints.

CHAPTER FOUR - INTERLEUKIN 1 PRODUCTION IN RHEUMATOID ARTHRITIS

4.1 Introduction

Historically, IL-1 was first used to describe macrophage-derived lymphocyte activation factor (LAF) (Gery and Waksman, 1972). However, long before the discovery of LAF, phagocytic cell products had been described and characterised as endogenous pyrogen (EP), which caused fever (Atkins, 1960) and leucocytic endogenous mediator, which induced neutrophilia, hypoferraemia, hypozincaemia and increased the hepatic synthesis of (APPs (Kampschmidt, 1981), which contribute to an elevated ESR. This constellation of responses are collectively referred to as the acute phase response (APR). Since upon purification these biological activities were inseparable from LAF activity, it became clear that IL-1 had other activities in addition to its ability to augment lymphocyte proliferation (for review see Dinarello, 1984a) (Fig. XXVII).

It appears possible that IL-1 fulfills ongoing roles in normal physiology. Such a 'non-inflammatory' role has become plausible with the detection of IL-1 activity in normal serum (Gahring, Sholly and Daynes, 1984) and urine (Kimball et al., 1984). However, others may disagree, for instance Dinarello (1984b) detected very little or no IL-1 in human plasma or serum obtained from healthy resting subjects. This could be explained by simultaneous production of inhibitors and masking the in vitro detection of serum or urinary IL-1. The normal serum levels observed by some (Gahring et al., 1984) are observed to vary, with increases occuring during exercise or even diurnal period of waking (Durum, Schmidt and Oppenheim, 1985). This therefore

raises the questions, do these basal IL-1 levels serve the organism in some beneficial way and will deficiencies lead to pathology, as with other hormones?

Interleukin 1 has attracted considerable interest in the past decade primarily as a mediator of host defence (Dinarello, 1984a) and in its pathogenic potential in inflammation and immune diseases (Duff, 1985).

Some of the effects of IL-1 on the host have an important and vital role in defence against infection and malignant transformation. These basal levels could well be essential in maintaining this aspect of host defence. For example, the increase in temperature, mediated by an abrupt increase in the synthesis of prostaglandins (PG), most notably PGE_2, in the hypothalmic thermoregulatory centre, has a direct effect on immunologic responses. Temperatures of 38 and 39°C dramatically augment the action of IL-1 on T-cell proliferative responses (Hanson et al., 1983), the generation of cytotoxic T-cells, B-cell activity, and Ig synthesis (Lipsky et al., 1983). In addition these febrile temperatures adversely affect the replication of several bacteria and viruses. The requirement for iron as a growth factor for micro-organisms increases at elevated temperatures (Bullen, 1981).

There is further evidence which suggests that IL-1 or its agonists could be beneficial in patients with deficient IL-1 production. Highly purified recombinant human IL-1 alpha, when administered intramuscularly, has been shown to enhance resistance, in a dose-dependent manner, of both normal and neutrophilic mice to infection with Pseudomonas aeruginosa and Klebsiella pneumoniae (Ozaki et al., 1987). Czuprynski et al. (1988) illustrated that administration of exogenous rIL-1 alpha accelerated the protective response of mice to infection by the facultative intracellular pathogen Listeria

monocytogenes. In a further study, Minami et al. (1988) have shown IL-1 to enhance resistance in mice to a wide variety of organisms, including S. aureus. Furthermore, IL-1 has been observed to protect rats from a fatal dose of Salmonella typhimurium (Dinarello, 1984a). Collectively these observations suggest a possible use for this cytokine in treatment of opportunistic infections in immunosuppressed patients. Therefore, any disturbance of the monocyte population or deficiencies in the production of IL-1 may render the host less resistant to infection.

Conversely, excessive IL-1 production may be detrimental to the host. For example, much evidence has implicated IL-1 in the pathogenesis of RA: high levels are found in RA joint effusions (Fontana et al., 1982; Wood et al., 1983; Nouri et al., 1984); RA synovial cells spontaneously produce biologically active IL-1 in vitro (Nouri et al., 1985); blood monocytes from patients with RA spontaneously secrete elevated levels of IL-1 in vitro (Nouri et al., 1985; Shore, Jaglal and Keystone, 1986); and in-situ hybridisation of messenger RNA shows that IL-1 gene expression is activated in vivo in RA synovial cells (Duff et al., 1988). Interleukin 1 was initially reported in joint fluids in 1968 by Bodel and Hollingsworth, who described a pyrogen, resembling EP, found not only in joint fluid of patients with RA but also in patients with rheumatic fever and pseudogout, as well as other disease states. Since EP or leucocytic pyrogen has now been shown to be indistinguishable from IL-1 (Rosenwasser and Dinarello, 1981), these results are quite consistent with those of subsequent researchers. In 1984, Tan et al. reported that patients with RA exhibit an enhancement of in vitro cytokine release. However they were unable to differentiate IL-1 from IL-2 since unseparated peripheral blood mononuclear cells (containing both monocytes and T-cells) were used to generate cytokines. More recently, the same group extended the study and showed enhanced spontaneous generation of

IL-1 by monocytes from recently active RA patients but normal IL-2 generation by their T-lymphocytes (Shore et al, 1986).

Additional evidence that overproduction of IL-1 is associated with pathological states, is that many activities of IL-1 are relevant to RA (Dinarello, 1984a). Interleukin 1 increases the release from synovial cells of vasoactive agents and mediators of tissue damage (eg. PGE_2, collagenase, reactive oxygen molecules and proteinolytic enzymes) (Dinarello, 1984a), and is a powerful stimulus of bone and cartilage resorption (Dinarello, 1986). It induces an increase in APPs (eg. CRP) (Dinarello, 1984b), neutrophilia and fever (Dinarello, 1986), the measurement of which are well-established procedures for monitoring many disease states, including RA. Also IL-1 may potentiate chronic inflammation by induction of lymphocyte growth factors such as IL-2 and its receptor (Durum et al., 1985). In addition, it is chemotactic for PMNs (Luger et al., 1983) and induces lactoferrin release from PMN specific granules, which in turn sequester iron, which consequently contributes to the anaemia often seen in patients with RA.

There is ample evidence that products formed from arachidonic acid by the fatty acid cyclooxygenase pathway (eg. PGs) are mediators of inflammation (Vane, 1971). Firstly, these compounds provoke many of the cardinal signs of inflammation (eg. erythema, fever, pain, oedema). Secondly, PGs are synthesised by phagocytic cells; macrophages are believed to be the major source of local PG formation in RA (Sturge et al., 1978) and finally, synthesis of stable PGs (eg. PGE_2) is inhibited by many anti-inflammatory drugs.

Indeed elevated PGs have been reported in synovial effusions from patients with RA (Sturge et al., 1978). In addition human rheumatoid synovial tissues

have been shown to produce approximately 10 times more PGE_2, during in vitro culture, than does normal synovial tissue (Robinson, Dayer and Krane, 1979).

The data suggests that these factors could contribute to the pathogenesis of RA, therefore the challenge is to identify and develop specific IL-1 antagonists that will provide potential therapeutic means of alleviating symptoms of RA, while allowing the many potentially beneficial actions of IL-1 to continue at a functional level.

There are 3 possible types of therapy that therefore might affect the pathogenesis of RA: 1) drugs that directly affect IL-1 production, 2) drugs that interfere with manifestations of the APR for instance, by inhibiting the cyclooxygenase system and thereby indirectly interfering with IL-1 production, and 3) drugs that modify the effect of IL-1 on target cells, ie. specificity may be a key issue. It may be possible to inhibit specific target sites of IL-1 stimulation and avoid inhibiting other vital target tissues. Clearly, it could be argued that to interfere with IL-1 at all could have profound deleterious effects, for example, in the amplification of the immune response.

At pharmacological concentrations, only a few agents have been shown to inhibit IL-1 production. Hydrocortisone at 10^{-5} to 10^{-7}M inhibits both the production and co-mitogenic effects of IL-1. This inhibitory effect may account in part for the anti-inflammatory effects of steroids (Oppenheim, 1986). Bondy and Bodel (1971) have also shown that corticosteroids are highly effective in reducing IL-1 production in vitro and in vivo. However, their side-effects are associated with recurrent infections, in support of the argument that interference with IL-1 production may be detrimental to the host.

On the other hand, therapeutic concentrations of NSAIDs such as indomethacin, do not reduce the production of IL-1 directly (Dinarello, 1984a). They fall into the second category of drugs. Indomethacin inhibits the conversion of arachidonic acid to biologically active PGs, which indirectly interferes with IL-1 production. This suggests that such inhibition may well explain the anti-inflammatory action of NSAIDS *in vivo* (Vane, 1971), but unlike the steroids, these are not associated with recurrent infections.

4.1.1 Aims of the study

The aim of the present investigation was to test rheumatoid and healthy blood monocytes for spontaneous and stimulated IL-1 release, to determine if patients with RA were perhaps susceptible to sepsis as a result of a deficiency in this particular host defence response. Also, to determine if rheumatoid joints were less protected from infection due to their monocytes showing a reduced IL-1 response either spontaneously or by stimulation, with LPS or *S. aureus*. Furthermore, the study was designed to investigate the effect of anti-rheumatic drugs on IL-1 production by monocytes from normals and patients with RA and normals.

4.2 MATERIALS AND METHODS

As the result of preliminary experiments (Chapter 3), IL-1-containing supernatants are generated under optimal conditions as follows: monocytes at a final concentration of $1.25x10^5$/ml are stimulated with LPS or S. aureus at final concentrations of 1μg/ml and $1x10^7$/ml respectively. All concentrations are made up in RPMI 1640 supplemented with 10% heat-inactivated FCS.

4.2.1 Induction of IL-1 production: rheumatoid arthritis versus normal monocytes

(i) Subjects

Peripheral blood was aspirated from 9 patients with definite or classical RA (Appendix A) (M:F = 4:5), with an average age of 52 years (range: 38-62 years), and collected into K/EDTA as previously described in Chapter 3 (3.2.1). Peripheral blood was also collected from 10 healthy volunteers (M:F = 5:5), with an average age of 28 years (range: 23-32 years). All patients received NSAIDs, and some received '2nd line' medication. None were prescribed steroids. Controls were free from any clinically detectable disease and were non-users of drugs.

(ii) Cell preparation

Peripheral blood monocytes were isolated, as described in Chapter 3 (3.2.3) and diluted to a working concentrations of $2.5x10^5$/ml.

(iii) IL-1 generation

To obtain monocyte supernatants for IL-1 determination, monocytes were stimulated with 1μg/ml LPS and S. aureus at a concentration of $1x10^7$/ml, as detailed in Chapter 3 (3.2.5).

(iv) Assay for IL-1 activity

The monocyte supernatants were assayed for IL-1 activity using the LBRM TG-6/HT2 bioassay system as detailed in Chapter 3 (3.2.6).

4.2.2 Induction of IL-1 production: PB monocytes versus SF monocytes

(i) Subjects

Seven patients with definite or classical RA (Appendix A), attending a rheumatology outpatient department were recruited.

(ii) Cell preparation

Peripheral blood monocytes were isolated, as previously described (3.2.3) and diluted to a working concentration of $2.5x10^5$/ml.

Matched SF was aspirated from an inflamed joint and collected into sterile universals containing K/EDTA (2mg/ml). Monocytes were separated from SF as described for peripheral blood with the exception of the 6% dextran sedimentation stage. The SF (5-6ml) was initially layered over Nycodenz Monocytes (3ml). If the SF was particularly viscous, it was diluted with an equal volume of RPMI 1640 (without FCS) prior to being layered onto the

Nycodenz Monocyte preparation. Monocytes were adjusted to a working concentration of $2.5x10^5$/ml.

(iii) IL-1 generation

Monocytes were stimulated with 1μg/ml LPS and S. aureus at a concentration of $1x10^7$/ml., as previously described in Chapter 3 (3.2.5).

(iv) Assay for IL-1 activity

Paired PB monocyte and SF monocyte supernatants were assayed for IL-1 activity using the LBRM TG-6/HT2 bioassay, described previously (3.2.6).

4.2.3 Interleukin 1 production by monocytes from patients with RA: before and after the in vivo administration of sulphasalazine

(i) Subjects

Eight patients with definite or classical RA (Appendix A), were recruited into this study. The group consisted of 6 females and 2 males with an average age of 50 years (range: 34-68 years). These patients had all previously received NSAIDs and a variety of '2nd line' drugs but had never been prescribed SASP. Each patient was prescribed a target dose of SASP between 2-5mg to be taken once daily for the treatment of their RA.

(ii) Cell preparation

Peripheral blood (10ml) was aspirated prior to SASP treatment and monocytes separated as previously discussed in Chapter 3 (3.2.3), and diluted to a working concentration of $2.5x10^5$/ml.

After 12 weeks of SASP treatment, the same patients were recalled to the clinic and were retested as above.

(iii) IL-1 generation

Monocytes were stimulated with 1μg/ml LPS and S. aureus at a concentration of $1x10^7$/ml, as previously detailed in Chapter 3 (3.2.5).

(iv) Assay for IL-1 activity

Monocyte supernatants were assayed for IL-1 activity using the LBRM TG-6/HT2 bioassay, described in detail in Chapter 3 (3.2.6).

4.2.4 Effect of drugs on Interleukin 1 production

(i) Drugs

The drugs studied were the same as those described in Chapter 2 (2.2.5 (v)); Tenidap, SASP, SP, 5-ASA, Hydrocortisone sodium phosphate and Indomethacin. They were prepared and stored as previously described. Assay concentrations of each drug were as shown in Table 2.5. The drugs were diluted to the appropriate working concentrations with RPMI 1640 (without FCS) culture medium just prior to use in the assay.

(ii) Subjects

Peripheral blood was aspirated from 7 patients with definite or classical RA (Appendix A) (M:F = 2:5), with an average age of 47 years (range: 35-62 years), and 7 healthy controls (M:F = 3:4) with an average age of 32 years

(range: 25-42 years). All patients were attending the rheumatology outpatient clinic at Glasgow Royal Infirmary and were receiving either NSAIDs or '2nd line' therapy at the time of bleeding. Controls were free from any clinically detectable disease and under no medication at time of testing.

(iii) Cell preparation

Blood monocytes were separated as previously described in Chapter 3 (3.2.3), and diluted to a working concentration of $5x10^5$/ml in RPMI 1640 supplemented with 20% FCS.

(iv) IL-1 generation

To induce IL-1 release, monocytes at $5x10^5$/ml (50ul) were stimulated with either LPS at 4μg/ml (50ul) or S. aureus at a concentration of $4x10^7$/ml (50μl), made up in RPMI 1640 plus 20% FCS. The drugs (at working concentrations) were added (100μl) to the cell cultures to give a final volume of 200ul and final assay concentrations of : monocytes $1.25x10^5$/ml; LPS 1μg/ml; S. aureus $1x10^7$/ml; and drugs (as shown in Table 2.5). The cultures were incubated at 37°C in a 5% CO_2 humidified incubator for 18 hours as described previously in Chapter 3 (3.2.5). Control cultures were prepared excluding the drugs but including the solvent used to dissolve the drug. Another set of control cultures were prepared excluding the LPS or S. aureus stimulants but including the various drug concentrations to examine if drug alone could stimulate IL-1 activity.

(v) Assay for IL-1 activity

The monocyte supernatants were assayed for IL-1 activity using the LBRM TG-6/HT2 bioassay as detailed in Chapter 3 (3.2.6).

4.2.5 Expression of results

A standard IL-1 preparation, as described in Chapter 3 (3.2.4) was included in each experiment to ensure the efficiency of the bioassay. The results of all studies in this chapter are expressed as a percentage of the maximum activity of the standard IL-1 preparation.

4.2.6 Statistical analysis

The data are expressed as the median plus upper and lower quartile values and were analysed using non-parametric tests; either the Mann Whitney U test, for unpaired data or the Wilcoxon rank test, for paired data. All significance was assessed at the 5% probability level.

4.3 RESULTS

The monocyte supernatants were found to have maximal IL-1 activity at a 1/200 dilution. Therefore, the data shown throughout this chapter are representative of this dilution.

4.3.1 Interleukin 1 production by monocytes from patients with RA and controls

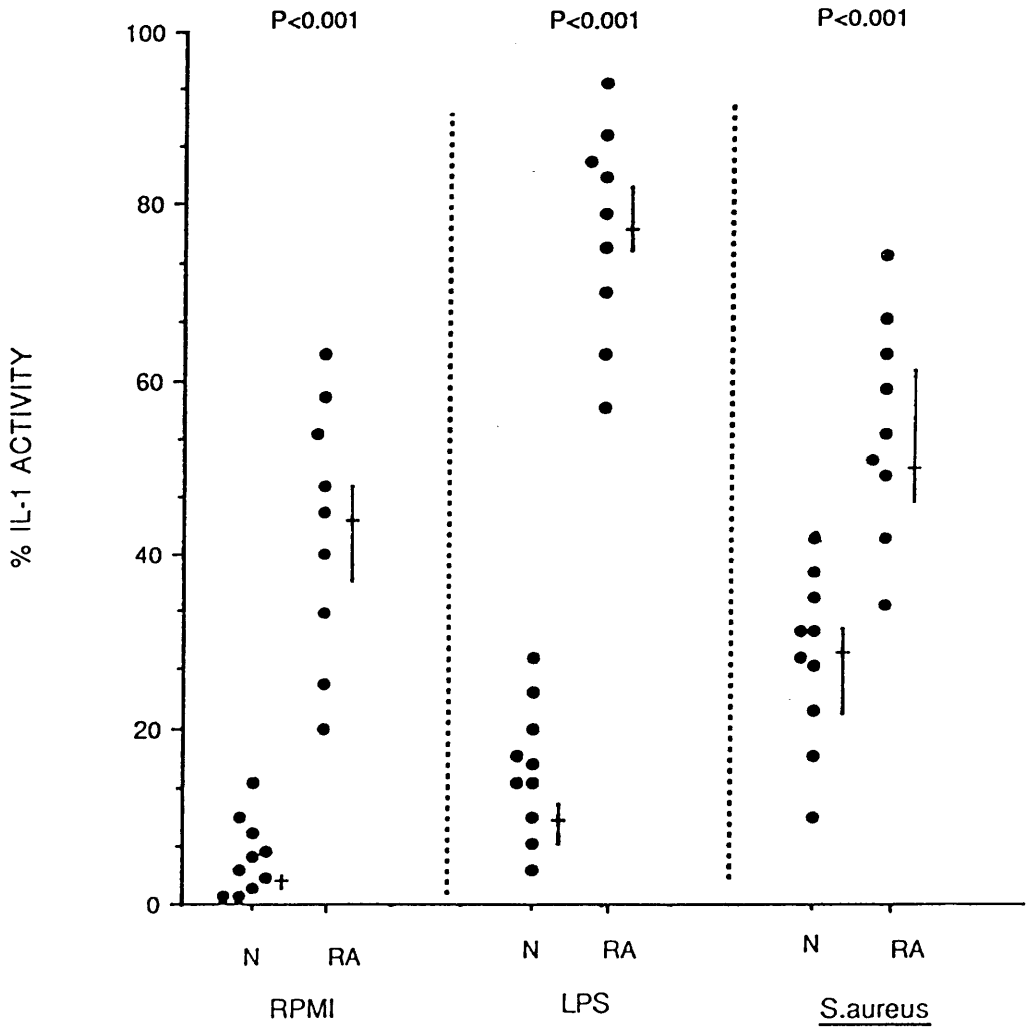
The results of IL-1 production by PB monocytes, spontaneously and after stimulation with 1μg/ml LPS or $1x10^7$/ml S. aureus, from 9 patients with RA and 10 control subjects are shown in Fig. XXXIII. Unstimulated RA cells produce significantly more IL-1 than normal monocytes ($p<0.001$) and this difference was maintained after LPS and S. aureus stimulation (LPS: $p<0.001$; S. aureus: $p<0.001$). In the case of both RA and control monocytes, LPS or S. aureus stimulation resulted in increased IL-1 production over the unstimulated cells (LPS: $p<0.01$, S. aureus: $p<0.01$ for RA; LPS: $p<0.01$, S. aureus: $p<0.01$ for normals).

It is interesting to note that in the case of RA monocytes, LPS appears to be the more potent stimulant over S. aureus ($p<0.01$), whereas for normal monocytes, S. aureus stimulates more IL-1 production ($p<0.01$).

4.3.2 Interleukin 1 production by paired PB and SF monocytes from patients with rheumatoid arthritis

When 8 paired PB and SF monocytes were compared, it was found that PB cells spontaneously produced significantly more IL-1 than corresponding SF

Fig. XXXIII Interleukin 1 production by monocytes from patients with rheumatoid arthritis and normals.



┼ median plus interquartile range

N	: normal
RA	: rheumatoid arthritis
RPMI	: unstimulated (spontaneous) IL-1 production
LPS	: lipopolysaccharide - stimulated IL-1 production
S. aureus	: Staphylococcus aureus - stimulated IL-1 production
IL-1	: interleukin 1
P	: significance level. Mann Whitney U test

monocytes, as shown in Fig. XXXIV (p=0.01). However, when spontaneous IL-1 production by SF monocytes was compared to spontaneous IL-1 production by control PB monocytes, the former was significantly higher ($p<0.001$).

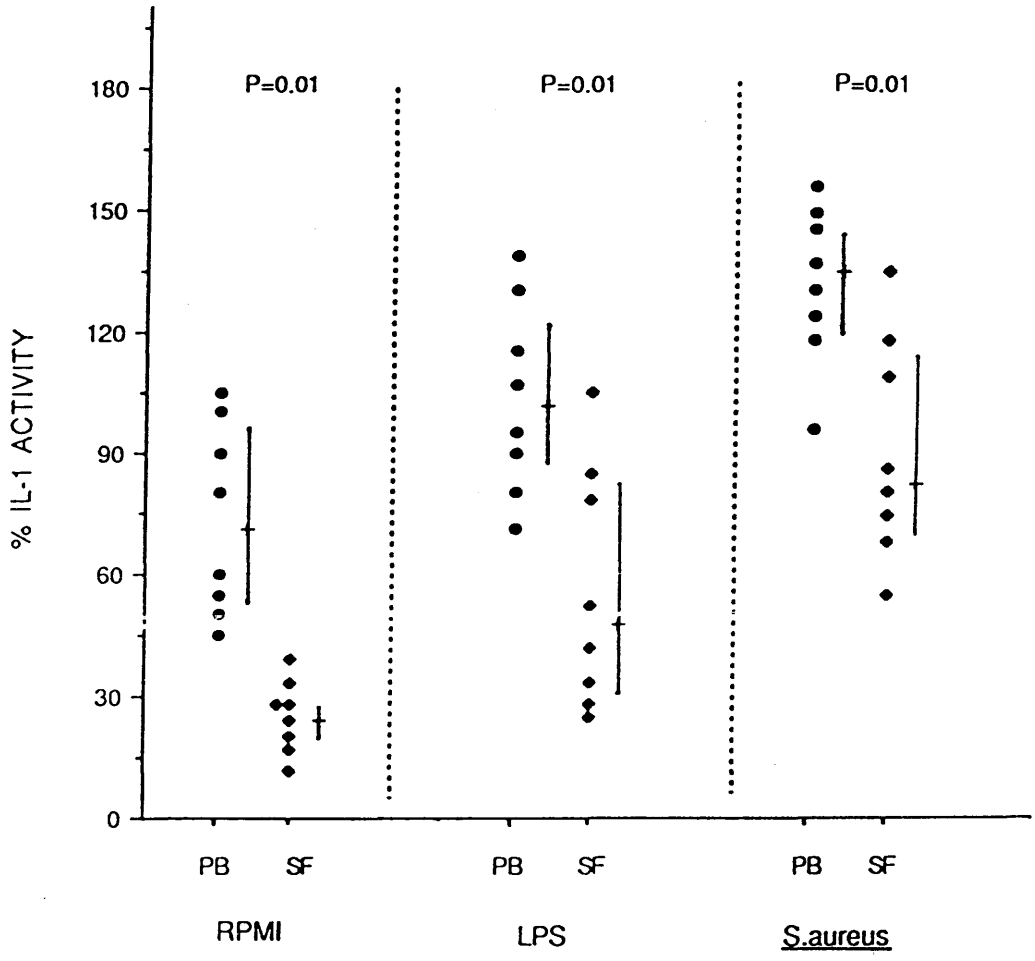
After LPS or S. aureus stimulation, both PB and SF monocytes produced significantly more IL-1 over spontaneous production (LPS: p=0.01; S. aureus: p=0.01 for both PB and SF monocytes). However, the PB monocytes produced significantly more stimulated IL-1 than corresponding SF monocytes in the case of both stimulants (Fig. XXXIV) (p=0.01).

It is interesting to point out that IL-1 production by RA SF and normal PB monocytes was also significantly different after stimulation with either LPS or S. aureus, a much greater response to both stimulants being shown by SF monocytes (LPS: $p<0.001$; S. aureus: $p<0.001$).

4.3.3 Effect of sulphasalazine in vivo on in vitro monocyte IL-1 production

As seen in Fig. XXXV, monocytes from 8 patients with RA released IL-1 spontaneously. The median value for spontaneous IL-1 release before SASP treatment was 20.2%, and this rose significantly to 29.9% in monocytes from these same patients, 12 weeks after commencing SASP treatment (p=0.01). This increased production was consistent in all 8 patients studied. Lipopolysaccharide stimulated IL-1 release in all patients as did S. aureus. The median value for LPS and S. aureus-stimulated IL-1 release prior to SASP treatment was 31.1% and 34.3% respectively. After 12 weeks of SASP treatment these rose to 36.2% and 37.8% respectively, however, this difference

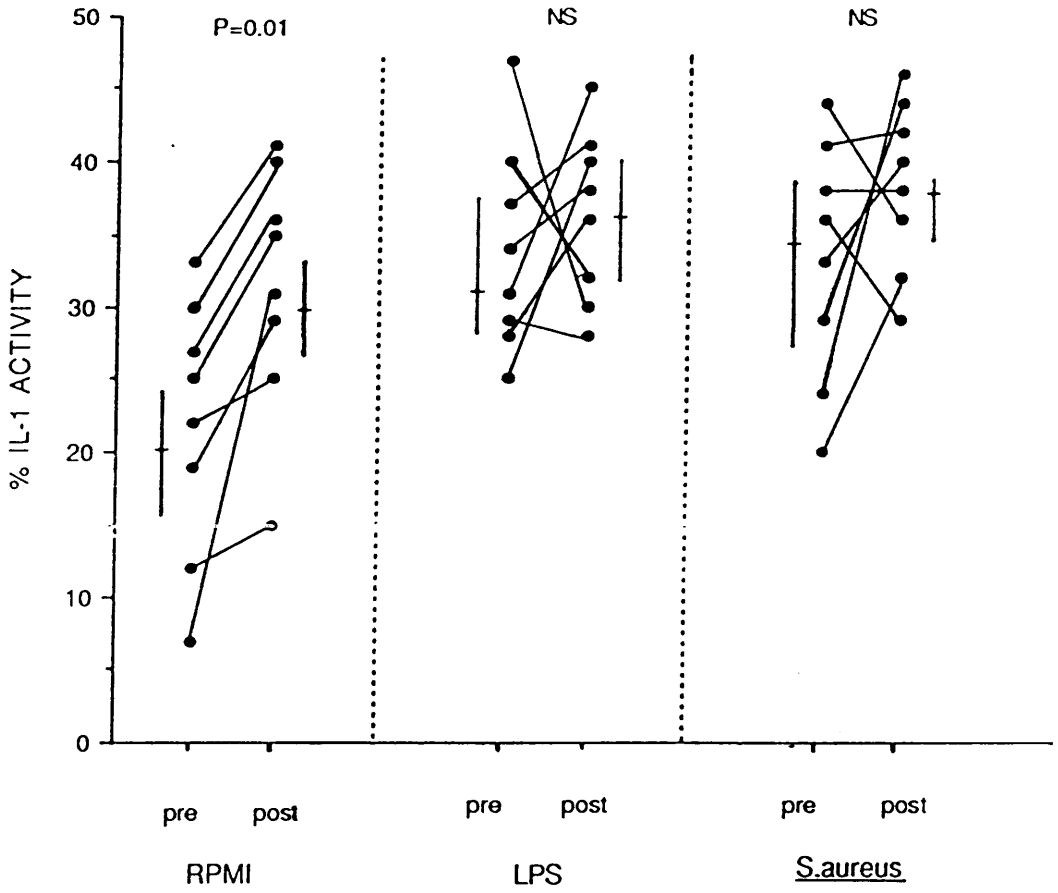
Fig. XXXIV Interleukin 1 production by paired PB and SF monocytes from patients with rheumatoid arthritis.



┼ median plus interquartile range

PB : peripheral blood
SF : synovial fluid
RPMI : unstimulated IL-1 production
LPS : lipopolysaccharide - stimulated IL-1 production
S. aureus : Staphylococcus aureus - stimulated IL-1 production
IL-1 : interleukin 1
P : significance level determined by the Wilcoxon rank test

Fig. XXXV Interleukin 1 production by PB monocytes from patients with rheumatoid arthritis: before and after sulphasalazine treatment



median plus interquartile range

PB	:	peripheral blood
pre	:	before sulphasalazine treatment
post	:	12 weeks after commencing sulphasalazine treatment
RPMI	:	unstimulated (spontaneous) IL-1 production
LPS	:	lipopolysaccharide - stimulated IL-1 production
S. aureus	:	Staphylococcus aureus - stimulated IL-1 production
IL-L	:	interleukin 1
P	:	significance level determined by Wilcoxon rank test
NS	:	not significant

was not significant (Fig. XXXV). While taking SASP, LPS-stimulated IL-1 release rose in 62.5% (5/8) and fell in 37.5% (3/8) of patients, and S. aureus - stimulated IL-1 release increased in 50% (4/8), decreased in 25% (2/8) and remained unchanged in 25% (2/8) of patients (Fig. XXXV).

4.3.4 Effect of drugs in vitro on monocyte IL-1 production

Monocytes obtained from 7 healthy volunteers and incubated directly with tenidap, SASP, SP, 5-ASA and indomethacin, at the concentrations shown in Table 2.5, did not release significantly more or less IL-1 than cells not exposed to these drugs (Table 4.1). The effect was variable and it did not follow a clear dose-response relationship. Upon stimulation with LPS or S. aureus, IL-1 release was significantly increased (LPS: $p=0.04$; S. aureus: $p=0.04$). None of the 5 drugs mentioned above exhibited an inhibitory action on monocytes, in respect to their ability to spontaneously release IL-1 (Table 4.1). However, monocytes incubated directly with hydrocortisone sodium phosphate, at concentrations of 20 and 30μg/ml, released significantly less IL-1 than untreated monocytes (Table 4.1) ($p=0.04$). Furthermore, in LPS and S. aureus-stimulated cultures, hydrocortisone sodium phosphate at all concentrations inhibited IL-1 release from monocytes. This effect was consistent in the 7 control subjects and followed a clear dose-response relationship.

Monocytes obtained from 7 patients with RA and subjected to these same 6 pharmacological agents demonstrated similar results (Table 4.2). Hydrocortisone sodium phosphate was the only agent to effectively reduce IL-1 .release, in a dose-dependent manner, both spontaneously ($p=0.02$) and in stimulated monocyte cultures ($p=0.02$). Upon stimulation with LPS or S. aureus, IL-1

	RPMI (spontaneous)	LPS stimulated	S. aureus stimulated
Monocytes (control)	19.4 (11.6-27.8)	56.2 (46.7-58.8)	69.1 (51.8-78.3)
Drug µg/ml			
TENIDAP			
1	15.9 (10.5-17.9)	46.4 (42.6-55.0)	65.7 (56.9-74.1)
2	16.6 (11.9-22.6)	48.2 (40.1-57.4)	64.7 (47.9-74.6)
5	23.4 (11.2-25.8)	48.1 (44.4-55.6)	61.7 (51.7-77.9)
10	13.3 (10.5-19.3)	50.2 (38.4-52.9)	68.6 (59.2-79.1)
SASP			
10	20.0 (16.5-21.6)	54.4 (44.5-55.5)	62.4 (56.0-80.1)
20	16.6 (8.5-21.6)	57.2 (51.6-61.5)	65.7 (57.1-68.4)
100	18.8 (9.4-22.0)	49.2 (47.4-62.6)	64.2 (58.2-68.3)
500	16.2 (15.5-22.6)	58.8 (50.9-60.9)	63.8 (50.9-72.3)
SP			
10	26.6 (21.0-30.0)	46.1 (37.9-57.5)	63.6 (49.7-82.9)
20	30.2 (17.4-34.8)	45.4 (42.0-64.1)	65.7 (44.9-79.1)
100	27.9 (10.4-30.9)	49.8 (44.6-50.6)	60.6 (50.6-81.0)
500	25.6 (18.2-27.6)	47.8 (42.3-58.0)	64.4 (51.0-81.1)
5-ASA			
10	19.4 (17.9-24.8)	46.8 (40.1-52.9)	66.8 (53.1-81.6)
20	18.8 (11.6-22.1)	48.6 (45.6-50.7)	64.1 (56.2-73.1)
100	22.6 (15.3-28.0)	47.4 (40.6-58.6)	67.8 (55.8-81.4)
500	20.8 (10.6-25.4)	51.9 (44.1-60.2)	59.6 (49.1-76.5)
HC			
10	11.4 (10.5-20.4)	*37.8 (29.7-47.1)	*50.9 (41.6-61.8)
20	*13.6 (9.7-16.8)	*29.4 (22.2-32.6)	*39.4 (31.6-47.1)
30	* 9.9 (8.9-14.2)	*19.8 (17.5-22.5)	*22.8 (19.6-30.6)
Indo			
1	16.4 (9.2-19.9)	58.9 (50.9-60.6)	65.5 (48.9-83.0)

All results are shown as median (range). * = $0.05 > p > 0.01$.
LPS: lipopolysaccharide, S. aureus: Staphylococcus aureus.
SASP: Sulphasalazine, SP: Sulphapyridine, 5-ASA: 5-Aminosalicylic acid,
HC: Hydrocortisone sodium phosphate, Indo: Indomethacin.

Table 4.1 In vitro drug effect on interleukin 1 production by monocytes from normal subjects

	RPMI (spontaneous)	LPS stimulated	S. aureus stimulated
Monocytes (control)	33.7 (32.4-49.0)	73.2 (69.4-75.7)	58.2 (54.5-64.3)
Drug µg/ml			
TENIDAP			
1	27.6 (22.4-40.1)	67.6 (62.9-88.8)	57.2 (52.6-60.6)
2	33.5 (31.6-52.5)	63.3 (61.2-87.2)	55.1 (48.2-63.6)
5	35.4 (32.8-48.5)	71.7 (68.9-84.2)	56.9 (55.2-59.6)
10	36.2 (31.8-44.9)	76.6 (62.4-81.7)	59.4 (51.6-60.0)
SASP			
10	41.6 (34.7-45.5)	69.8 (68.5-79.7)	60.0 (55.0-66.6)
20	42.9 (34.4-48.9)	72.2 (68.6-75.4)	56.9 (53.1-65.1)
100	39.6 (35.6-48.8)	72.6 (70.3-73.5)	57.7 (51.2-67.2)
500	35.7 (26.7-39.6)	72.9 (62.0-76.7)	57.3 (45.4-63.6)
SP			
10	38.0 (29.6-47.5)	67.8 (46.9-80.6)	54.3 (49.7-67.2)
20	39.8 (38.1-48.8)	73.2 (65.4-79.9)	56.3 (51.2-65.2)
100	41.9 (33.2-50.2)	72.0 (69.2-76.5)	54.4 (48.6-61.5)
500	40.3 (39.4-46.9)	70.0 (68.0-80.0)	57.3 (54.1-60.5)
5-ASA			
10	44.7 (32.4-50.6)	73.1 (62.1-77.0)	60.1 (51.6-63.3)
20	47.6 (34.2-48.5)	71.4 (63.5-80.9)	57.0 (51.5-71.6)
100	45.6 (41.4-50.4)	68.4 (62.5-74.9)	53.8 (45.2-66.7)
500	42.6 (40.4-49.0)	67.2 (60.0-73.0)	56.4 (46.7-67.6)
HC			
10	26.2 (23.0-37.7)	*45.9 (30.6-52.6)	*32.4 (26.2-35.5)
20	*21.6 (17.9-32.5)	*39.6 (29.1-54.2)	*26.5 (25.0-29.6)
30	*18.2 (14.6-29.5)	*30.2 (27.7-49.6)	*24.1 (17.6-26.0)
Indo			
1	33.5 (26.1-42.8)	62.5 (54.9-69.7)	54.9 (46.4-59.8)

All results are shown as median (range). * = $0.05 > p > 0.01$.
LPS: lipopolysaccharide, S. aureus: Staphylococcus aureus.
SASP: Sulphasalazine, SP: Sulphapyridine, 5-ASA: 5-Aminosalicylic acid,
HC: Hydrocortisone sodium phosphate, Indo: Indomethacin.

Table 4.2 In vitro drug effect on interleukin 1 production by monocytes from rheumatoid arthritis subjects

release from RA monocytes, was significantly increased (LPS: p=0.02; S. aureus: p=0.02).

A significantly higher degree of spontaneous and LPS-stimulated, but not S. aureus-stimulated, IL-1 production was evident in monocytes from RA patients compared to the control group ($p<0.01$).

4.4 CONCLUSIONS

The results of this study have shown that:

1) PB monocytes of patients with RA produce significantly more IL-1 than normal controls both spontaneously and after LPS or S. aureus stimulation.

2) Synovial fluid monocytes spontaneously produce less IL-1 than paired PB monocytes, and such a difference was maintained after LPS or S. aureus stimulation.

The findings of the first study confirm those of Nouri et al. (1985) and Shore et al. (1986). However, the observations made in the latter study are in disagreement with Nouri et al. (1985). He observed higher spontaneous IL-1 production from SF mononuclear cells than from corresponding PB cells, however this was not observed after stimulation. In the present study, the possibility remains that the apparent lower level of IL-1 production by monocytes from SF, may have been due to the presence of IL-1 inhibitors.

Spontaneous IL-1 production by monocytes has been previously described (Shore et al., 1986) and in all cases may be due to trauma during isolation

procedures. This could explain enhanced spontaneous IL-1 production by SF mononuclear cells in the study by Nouri et al. (1985). He included a centrifugation and resuspension step to remove the cells from the SF. This step was omitted during the separation of mononuclear cells from peripheral blood. Possibly, this additional treatment has overstimulated the SF mononuclear cells. In the present study, SF was diluted 1:1 with culture medium before being layered directly onto Nycodenz Monocytes. Other than this it was treated in exactly the same way as the PB samples. Furthermore, there was no mention of any attempts to limit endotoxin contamination in Nouri's studies. Endotoxin could have been a significant contributing factor to the amount of IL-1 produced.

Another explanation for the controversial results may have been due to the use of LPS or S. aureus as stimulants rather than PHA, as used by Nouri and his colleagues (1985). Mononuclear cells contain T-cells, B-cells and monocytes. Relevant to this Shore et al. (1986) have found that IL-2 generation by PB mononuclear cells in response to PHA is highly variable. This may explain why Nouri and his colleagues (1985) did not observe any difference in the mouse thymocyte assay between SF and PB mononuclear cells after stimulation, since the mouse thymocyte assay is sensitive to IL-2 contaminating IL-1 supernatants.

Enhanced spontaneous generation of IL-1 in the RA patient group suggests in vivo activation of their monocytes. Monocytes from patients with RA are known to have increased numbers of Fc receptors and elevated activity of their hexose monophosphate shunt (Shore et al., 1986), both features indicative of activation. Furthermore, Eastgate et al. (1988) have found messenger RNA for IL-1 beta, IL-1 alpha and TNF by in-situ hybridisation in synovial sections

from RA joints. These observations indicate that IL-1 gene expression is activated in vivo within the rheumatoid synovium.

The question which one asks is, what is the mechanism of this activation? There are a few possibilities. Firstly, purified human C5a has been reported to stimulate IL-1 production in human monocytes (Goodman, Glenweth and Weigle, 1982) ie. complement activation. Secondly, ICs have the potential to stimulate IL-1 production (Arend, Joslin and Massoni, 1985). Moreover ICs are known to be produced and phagocytosed locally in the joint. Arend et al. (1985) suggest that complement fixation by ICs generates IL-1 inducing activity. While Arend et al. (1985) were unable to detect direct stimulation of IL-1 production by various forms of ICs in the absence of complement, other investigators have (Dayer et al., 1980).

Fever, elevated APPs and leucocytosis are some responses to systemic IL-1 that make it a key mediator of inflammation. However, clinical observations have revealed that not all patients with RA have a characteristic systemic response. For instance, fever is often transient and low grade, leucocytosis is not always detectable; a wide range of SF leucocyte counts (6,800 to 258,000mm^3) has been demonstrated, and APPs are not always elevated (Rosin and Goldberg, 1962). This could be explained by a reduced IL-1 response by SF monocytes. The results illustrated in this study would support this possibility.

A possible explanation for this could be that some inhibitory substance present in SF has downregulated the cells' mechanism for producing IL-1 when in vivo. Consequently when stimulated in vitro the cells have an impaired response to IL-1 production. At present, no such inhibitory factor has been characterised.

One possibility could be the acidic pH of SF, which may be detrimental to monocytes and their receptors.

The marked leucocytosis characteristic of a profound bacterial infection may not occur in the presence of concurrent RA (Rosin and Goldberg, 1962). Consequently, these patients may be more susceptible to bacterial infection in joints and tissues affected by rheumatoid changes. Furthermore, Ozaki et al. (1987) have encountered debilitated patients who have signs of bacterial infection without elevated body temperature. Some clinicians have the view that the prognoses of these patients are generally poorer than the prognoses of those who manifest fever. In support of this observation, Keenan et al. (1982) reported that infected patients who produce EP had a better survival rate than those who lack the ability to produce them. This suggests that RA patients who do not show a systemic response and develop an infection within a joint may be more susceptible to fatal sepsis than those who manifest a systemic response.

Wood and his associates (1983) reported that the amount of IL-1 recovered from SF did not correlate with the numbers of monocytes observed in the fluid. The joint fluid IL-1 may thus be produced not only by free monocytes but by those immobilised in the pannus. Therefore, the possibility that in vitro results may not necessarily truly reflect what happens in vivo, cannot be dismissed. This could be a potential explanation why, in this study, monocytes from SF produced less IL-1 than monocytes from the PB, even although high levels of IL-1 have been detected in SF from patients with RA (Fontana et al., 1982).

Since bacterial products are potent stimulators of IL-1 production by macrophages (Wood et al., 1983), it is possible that the presence of IL-1 in SF

may reflect the presence of persistent bacterial debris. The results presented herein, that monocytes from SF produce less IL-1 than monocytes from PB when stimulated with S. aureus, could be due to previous occupation of receptors on SF monocytes by S. aureus from within the joint cavity and therefore, fewer free receptors are available for in vitro stimulation.

It was confirmed in these studies that hydrocortisone, in a dose-dependent manner, is a highly effective IL-1 inhibitor in vitro. This is also apparently true of its action in vivo (Bondy and Bodel, 1971). Therefore, a joint treated with a steroid injection may be more susceptible to septic arthritis. Therapeutic concentrations of NSAIDs such as indomethacin have been found not to reduce the production of IL-1 (Dinarello, 1984a) which is in agreement with this study. It would be expected that the disease-modifying drugs, which, along with clinical improvement, are associated with a marked and sustained fall in the level of APPs, should slow down erosive progression of RA as measured x-radiographically. The fall in APPs could be the consequence of reduced IL-1 production, since IL-1 is known to induce the production of APPs. The results presented herein do not support this theory, as IL-1 production was not depressed by either SASP or tenidap (a new agent from Pfizer), the two representatives of '2nd-line' drugs in this study.

CHAPTER 5 - DISCUSSION AND FUTURE RESEARCH

The aim of these studies was to investigate a possible association between susceptibility of RA patients to bacterial infection (Gristina et al., 1974) and impaired immune responses in these patients. The research herein concentrated on two aspects of immunity in particular; the phagocytic capacity of PMNs and the capacity of monocytes to produce IL-1.

Rheumatoid arthritis and its therapy provide portals of entry for invading organisms via skin ulcers, joint injections, aspirations and surgery. The first line of non-specific cellular defence, that is PMNs, has been reported to be impaired in these patients (Wilton et al., 1978; Attia et al., 1982b). Those researchers who reported such an intrinsic defect in RA PMNs have been criticised for failing to separate PMNs from autologous plasma or SF (Turner et al., 1973) and consequently what they detected could have been the result of an extrinsic defect. Indeed, investigations on the effect of RA serum and SF on phagocytosis by normal PMNs revealed that the defect in RA PMNs was of an extrinsic nature (Turner et al., 1973).

In this investigation the phagocytic uptake of S. aureus and P. mirabilis by RA PMNs was significantly depressed in comparison to normal PMNs. This is suggestive of an intrinsic defect. Serum from seropositive and seronegative RA patients did not inhibit the phagocytic uptake of S. aureus by normal PMNs significantly more so than serum from normal subjects. On the other hand SF from RA patients did inhibit phagocytic uptake in a dose-dependent manner, and significantly more so than SF from patients with other arthritic diseases. Since PMNs circulate from serum into SF and back into serum, it is possible

that the intrinsic defect could be a measurement of the response of PMNs which had not yet recovered from the inhibitory effect of RA SF in vivo.

The nature of the inhibitor(s) of PMN phagocytosis in SF of RA patients remains unclear. It is unlikely that inhibition was mediated by simple blockage of receptors, as all PMN preparations were washed after exposure to SF. There is some evidence that ICs, present in both RA serum and SF, may be responsible for the inhibition of PMN phagocytosis (Turner et al., 1973). Phagocytosis of ICs requires the internalisation of bound C3b and Fc receptors which may also involve the removal of unbound receptors for opsonised bacteria. Serum from patients with RA was found to contain significantly greater concentrations of both IgG and IgM ICs, than normal serum. Yet RA sera, in comparison to normal sera did not significantly depress normal PMN phagocytic capacities, irrespective of the RF titre. Furthermore, normal PB-PMNs incubated with purified RF, did not exhibit defective phagocytic uptake. Therefore, the foregoing theory of ICs being responsible for depressed phagocytosis seems an unlikely explanation in this study.

The lack of correlation between parameters such as RF titres and levels of complement components suggest that inhibitory agents, other than ICs, may be involved. Rheumatoid arthritis SF also contains hyaluronic acid and free iron (Gutteridge et al., 1982) both of which have a detrimental effect on phagocytosis (Brandt, 1974; Sweder van Asbeck et al., 1984). Furthermore, SF is known to contain high levels of IL-1 (Wood et al., 1983). Therefore, it most possibly contains other mediators secreted by activated mononuclear phagocytes, such as interleukin 6, colony stimulating factor, TNF and gamma interferon (Unanue, 1986).

Further research in this aspect, should involve the characterisation of the inhibitory nature of SF. One possibility is to characterise it according to its molecular size. Synovial fluids could be run through a sephadex column designed to remove a particular molecular weight fraction. The remainder of the fluid could be retested in the phagocytic assay to determine its inhibitory capacity. By removing a variety of molecular weight fractions and reassaying the fluids, one might be able to identify a specific fraction which significantly contributes to the inhibitory action of SF. The nature of this fraction could then possibly be determined by gel electrophoresis techniques.

It would also be interesting to measure the concentration of hyaluronate, free iron and other metal ions in the SFs and to correlate these with the effect of SF on PMN phagocytic uptake. Either one of these may contribute to the inhibitory action of SF.

Another area of research which could be pursued would be to investigate the effect of incubating IL-1, IL-6, TNF, colony stimulating factor and gamma interferon - individually and in all possible combinations - with normal PMNs. In this way it is possible to determine whether one of these factors alone is the inhibitor or whether they act synergistically or antagonistically to inhibit PMN phagocytosis. Synovial fluid from patients with RA may also indirectly inhibit phagocytosis by a lack of opsonins. In this study, and others, SF from RA patients showed decreased concentrations of complement components and heat-labile opsonic activity.

In terms of providing a mechanistic explanation for the protective effects of IL-1, the most obvious possibility might be that IL-1 directly activates antibacterial cellular defence mechanisms.

Ozaki and his colleagues (1987) among others (Minami et al., 1988) demonstrated that purified recombinant human IL-1 alpha, in a dose-dependent manner, significantly increased the survival rate of experimentally infected mice. The ineffectiveness of heat-inactivated IL-1 alpha given at the same protein concentration on the same schedule as that of native IL-1 alpha suggests that the protective effect of IL-1 against infection was due to specific biological activities.

In addition, other studies of in vitro IL-1 production, from blood leucocytes of patients with various conditions such as cancer, large tumour burdens, fatal sepsis and malnourishment, that have demonstrated reduced IL-1 production (Oppenheim, 1986) emphasize its importance.

Interleukin 1 is chemotactic for PMNs, monocytes and lymphocytes (Hunninghake et al., 1987), and local concentrations of IL-1 in inflammatory sites, such as the rheumatoid joint, probably contribute to cellular infiltration. Interleukin 1 has been reported to activate PMNs (Murphy, 1985) upon which they release toxic oxygen radicals and enzymes into the surrounding mileu. These toxic oxygen radicals and proteolytic enzymes cause tissue damage and may damage PMN surface Fc receptors thereby preventing them from participating in phagocytosis.

Monocytes from the SF of patients with RA have been shown in this study to have a lower capacity to produce IL-1 in vitro. This may be the result of pre-stimulation and exhaustive production in vivo. Although IL-1 is chemotactic for PMNs, this study has shown SF from RA patients to be inhibitory to PMN phagocytosis. In addition Mowat and Baum (1971) described RA serum to inhibit PMN chemotaxis. It is possible that chemotaxis inhibition extends to

the SF, which would render the joint susceptible to infection not only due to impaired PMN phagocytosis but also to a reduced PMN cell population.

Since some microbial surface structures (such as mucopeptide of S. aureus) act to resist phagocytosis (Minami et al., 1988), higher doses of IL-1 might be necessary to eliminate such organisms. As IL-1 production by SF monocytes is lower than that produced by PB monocytes in vitro, these organisms may multiply without much interference from IL-1. If this is the case in vivo it is interesting to note that S. aureus is one of the most common organisms to be isolated from cases of septic arthritis (Kellgren et al., 1958; Goldberg and Cohen, 1976).

It seems reasonable to suggest that the infecting dose in vivo may be important. If large numbers of bacteria infect the joint, it may overcome the capacity of IL-1 to protect the host. In addition if the phagocytic capacity of PMNs in the joint space is depressed as a result of the inhibitory action of SF, as previously reported herein, the joint is consequently rendered more susceptible to infection by lower numbers of bacteria.

It would be interesting to isolate monocytes from the SF and measure their production of IL-1, produced both spontaneously and after stimulation. The same SF could be tested for the presence or absence of staphylococcal Ags, which would be evidence of past or recent infection, and might provide an explanation for the changes in IL-1.

Whatever the cause of defective phagocytosis in the joint, and although substantial amounts of IL-1 are produced by SF monocytes in comparison to

normal monocytes, the consequences of this may be tissue damage and a susceptibility to infection of the RA patients.

Experience to date with disease-modifying drugs of RA is that by and large they have not significantly compromised the patient, as confirmed in this study. This unfortunately is not the case with steroids, which inhibit IL-1 production and at high concentrations may possibly be detrimental to PMN phagocytosis. The observed increase in susceptibility to infection associated with steroid treatment favours the theory that some of the effects of IL-1 on the host have an important and vital role in defence against infection and therefore treatment may be contra-indicated. Steroids are administered sparingly now, often as a last resort and only to patients with severe persistent arthritis who have been unresponsive to alternative therapy.

In summary, although inhibitors of phagocytosis do exist, as yet to be clearly characterised, I feel that future research should be directed towards:

1. Measuring the concentration of hyaluronate and metal ions in SF and correlating these to the inhibitory action of SF on PMN phagocytosis,

2. isolation of inhibitors in SF using gel chromatography,

3. studying the dose/response of interleukins on PMN phagocytosis, and

4. investigation of other PMN functions in response to incubation with SF, would also be of interest, eg. chemotaxis and bactericidal activity.

APPENDIX A

Diagnostic criteria for rheumatoid arthritis according to the American Rheumatism Association (ARA).
(Taken from Ropes et al, 1959)

Patients described as classical RA satisfied at least 7 of the following criteria, while the diagnosis of definite RA required 5.

1. Morning stiffness.

2. Pain on motion or tenderness in at least one joint.

3. Swelling or soft tissue or fluid (not bony outgrowth alone) in at least one joint.

4. Swelling of at least one other joint (any interval free of joint symptoms between the 2 joint involvements may not be more than 3 months).

5. Symmetrical joint swelling with simultaneous involvement of the same joint on both sides of the body.

6. Subcutaneous nodules over bony prominences, on extensor surfaces or in juxtra articular regions.

7. X-ray changes typical of RA (which must include at least bony decalcification localised to or greatest around the involved joints and not just degenerative changes.

8. Positive agglutination test - demonstration of rheumatoid factor.

9. Poor mucin precipitate from SF.

10. Characteristic histological changes in synovial membrane with 3 or more of the following:

    a) marked villous hypertrophy
    b) proliferation of superficial synovial cells often with palisading
    c) marked infiltration of chronic inflammatory cells.
    d) deposition of compact fibrin
    e) foci of cell necrosis.

11. Characteristic histological changes in nodules.

Note: for criteria 1-5 the joint signs and symptoms must have been continuous for at least 6 weeks.

None of the RA patients displayed any of the features listed below:

1. The typical rash of disseminated lupus erythematosus.
2. High concentrations of lupus eruthematosus cells.
3. Histological evidence of periarteritis nodosa or sarcoid or a positive Kveim test.
4. Weakness of neck, trunk and pharyngeal muscles, or persistent muscle swelling of dermatomyositis.
5. Definite scleroderma.
6. Tophi.
7. Tubercle bacilli in joints or histological evidence of joint tuberculosis.
8. Homogentisic acid in the urine.
9. Multiple myeloma.
10. Characteristic skin lesions of erythema nodosum.
11. Leukemia or lymphoma.
12. Agammaglobulinaemia.
13. A clinical picture characteristic of rheumatic fever, gouty arthritis, acute septic arthritis, Reiter's syndrome, shoulder hand syndrome, hypertrophic pulmonary osteoarthropathy or neuroarthropathy.

APPENDIX B

Standardisation of phagocytosis methodology

Standardisation of bacteria

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

1. Staphylococcus aureus, Cowan strain

2. Proteus mirabilis, laboratory strain

A suspension 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 was prepared in phosphate buffered saline, and 10-fold dilutions were made from this. 10µl from each dilution were then carefully dropped onto an agar plate and incubated overnight at 37°C. The following day, bacterial colonies were counted and the concentration of bacteria per millilitre in the original sample could be calculated.

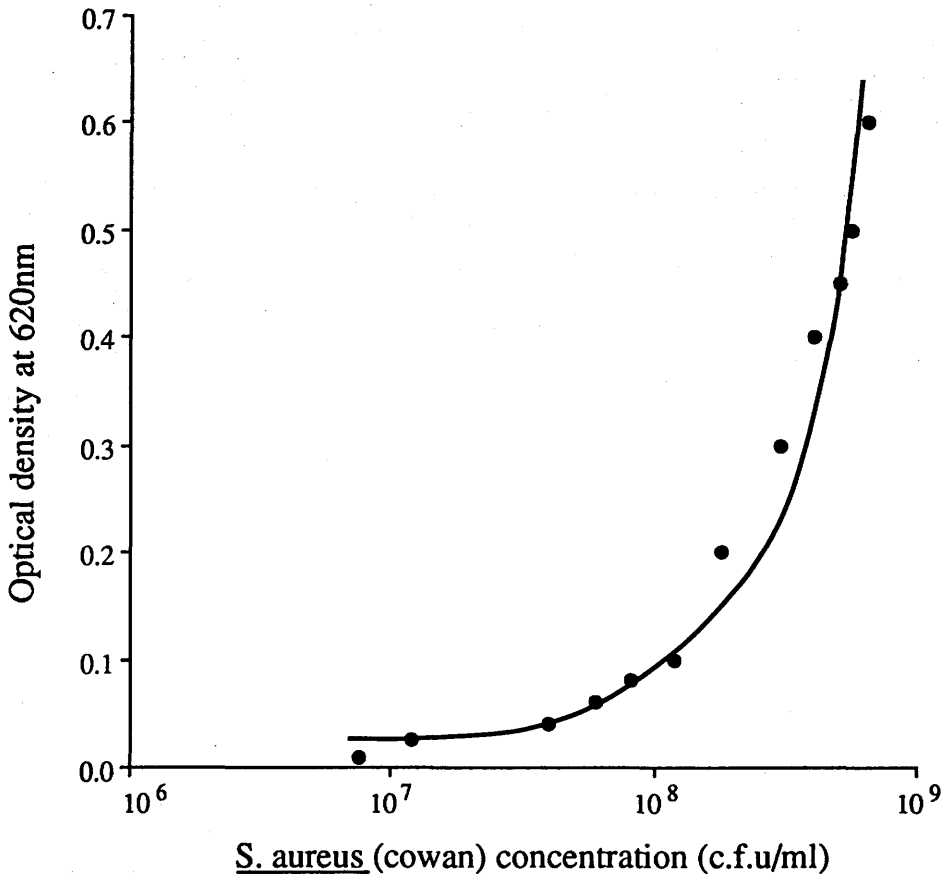
For example, if 10 colonies were counted at 10^{-4} dilution, the c.f.u./ml would be calculated from the following equation:

$$\text{c.f.u./ml} = 10 \times 10^4 \times 10^2$$

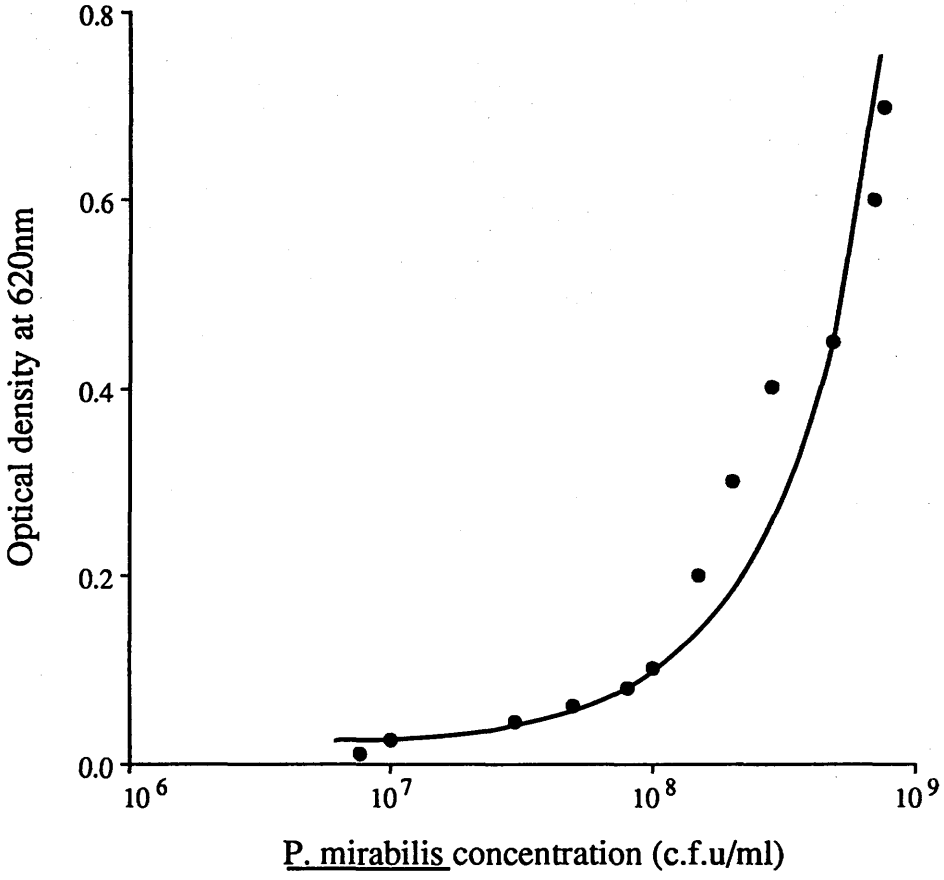
Multiply by a factor of 10^2 since only added 10µl of bacterial suspension to the plate and the desired concentration is per millilitre.

This experiment was repeated 8 times for each of the bacteria and the median c.f.u./ml calculated and illustrated graphically as shown overleaf.

Correlation between optical density and concentration of Staphylococcus aureus



Correlation between optical density and concentration of Proteus mirabilis



% serum used as opsonin		phagocytic uptake (%)		
5	*	34.2	**	(29.7 - 41.1)
10		53.1		(31.1 - 65.9)
20		38.5		(31.0 - 58.1)
50		12.2		(5.9 - 20.7)
100		5.9		(1.5 - 22.4)

I Staphylococcus aureus

% serum used as opsonin	phagocytic uptake (%)	
5	5.0	(1.5 - 9.9)
20	29.2	(21.4 - 41.2)
50	57.2	(40.1 - 65.5)
75	53.3	(49.8 - 60.4)
100	52.1	(29.9 - 59.9)

II Proteus mirabilis

* Median of 5 separate experiments performed with 5 different polymorphonuclear leucocyte.

** Range.

Effect of the concentration of serum used during opsonisation of, I. Staphylococcus aureus and II. Proteus mirabilis, on phagocytic uptake.

		Phagocytic uptake (%)	
Bacteria : PMN ratio		S.aureus	P. mirabilis
1:1	*	62.3	54.1
	**	(25.5 - 68.1)	(34.6 - 67.8)
5:1		43.1	48.6
		(23.2 - 49.9)	(21.6 - 54.9)
10:1		24.1	45.9
		(15.4 - 35.1)	(14.2 - 57.8)
20:1		10.4	27.2
		(8.0 - 15.7)	(15.6 - 37.1)
50:1		5.1	11.0
		(4.5 - 8.7)	(10.1 - 21.7)
100:1		3.2	10.6
		(3.0 - 4.4)	(5.4 - 17.9)

* Median of 5 experiments with 5 different PMN preparations.
** Range.
PMN: polymorphonuclear leucocyte.
S. aureus: Staphylococcus aureus
P. mirabilis: Proteus mirabilis

Effect of bacterial concentration on phagocytic uptake.

% synovial fluid used as opsonin	phagocytic uptake of S. aureus (%)	% precision
10	* 18.7 ** (6.4 - 42.3)	52.7
20	20.3 (10.6 - 48.2)	53.6
25	17.6 (13.8 - 33.7)	37.9
50	20.2 (13.4 - 30.6)	31.4
75	21.1 (6.6 - 35.4)	47.0

* Median of 8 experiments performed with 8 different polymorphonuclear leucocyte preparations.

** Range.

S. aureus: Staphylococcus aureus.

Effect of the concentration of synovial fluid used during opsonisation of S. aureus, on phagocytic uptake.

	Staphylococcus aureus	Proteus mirabilis
*	45.4	43.7
**	39.7 - 50.7	37.1 - 49.0

* Median of 5 experiments performed on 5 separate days using polymorphonuclear leucocytes from one normal volunteer.

** Range.

Reproducibility of the phagocytosis assay.

APPENDIX C

Statistical analysis

Non-parametric statistics were used throughout this study as these tests make relatively few assumptions about the nature of the population distribution, ie. they take into consideration that the data may not be normally distributed. The tests were performed using the statgraphics package (version 2.6) on an Amstrad personal computer.

1. The Mann Whitney U test

The Mann Whitney U test was used to determine whether there was a significant difference between the values found in 2 separate sets of data. The 2 samples are combined and all sample observations are ranked from smallest to largest. Tied observations are assigned the mean of rank positions that they would have occupied if there had been no ties. When both n_1 or n_2 is less than 20, the test statistic is

$$U = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1 \qquad (I)$$

or

$$U = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - R_2 \qquad (II)$$

Where R_1 = sum of ranks assigned to the group whose sample size is n_1 and R_2 = sum of ranks assigned to the group whose sample size is n_2. If the U calculated is larger than $\frac{n_1 n_2}{2}$ than it is U^1 and can be transformed to U using the formula

$$U = n_1 n_2 - U^1 \qquad (III)$$

If U is equal to or less than the value given in a table of critical values of U (Leach, 1983), then there is a significant difference between the two groups with the level of significance indicated at the head of that table.

When n_1 or n_2 is larger than 20 the probability associated with a value as extreme as the observed value of U may be determined by computing the value of Z.

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{(n_1)(n_2)(n_1+n_2+1)}{12}}} \quad \text{(IV)}$$

This value is tested by referring to a table of probabilities (Leach, 1983). If the observed value of U has an associated probability equal or less than 0.05, then there is a significant difference between the 2 groups. If the associated probability is less than 0.01, then the difference is highly significant. If there is a large proportion of ties then the corrected formula for Z is

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\left(\frac{n_1 n_2}{N(N-1)}\right)\left(\frac{N^3 - N}{12} - \sum T\right)}} \quad \text{(V)}$$

Where $N = n_1 + n_2$

$$T = \frac{t^3 - t}{12}$$

t = the number of observations tied for a given rank.

2. <u>The Wilcoxon rank sum test</u>

The Wilcoxon rank sum test was used to determine whether the values from one sample are significantly different from the values from another paired sample. The

observations in the two samples are combined into a single series and ranked in order. The lowest score starting with a rank of 1, the next getting a rank of 2 and so on. When n_1 and n_2 are both less than 26 the test statistic is

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

where R = sum of ranks in smaller sample

N = total number of values

n_1 = sample size of larger group

n_2 = sample size of smaller group

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

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

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

where Sc = S-1 or S+1

c = continuity correction

mean = mean of S = 0

SD = standard deviation of S

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

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

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

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

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

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

U = the column marginal totals from an ordered contingency table (Leach, 1983).

3. The Spearman rank correlation

The Spearman rank correlation coefficient was calculated to investigate the degree of association between 2 variables. Each observation from each of the populations (X and Y) is ranked from smallest to largest, in order of magnitude

relative to all other observations in that one population. If ties occur in either population each tied value is assigned the mean of the rank positions for which it is tied. The test statistic is

$$r_s = 1 - \frac{6\sum di^2}{n\,(n^2 - 1)} \qquad \text{(VI)}$$

where $di^2 = \sum_{i=1}^{n} [R(x_i) - R(y_i)]^2$

$R(x_i)$ = the rank of the ith value of x

$R(y_i)$ = the rank of the ith value of y

n = the number of paired variables

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

$$r_s = \frac{\sum x^2 + \sum y^2 - \sum di^2}{2\sqrt{\sum x^2 \sum y^2}} \qquad \text{(VII)}$$

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

$y^2 = \frac{n^3 - n}{12} - \sum Ty$

$$T_x = \frac{t_x^3 - t_x}{12}$$

$$T_y = \frac{t_y^3 - t_y}{12}$$

T_x and T_y = the number of x and y observations that are tied for a particular rank.

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

$$t = r_s \sqrt{\frac{n - 2}{I - r_s^2}} \qquad \text{(VIII)}$$

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

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