CELL-ASSOCIATED INTERLEUKIN 1 IN RHEUMATOID ARTHRITIS

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by

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The work described in this thesis was stimulated by the hypothesis that interleukin 1 (IL-1) plays an important role in rheumatoid arthritis (RA) and mediates many of the characteristic features of this autoimmune joint disease. The evolution of this study included the development of a new method, the application of this method to study cell-associated IL-1α and IL-1β in normal and rheumatoid blood and the investigation of possible patho-physiological inducers of the cell-associated IL-1α found in the peripheral blood of patients with RA. The impetus for this work arose from my initial studies as a research fellow on differential Class II molecule expression in the synovial membrane of RA patients using well characterised monoclonal antibodies and immunohistological techniques. The results of this study were published and the paper is attached (appendix 1). This work coincided with the development by other laboratories of monoclonal antibodies to various cytokines. Monoclonal antibodies to IL-1α and IL-1β became available and I attempted to extend the immunohistological study using these antibodies to identify and localise cells producing IL-1 in the rheumatoid synovial membrane. Immunostaining of tissue sections with monoclonal antibodies to IL-1α and IL-1β had not been previously reported and I experienced extensive technical difficulties using these antibodies for immunostaining in synovial membranes. In an attempt to overcome these difficulties and to characterise the monoclonal antibodies and conditions required for staining, I developed a
method for identifying cell-associated IL-1α and IL-1β at the single cell level. This involved experiments on normal peripheral blood activated with mitogen in vitro. These produced interesting results and the remainder of the work stemmed from these studies. I performed all the experimental work discussed in this thesis unless otherwise indicated.
ABSTRACT

This thesis outlines the development of a specific method for identifying cell-associated IL-1α and IL-1β at the single cell level and the application of this method in normals and patients with RA. The findings are summarised below:

1. In normal peripheral blood mononuclear cells (PBM) activated with mitogen in vitro distinct populations of cells produce the two subtypes IL-1α and IL-1β.

2. Significantly more cell-associated IL-1α is seen in the unactivated blood of patients with RA compared to normals and controls. The percentage of bright IL-1α staining cells correlates with CRP. No IL-1β staining is seen.

3. Significantly fewer IL-1α staining cells are seen in the blood of RA patients receiving parenteral gold therapy.

4. Incubation of normal PBM with GM-CSF reproduces the picture of staining seen in RA blood. This is markedly inhibited by gold in vitro.

The above findings have not been reported before. The method developed allows the study of the two IL-1 subtypes at the single cell level and may be useful in the future to study IL-1 production by different cell types in health and disease. The results in normal blood may be important in understanding the reason for the existence of two forms of IL-1 with identical biological activities. Cell-associated IL-1α has not been previously reported in RA blood and may play an important role in the disease pathogenesis. GM-CSF may be involved in the induction of cell-associated IL-1α in vivo in RA and gold may exert its therapeutic effect via inhibition of this IL-1α production. The findings in RA blood may be important in understanding the role of IL-1α in this disease and suggest possible sites of action for novel therapeutic agents.
CONTENTS

CELL-ASSOCIATED INTERLEUKIN 1 IN RHEUMATOID ARTHRITIS

CHAPTER 1:

INTRODUCTION

1.1 General 12
1.2 Interleukin 1 25
1.3 Interleukin 1 in joint disease 33

CHAPTER 2:

DEVELOPMENT OF A METHOD FOR DETECTION OF CELL-ASSOCIATED INTERLEUKIN 1α AND INTERLEUKIN 1β

2.1 Introduction 38
2.2 Aims 39
2.3 Characterisation of antibodies
   Methods 40
   Results 43
   Discussion 47
2.4 Study of in vitro activated blood
   Materials and methods 48
   Results 56
   Discussion 69
2.5 Conclusions 75
ABBREVIATIONS

AG antigen
APC antigen presenting cell
AS ankylosing spondylitis
CPM counts per minute
CRP C-reactive protein
C_{5a} complement component C_{5a}
DMD disease modifying drug
ELISA enzyme linked immunoabsorbent assay
FCS foetal calf serum
FITC fluoroscein isothiocyanate
GM-CSF granulocyte-macrophage colony-stimulating factor
^{3}H-thymidine tritiated thymidine
HLA human leukocyte antigen
IFN_{\gamma} interferon \gamma
IL interleukin
Ig immunoglobulin
KD kilodalton
LPS lipopolysaccharide
M molar
\mu Ci micro-curie
MHC major histocompatibility complex
MLR mixed lymphocyte reaction
mRNA messenger RNA
MW molecular weight
NGS normal goat serum
NK cells natural killer cells
OA osteoarthritis
PBM peripheral blood mononuclear cells
PBS phosphate buffered saline
PHA phytohaemagglutinin
PMA phorbol myristate acetate
PGE_{2} prostaglandin E_{2}
RA rheumatoid arthritis
rIL-1 recombinant IL-1
RF rheumatoid factor
TNF tumour necrosis factor
List of Tables

1.1 Biological activities of IL-1

2.1 Monoclonal antibodies for cellular identification

2.2 Kinetics of IL-1α and IL-1β production

2.3 Effect of using anti-IL-1α and anti-IL-1β together on the percentage of activated PBM showing staining.

2.4 Inhibition of staining with excess antigen

3.1 Clinical details of RA patients

3.2 Clinical details of non RA patients

3.3 Clinical details of sarcoidosis patients

4.1 Stimuli for IL-1 production and release

4.2 Viability of cells following 22 hour culture +/- gold salts
List of Figures

1.1 IL-1 and T cell activation

2.1 ELISA results using the anti-IL-1 monoclonal antibodies and rIL-1α and rIL-1β as antigens

2.2 Immunoblot of anti-IL-1α

2.3 Immunoblot of anti-IL-1β

2.4 Preparation of PBM for immunostaining

2.5 Indirect immunofluorescence method

2.6 Kinetics of IL-1α and IL-1β production

2.7 Indirect immunofluorescence with anti-IL-1α on unstimulated PBM (a) and stimulated PBM; T=24 hours (b) T=44 hours (c)

2.8 Indirect immunofluorescence with anti-IL-1β on unstimulated PBM (a) and stimulated PBM; T=20 hours (b) T=44 hours (c)

2.9 Kinetics of IL-1 production: additive effect of using anti-IL-1α and anti-IL-1β together
2.10 Indirect immunofluorescence with anti-IL-1α and anti-IL-1β used together on stimulated PBM (T=22 hours)

2.11 Inhibition of staining with excess antigen

2.12 Indirect immunofluorescence using anti-IL-1α on T=24 hour activated PBM; (a) alone (b) preincubated with rIL-1α (c) preincubated with rIL-1β

2.13 Indirect immunofluorescence using anti-IL-1β on T=20 hour activated PBM; (a) alone (b) preincubated with rIL-1β (c) preincubated with rIL-1α

2.14 Indirect immunofluorescence with monoclonal anti-HLA-DR on activated PBM showing different morphology of IL-1α and IL-1β positive cells

2.15 Latex particle injection by a) IL-1α staining cells and b) IL-1β staining cells

3.1 Scattergram of IL-1α % positive cells in normals and RA

3.2 Indirect immunofluorescence using anti-IL-1α on unactivated PBM of RA patients (a)-(f)

3.3 Scattergram of IL-1α positive cells in RA patients on gold and RA patients on other therapies
3.4 Indirect immunofluorescence using anti-IL-1α on unactivated PBM of an RA patient receiving parenteral gold therapy

3.5 Graph showing % IL-1α positive cells vs C-reactive protein

3.6 Graph showing % IL-1α positive cells vs ESR

3.7 Graph showing % IL-1α positive cells vs haemoglobin

3.8 Scattergram of % IL-1α positive cells in RA patients and in patients with other rheumatic disease

3.9 IL-1α and IL-1β positive cells in sarcoidosis patients

4.1 IL-1α and IL-1β positive cells following 22 hour culture of normal PBM with different inducers (+/- gold)

4.2 Indirect immunofluorescence showing an IL-1α positive cell induced by GM-CSF

4.3 IL-1 bioactivity (supernatants/cell lysates) following 22 hour culture of normal PBM with different inducers (+/- gold)

5.1 Hypothesis for disease perpetuation in RA
CHAPTER 1

INTRODUCTION

1.1 General

Rheumatoid arthritis is a chronic inflammatory polyarthritis of unknown cause. It is the most prevalent of the autoimmune rheumatic diseases, affecting 0.5% to 1% of the population in Great Britain with a marked female preponderance. Clinically it is characterised by inflammation of the synovium with erosion and destruction of the joint. The disease process is not restricted to the joint and systemic extra-articular features occur. There is no diagnostic test with high specificity or sensitivity and the diagnosis of RA is based on the presence of certain clinical and laboratory features. The American Rheumatism Association criteria are now widely accepted (Arnett et al, 1988).

AETIOLOGY OF RA

Despite the high prevalence of RA in the population the aetiology remains unknown. Early family and mono and dizygotic twin studies suggested a weak genetic factor (Lawrence, 1970). In the 1970’s techniques became available to type the HLA-D related DR antigens and the importance of the MHC Class II molecules in the immune response became apparent. A strong association between HLA-DR4 and patients with RA was subsequently noted, with DR4 being present in 70% of the cases studied (Batchelor et al, 1977). This finding is generally true for RA in Caucasians, Japanese and Negroes, but in some
populations (eg Jews, Greeks) it is the DR1 molecule which is present at greater frequency (Stastny, 1980; Woodrow et al, 1981). More recent studies have identified a sequence on the third hypervariable domain of DR1 and the Dw4, Dw14 and Dw15 subgroups of DR4 which have been associated with RA. This sequence is not present on the Dw10 subgroup of DR4 which is not associated with RA (Winchester et al, 1988; Merryman et al, 1989). These studies suggest a powerful genetic factor in RA with a definite link with HLA-D Class II molecules. However the association is not absolute, with only a small proportion of people with this sequence developing the disease and some individuals having RA without possessing the sequence. This suggests that factors other than genetic predisposition play an important role in the aetiology of RA.

The synovial membrane in RA has been likened to a hyperactive, immunologically stimulated lymphoid organ (Janossy et al, 1981). The inflammatory process shows lymphocytic cell infiltrates and an influx of cells of the monocyte/macrophage and dendritic cell lineage. The picture is one of a chronic immune response. This has led to two hypotheses concerning the aetiology of RA, the infective and the autoimmune. Many bacteria, viruses and fungi are known to be arthritogenic in animal models and in man (Cole et al, 1973) and multiple attempts have been made to isolate and identify a micro-organism as the causitive agent in RA. Since no organism has been convincingly isolated, an infective aetiology must be explained either by the responsible organism being as yet unrecognised (eg. an unknown species of retrovirus) or perhaps more likely that a "hit and run"
phenomenon is occurring - by the time of clinical presentation of RA the offending infective agent has been eliminated from the system but has started a self perpetuating "autoimmune" process. It is now fairly widely accepted that a combination of a genetic predisposition and an exogenous agent, probably a virus, initiates an autoimmune process which is responsible for the chronic inflammation (with a predilection for the synovial joints) seen in RA. It is possible that RA patients are peculiarly reactive to certain infective agents because the DR molecules of RA bind their specific peptides. This would predispose the patient to the development of autoimmunity based on homologies between the bound exogenous peptides (eg viral proteins) and peptides found within self proteins. This "molecular mimicry" theory is currently under extensive study in RA and in many autoimmune conditions - a cross reactivity between HLA-B27 and Klebsiella has already been demonstrated in ankylosing spondylitis (Schwimmbeck et al, 1987).

IMMUNOPATHOLOGY OF RA
The immunopathology of RA has been partially elucidated. Many immunological features are present both systemically and locally in the joint. There is evidence of both humoral and cellular abnormalities. It is not clear which of these features are pathogenic and which are epiphenomena. The identification of the features involved in the disease pathogenesis is important as modification of these by therapy would prevent disease progression and provide clues for the development of novel therapies. The immunological abnormalities which have been suggested to be pathogenic
include the presence of autoantibodies; increased numbers of immature type lymphocytes; excessive class II expression in the joint; and imbalance of the cytokine network.

Autoantibodies
Most patients with RA have autoantibodies in their serum. If the causitive agent in RA is only transiently present, some ever-present endogenous factor may be involved in stimulating the immune reaction and the maintenance of chronicity. Autologous IgG (Roitt et al, 1982; Carson, 1985) and collagen (Holmdahl et al, 1987) are suggested candidates.

Rheumatoid factors (RFs) are autoantibodies, most commonly of IgM or IgG subclass which bind the Fc portion of IgG. Using the Latex agglutination test (which tends to select for IgM rheumatoid factors) 70% of patients with RA have rheumatoid factor antibodies. However RFs are not specific to RA and are seen in cryoglobulinaemias, infections and following immunisations. In these cases sustained RF production depends on continued immunological stimulation without which RF titres decline rapidly (Carson et al, 1978). It is not clear why in RA RFs are produced in large amounts and often for a lifetime. This may occur due to autosensitisation to aggregated IgG secondary to abnormal glycosylation of IgG molecules in RA (Parekh et al, 1985). It has been suggested that agalactosyl chains in RA serum IgG contribute to the autoantigenicity of IgG and may facilitate the self association of IgG/RF (Roitt et al, 1988). The pathogenicity of RFs in RA may be due to their ability to form form self-associated immune complexes (particularly IgG RFs) (Mannik et al, 1971). Immune
complexes are known to be present in both the blood and
synovial fluid of patients with RA (Hannested, 1967; Zvaifler,
1974; Zubler et al, 1976; Hay et al, 1979) and their presence
in the joint may give rise to joint damage via pathways
involving complement fixation and neutrophil recruitment
(Winchester et al, 1970); cytokine production (Chantry et al,
1989); and release of hydrolytic and proteolytic enzymes from
lysosomes (Cooke et al, 1984). The pathogenicity of RFs in RA
remains unproven. Although seropositive RA patients tend to
have more erosive and extra-articular activity, in seronegative
patients rheumatoid inflammation and joint destruction occurs
in the absence of RF or immune complexes.
Collagen has also been suggested to function as an autoantigen
in RA (Steffan et al, 1970). Collagen is able to induce an
erosive arthritis in experimental animals (Trentham et al,
1977). The arthritis can be transferred by immune cells
(Trentham et al, 1978) or by serum (Stuart et al, 1982).
Increased levels of anti-collagen antibodies have been reported
in RA by several groups, both in serum and in synovial fluid
(Andriopoulos et al, 1976; Menzel et al, 1976; Morgan et al,
1987; Rowley et al, 1987). Some groups have reported
antibodies to both native and denatured collagen (Clague et al,
1981). Other groups have found only antibodies to denatured
collagen (Rowley et al, 1986) and this may simply be a marker
of cartilage destruction (Stuart et al, 1983). The incidence
of serum antibodies to collagen in RA varies considerably
between studies (3-71%). The finding does not appear to be
specific to RA and these antibodies are also found in patients
with Dupuytren's contracture (Pereira et al, 1986), relapsing
polychondritis, (Ebringer et al, 1981) and psoriatic arthritis and osteoarthritis, graft versus host disease and gout (Trentham et al, 1981; Choi et al, 1988). The lack of disease specificity coupled with the observation that antibodies against native type II collagen do not precede the onset of rheumatoid arthritis (Möttönen et al, 1988) suggest that these antibodies do not play a primary role in the disease. It is not clear yet whether anti-collagen antibodies are an epiphenomenon secondary to a generalised disturbance of immunoregulation and cartilage destruction or whether autoreactivity to collagen plays a role in the perpetuation of rheumatoid inflammation. There is evidence that lymphocyte-collagen interactions occur within the joint (Tarkowski et al, 1989) and these may lead to the release of humoral or cellular products that are capable of contributing to the connective tissue inflammation. Epitopes present on native collagen II molecules are exposed in cartilage from rheumatoid joints compared to OA (Klareskog et al, 1986) and collagen immune complexes are known to be present in the joint (Jasin, 1985). Local cellular and humoral immunity to collagen may contribute to the activation of macrophages in the joint and thus disease perpetuation in RA although this remains speculative. Other cytoskeletal antibodies have been reported in the serum of some patients with RA. As yet there is no evidence that these have a pathogenic role in rheumatoid inflammation (Miossec et al, 1982; Osung et al, 1982).

Cell populations
Abnormalities of cellular immunity and its regulation have also
been suggested to play a pathogenic role in RA. Differences in sub-populations of chronic inflammatory cells have been reported in RA, both in blood and synovium. An increase in T lymphocyte helper-suppressor ratio has been demonstrated in RA blood and synovium (Veys et al. 1981; Janossy et al., 1981) and more recently increased numbers of foetal type T cells bearing the γδ T cell receptor have been described in RA blood and synovial fluid (Brennan et al., 1988). Increased numbers of a distinct lineage of B cells (CD5 positive B cells) are seen in RA blood (Plater-Zyberk et al., 1985). These are rarely seen in the synovium, as few B cells are present in the synovial membrane. This may reflect strong differentiation pressures as plasma cells are numerous in RA synovium. The significance of these CD5 positive B cells is not known although it has been suggested that they may be important in producing pathogenic IgG autoantibodies. Their frequency in RA blood correlates with the frequency of γδ T cells (Plater Zyberk et al., 1989).

Dendritic cells with potent antigen presenting capacity are found in increased numbers in the synovial fluid (Zvaifler et al., 1985; Harding et al., 1986) and synovial membrane (Poulter et al., 1981) of patients with RA and it has been suggested that these may be involved in the presentation of self antigen within the joint. The differences in cell populations are not specific to RA with similar lymphocyte populations seen in Sjogrens syndrome (Plater Zyberk et al., 1989) and increased dendritic cell numbers seen in synovium from patients with ankylosing spondylitis (Duke et al., 1985) and reactive arthritis (Barkley et al., 1989). As yet there is no evidence to suggest that the increase in these cell populations alone
plays a major role in the pathogenicity of rheumatoid inflammation. Further studies on factors (cytokines, growth factors, antibodies) released by these cells in rheumatoid patients may indicate a pathogenic role. Functional studies have shown abnormalities in T and B lymphocyte and natural killer cell responses in RA: T cells from RA patients show a defective response to T cell mitogens in vitro (Burmester et al, 1978; Bacon, 1988) and depressed autologous and allogenic MLR (Burmester et al, 1981), B cells from RA patients show increased reactivity with Epstein-Barr virus (Stierle et al, 1981) and cells with natural killer function are selectively increased in the joint in RA (Reinitz et al, 1982). The significance of these functional differences in RA are not fully understood.

MHC class II expression

Abnormal or excess presentation of exogenous or self antigen has been proposed as a mechanism for the perpetuation of rheumatoid inflammation. Under normal circumstance antigens are processed by antigen presenting cells (APC) such as macrophages or dendritic cells. The surface expression of MHC class II molecules by these cells appears to be essential for antigen presenting function. Following processing, the appropriate part of the antigen peptide is presented to T cells on the surface of the APC in association with these MHC class II molecules (DR, DP, DQ). Over-expression of MHC class II molecules and lymphocytic infiltration have been demonstrated in the target tissue of several human autoimmune diseases (eg. Graves´ and Hashimoto´s thyroiditis, diabetes mellitus,
rheumatoid arthritis, primary biliary cirrhosis, multiple sclerosis). This finding has led to the suggestion that MHC class II molecules may be important in autoimmune disease (Bottazzo et al, 1983). The hypothesis that excessive or aberrant (present on cells not normally expressing class II) class II expression leads to presentation of autoantigen to autoreactive T cells which in turn produce molecules augmenting MHC class II expression has been verified using the thyroid as a model: Throcytes in Graves' disease show aberrant expression of HLA-DR (Hanafusa et al, 1983). Interferon γ is able to induce aberrant class II expression on thyrocytes (Todd et al, 1985). These cells are able to present antigen to T cells (Londei et al, 1984) and in autoimmune thyroiditis many of the infiltrating T cells specifically recognise class II expressing thyrocytes (Londei et al 1985). The same vicious cycle occurring in RA synovium is supported by the finding of increased expression of class II molecules in RA synovium, both by cells that constitutively express these molecules (Janossy et al, 1981; Burmester et al, 1982; Poulter et al, 1981) and by endothelial cells (Palmer et al, 1985; Klareskog et al, 1981). Studies at mRNA level have confirmed this and have also shown that raised levels of MHC class II mRNA persist in cultures of synovial cells suggesting the presence of a substance in RA synovium capable of inducing class II molecules (Kissonerghis et al, 1989). Low levels of γIFN mRNA have been identified in the rheumatoid joint (Buchan et al, 1988a). Many of the studies have concentrated on expression of HLA-DR in synovium. However HLA-DR expression by synoviocytes and endothelium has been demonstrated in normal synovium suggesting that HLA-DR
alone is unlikely to play a major role in the perpetuation of rheumatoid inflammation. Our recent study suggests that aberrant HLA-DQ expression may be more important in rheumatoid inflammation. (Barkley et al, 1989).

Cytokines
There is now considerable interest in the role of cytokines in the regulation of antigen presentation and the immune response. The importance of these soluble protein mediators in rheumatoid arthritis and other diseases characterised by chronic immune activation is currently under study by many laboratories. Cytokines include lymphokines and monokines and are soluble protein mediators released by lymphocytes and monocytes/macrophages respectively (this nomenclature is confusing as it is becoming apparent that a wide variety of cells are capable of producing these mediators). Cytokines mediate effector functions of the immune system, amplify the immune response and allow communication between cells of the immune system and cells outside the immune system. They are involved in many biological processes including growth, differentiation, inflammation, immunity, repair and healing. The network of cytokine interactions is complex and delicately balanced. Under normal circumstances cytokines mediate the controlled processes of the immune response but it has been suggested that they may function pathologically in immune mediated inflammation. They do not appear to be produced by resting cells and are usually indicative of cell activation or sensitization. Many cells in the synovial membrane are thought to be activated and the presence of cytokines in the rheumatoid
joint has received considerable attention. Increased levels of two cytokines IL-1 and TNFα have been demonstrated in the synovial fluid of patients with RA (Hopkins et al, 1988; Saxne et al, 1988; Duff et al, 1988). Both these molecules have also been demonstrated in situ in RA synovial membrane (Duff et al, 1987; Husby et al, 1988) and raised IL-1β levels have been demonstrated in the blood of RA patients (Eastgate et al, 1988). TNF and IL-1 have similar biological properties with the exception that IL-1 has immunostimulatory effects (on T and B cells) and TNFα does not. IL-1 and TNFα have effects on connective tissue which make them of particular interest in rheumatoid arthritis (see chapter 2 & 3).

IL-6 is a cytokine produced by a wide variety of cells. It has many growth and differentiation activities and has been suggested to play an important role in B cell differentiation and immunoglobulin secretion in the RA synovium. IL-6 has been demonstrated in the synovial fluid in RA (Houssiau et al, 1988) although this is not specific to RA as similar levels were found in other inflammatory arthritides.

The T cells in RA synovium bear markers of activation (class II, the Tac component of the IL-2 receptor and the transferrin receptor) but the full range of T cell lymphokines are not detectable. mRNA for both IL-2 and IFNγ has been demonstrated in increased amounts in the rheumatoid joint (Buchan et al, 1988a) but there has been no convincing demonstration of the protein for these cytokines (Husby et al, 1985; Egeland, 1987; Firestein et al, 1987). It has been suggested that activation of T cells in RA is abnormal and "incomplete" (Pitzalis et al, 1987) but analysis of cytokine protein in
rheumatoid joints is beset with problems and it is not yet clear whether the absence of these T cell products reflects non-translation, absorption or insensitivity of assays. Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a haemopoietic growth factor which is present in RA synovial fluid (Xu et al, 1989). This cytokine has potent stimulating effects on mature granulocytes and macrophages and enhances antigen presentation by accessory cells (Morrissey et al, 1987). GM-CSF is also of particular interest in RA as it has recently been described to be a powerful inducer of class II molecules (Alvaro-Garcia et al, 1988). As only very low levels of IFNγ have been detected in the RA synovium GM-CSF be may the cytokine which plays a major role in the macrophage activation and over-expression of class II seen in RA.

RA is a crippling autoimmune disease. The aetiology remains unknown, although it seems likely (based on HLA-DR4 or DR1 association but discordance between identical twins) that it occurs following exposure to an exogenous agent (eg a virus) in a genetically predisposed individual. Many immunological abnormalities have been demonstrated. Although none of these alone is likely to be the primary cause of disease, identification of features which play a major role in the progression of the disease will allow the rational development of therapies to prevent the clinical manifestations of RA. An imbalance in the cytokine network has been suggested to be such a feature and interleukin 1 has received particular attention in RA. Although levels of cytokines may be raised in RA synovitis simply as sequelae of the inflammatory reaction and
complicated synergies between different cytokines may exist, interleukin 1 is of particular interest in rheumatoid inflammation. It is central to T cell activation and has multiple biological activities both on the immune system, systemically and on connective tissues suggesting that it might mediate many of the features seen in RA. The following sections consider interleukin 1 in detail.
1.2 Interleukin 1

Interleukin 1 is a polypeptide immunoregulatory cytokine produced primarily by cells of the mononuclear phagocyte lineage. It has a very wide range of biological activities, both on cells of the immune system and non-immune cells.

HISTORY

IL-1 activity was first described in 1948 when a pyrogenic substance was extracted from polymorphonuclear leukocytes (Beeson, 1948). This substance was found to be a heat labile protein and was termed "leukocytic pyrogen". A substance with similar activity, termed "endogenous pyrogen" was described some years later when a factor in the serum of rabbits following vaccination was noted to cause fever when injected into experimental animals (Atkins, 1960). In the 1970s a soluble factor in the supernatants of leukocyte cultures was found to augment the responses of thymocytes to mitogen and antigen (Gery et al, 1972) and was termed "lymphocyte activating factor" (LAF). This substance was purified around this time (Murphy et al, 1974; Dinarello et al 1977) and it became apparent that it had multiple biological activities. It was known to be produced by one leukocyte population and have effects on another leukocyte population and therefore in 1980 it was termed interleukin 1. Biochemical characterisation of several substances named for their biological activities has subsequently shown them to be identical to IL-1. These are leukocyte endogenous mediator (Kampschmidt, 1981), mononuclear cell factor (Krane et al,
catabolin (Saklatvala et al, 1984), osteoclast activating factor (Dewhirst et al, 1985) and haemopoetin-1 (Moore et al, 1987). In 1984 two distinct subtypes of IL-1, IL-1α and IL-1β were identified, cloned and sequenced (Auron et al, 1984; Lomedico et al, 1984).

**IL-1α AND IL-1β**

The two subtypes of IL-1 were originally identified on isoelectric focusing as a pI 7 (IL-1β) and a pI 5 (IL-1α). The two forms are encoded for by separate genes both located on the long arm of chromosome 2. Each IL-1 gene contains 7 exons coding for the processed mRNA (March et al, 1985). Both forms are produced as 33 KD precursor polypeptides which are cleaved to mature 16-18 KD IL-1α or IL-1β. The processing and mechanism of secretion of IL-1 is not fully understood (Singer et al, 1988). The precursor NH₂-terminal domains are removed during processing but they do not have a hydrophobic leader sequence characteristic of secretory proteins (Furutani et al, 1985). This lack of leader or membrane anchor sequence and the observed intracellular accumulation of IL-1 within activated monocytes has lead to the suggestion that IL-1 processing and secretion may be unique in comparison to other secreted proteins. There is evidence to suggest that the 33 KD precursor is secreted and cleaved extracellularly to the mature 17 KD form by enzymes, the nature of which remain unknown, released at the same time (Hazuda et al, 1988). Human IL-1α and IL-1β share only 26% amino acid homology but bind to the same receptor and have identical biological activities (Kilian et al, 1986; Rupp et al, 1986). There are five small regions of
homology between human and murine precursors for IL-1α and IL-1β (A-E) (Auron et al, 1985). Region A and B are contained in the precursor sequence which is cleaved in processing to the mature form, therefore it follows that the important biologically active regions of homology for the two mature forms must be located in the carboxyl C, D and E regions. These regions have been suggested to represent the receptor binding site of the IL-1 molecule (Mosley et al, 1987). This is under further study following the crystalisation of IL-1β and the analysis of its tertiary structure (Priestle et al, 1988). It is unclear why the two forms of IL-1 exist as there are no biological effects unique to one form and both bind to the same receptor. Since there is considerable homology between species for individual subtypes it is likely that the two forms have arisen from a gene duplication before or during vertebrate evolution (Lomedico et al, 1986). As the two genes are maintained in evolution it is likely that both forms are required although as yet functional differences between the two forms are poorly understood. There is some evidence suggesting that the cellular distribution may be different for each subtype and that this may have some functional significance (Conlon et al, 1987). IL-1β appears to be readily secreted from activated monocytes whereas IL-1α remains cell-associated and is detectable on the cell surface (Hazuda et al, 1988). IL-1β is only active in its cleaved secreted form whereas IL-1α is active in both its cell-associated precursor and secreted cleaved form (Mosley et al, 1986). Membrane associated IL-1 is thought to be functionally relevant and is required for the activation of T cells (Hurme, 1987). IL-1β can therefore be
thought of as the secreted form and IL-1α as the predominantly cell-associated form. It is possible that IL-1α is important in cell-cell interactions involved in antigen presentation and that IL-1β mediates the more "distant" activities (eg. cartilage breakdown by synoviocyte derived IL-1) explaining the existence of two types although this remains speculative only.

SOURCE OF IL-1
The major source of IL-1 is from cells of the macrophage/monocyte lineage. However there have been many reports that several non-macrophage cells can also produce this cytokine. Cells as diverse as B cells (Scala et al, 1984), dendritic cells (Duff et al, 1985), endothelial cells (Windt et al, 1984), astrocytes, mesangial cells and keratinocytes (Blanton et al, 1989) have been reported to produce IL-1 with an appropriate stimulus. These cells have in common the ability to present antigen to T cells under certain circumstance and this is reflected in their ability to produce IL-1 which is central to T cell activation. It is not known whether all these cells produce both IL-1α and IL-1β or whether under some circumstances distinct populations produce the two subtypes. It is known that some cells produce more of one type than other cells. IL-1β is the predominant type in human peripheral blood mononuclear cells with approximately 10x more IL-1β mRNA than IL-1α mRNA detectable following activation (Demczuk et al, 1987). This ratio is reversed in human umbilical vein endothelium, with significantly more IL-1α mRNA produced following LPS activation (Stern et al, 1985). The significance of these differences is not understood currently.
BIOLOGICAL EFFECTS

IL-1 has multiple and diverse biological activities (Table 1.1). The biological effects can be divided into those on immunocompetent cells; the generalised systemic effects; and the effects on connective tissue. IL-1 acts locally in a paracrine fashion augmenting T, B and NK cell responses. It is central to T cell activation (Figure 1.1) although it remains unclear whether it is an absolute requirement. Activation of class I restricted CD8+ cytotoxic T lymphocytes has been reported to be independent of IL-1 (Lowenthal et al, 1986) although this appears to be restricted to a subpopulation of these cells (Klarnet et al, 1989). IL-1 has been suggested to have an enhancing rather than obligatory role in T cell activation (Mizel, 1987). IL-1 is involved in B cell growth and differentiation (Pike et al, 1985). IL-1 activates macrophages for tumour killing (Durum et al, 1985) and stimulates macrophages to produce PGE2 (Dinarello et al, 1983). It stimulates neutrophil migration and activation (Luger et al, 1983; Klempner et al, 1978) and basophil and eosinophil degranulation (Pincus et al, 1986; Subramanian et al, 1987). IL-1 also increases endothelial cell-leukocyte adhesion via upregulation of the expression of an intercellular adhesion molecule, ICAM-1 by endothelial cells (Dustin et al, 1986). This results in the trafficking of leukocytes to sites of inflammation.

IL-1 act as a soluble mediator to exert some of its systemic effects. These include induction of fever via an action on the hypothalamus (Dinarello et al, 1981) and promotion of sleep (Krueger et al, 1984). IL-1 has a direct action (and an
### TABLE 1.1

**SOME OF THE BIOLOGICAL EFFECTS OF IL-1**

<table>
<thead>
<tr>
<th>TARGET CELL</th>
<th>ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effects on immunocompetent cells</strong></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>Stimulation and IL-2 release</td>
</tr>
<tr>
<td>B cells</td>
<td>Cofactor in proliferation/differentiation</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Chemotaxis, degranulation and bone marrow release</td>
</tr>
<tr>
<td>Endothelium</td>
<td>Increased adhesion of leukocytes</td>
</tr>
<tr>
<td><strong>Systemic effects</strong></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Induction of fever</td>
</tr>
<tr>
<td>Unknown</td>
<td>Induction of sleep</td>
</tr>
<tr>
<td>Liver</td>
<td>Synthesis of acute phase proteins</td>
</tr>
<tr>
<td></td>
<td>(direct &amp; indirect action via IL-6)</td>
</tr>
<tr>
<td><strong>Effects on connective tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>Bone resorption</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>Cartilage breakdown</td>
</tr>
<tr>
<td>Synovium</td>
<td>Collagenase release, PG release</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Collagenase release, growth, PG release</td>
</tr>
</tbody>
</table>

(This table outlines some of the major effects of IL-1 with relevance to inflammatory arthritis. IL-1 has numerous other biological effects, both directly and indirectly via other cytokines, which are not discussed in this thesis).
IL-1 is released from antigen presenting cells (APC) when processed antigen is presented to T cells in association with MHC class II molecules. IL-1 provides an activation signal to T cells and this is followed by their proliferation.
indirect action via IL-6) on hepatocytes stimulating the production of acute phase reactants from the liver, eg. C-reactive protein, serum amyloid A (Sztein et al, 1981). It also causes decreased synthesis of metallo-proteinases by the liver resulting in hypoferraemia and hypozincaemia (Kampschmidt et al, 1973).

IL-1 has many local effects on connective tissue and this has prompted the considerable interest in this cytokine in inflammatory arthritis. It is known to stimulate collagenase release and PGE$_2$ release from rheumatoid synovial fibroblasts (Mizel et al, 1981). It stimulates cartilage breakdown (Gowen et al, 1984) and bone resorption in vitro (Gowen et al, 1983). IL-1 is therefore a possible mediator of the characteristic erosion of bone and cartilage seen in RA.
The role of IL-1 in rheumatic diseases, particularly RA, has received considerable attention over the past few years. As discussed in previous sections, with its diverse range of activities IL-1 has the potential to mediate the immune activation, systemic features and progressive erosion of bone and cartilage seen in RA.

ANIMAL STUDIES

Most of the studies on biological activities have been performed \textit{in vitro}. Animal studies on IL-1 \textit{in vivo} have supported its role in joint damage in inflammatory arthritis; Intra-articular injections of IL-1 in rabbit knee joints induces a transient synovitis with inflammatory cell infiltrates, and loss of proteoglycan from articular cartilage (Pettipher et al, 1986; Dingle et al, 1987). Repeated doses of intra-articular IL-1 are known to promote chronic erosive synovitis in rat joints previously injured by bacterial cell wall polymers (Stimpson et al, 1988). In contrast erosion of articular cartilage is not seen with intra-articular TNF which has also been suggested to play a pathogenic role in RA (Henderson et al, 1989).

IL-1 IN RA

Evidence suggesting a role of IL-1 in human arthritis began over two decades ago with the report that pyrogen was released from synovial fluid exudate cells (Bodel et al, 1968). This
was followed by the finding in 1977 that a soluble factor produced by rheumatoid synovial lining could stimulate chondrocytes to degrade their own extracellular matrix (Fell et al, 1977). The activity released by synovial lining tissue, then termed "catabolin" was subsequently shown to be interleukin 1 (Saklatvala et al, 1985). Raised levels of IL-1 bioactivity have been detected in the joint effusion fluids in several arthritic conditions including RA (Stastny et al, 1975; Fontana et al 1982; Wood et al, 1983; Nouri et al, 1984) osteoarthritis, ankylosing spondylitis, psoriatic arthritis (Nouri et al, 1984) and gout (Woolf et al, 1985). Cells isolated from RA synovial membrane have been shown to have increased spontaneous IL-1 production when compared to osteoarthritis. This has been studied both at the mRNA level (Buchan et al, 1988b) and at the protein level by bioassay (Miyasaki et al, 1988). Rheumatoid synovial cells have been reported to spontaneously produce an IL-1-like factor even after long term culture although this may be in part due to IL-6 production rather than IL-1 (Goto et al, 1987). In this latter study synovial cells with dendritic morphology produced more IL-1 like activity than macrophages, with fibroblasts producing the least. Duff et al (1985) have shown IL-1 production by RA synovial dendritic cells although this has been disputed (Bhardwaj et al, 1988). RA is a systemic disease, but most studies have concentrated on IL-1 in the joint. Studies on IL-1 in rheumatoid blood have been limited. This has partly been due to lack of appropriate methods which is discussed in the next chapter. The limited studies have shown that peripheral blood monocytes from patients with
juvenile arthritis produce greater amounts of IL-1 than controls. This increase was more marked in patients with active disease (Martini et al, 1986). IL-1 secretion by peripheral blood monocytes in culture is increased in RA patients compared with osteoarthritis patients and RA patients on gold (Danis et al, 1987). However these studies may not reflect the situation in vivo as the culture conditions could have profound effects on IL-1 production. With the availability of a polyclonal antibody specific to IL-1β used in an ELISA, Eastgate et al (1988) have shown increased levels of IL-1β in the plasma of RA patients compared to normal controls. This study did not involve culture and may therefore reflect the levels of IL-1 present in the circulation. IL-1β is protein bound in the circulation and chloroform extraction was required for this assay. It is not clear whether this immunoreactive IL-1β represents biologically active IL-1 although the levels of IL-1β following chloroform extraction correlated well with disease activity.

IL-1 AND THERAPEUTIC AGENTS USED IN RA

The studies reviewed above implicate IL-1 as an important mediator in RA. This is further supported by reports that many of the disease modifying drugs used in RA have effects on IL-1 production. Gold is a powerful remission inducing agent used in the treatment of RA. In vitro experiments have shown that gold compounds can inhibit mitogen induced T cell proliferation at levels found in the serum/tissue of patients receiving gold therapy (Lipsky et al, 1977). It has been suggested that this is due to inhibition of an early critical step in lymphocyte
activation - eg. inhibition of the production or release of IL-1 by antigen presenting cells (Salmeron, 1982). There is now accumulating evidence that gold compounds can inhibit mitogen stimulated IL-1 production in vitro (Remvig et al, 1988) as well as inhibiting the proliferative response of T cells to exogenous IL-1 by an unknown mechanism (Drakes et al, 1987; Haynes et al, 1988;). Corticosteroids, chloroquine and methotrexate are agents with marked suppressive action on rheumatoid inflammation. It has been suggested that these agents, like gold, might exert their therapeutic effect in RA via inhibition of IL-1. Corticosteroids are known to block IL-1 transcription and to have an effect on post-transcriptional synthesis (Knudsen et al, 1987; Kern et al, 1988). Chloroquine has been shown to inhibit interleukin 1 induced cartilage degradation in organ culture (Arner et al, 1987). Methotrexate inhibits the lymphoproliferative activity of IL-1 in vitro but does not appear to inhibit its synthesis (Segal et al, 1989). Inhibition of production/action by therapeutic agents used in RA is not unique to IL-1. Reports on other cytokines are rather limited although this may simply reflect areas of current interest. There have been reports of inhibition of LPS induced TNFα production by dexamethasone and gold compounds (Evans et al, 1989). It is also of interest that dietary supplementation with omega-3 fatty acids has led to improvements in patients with RA (Kremer et al, 1987) and a recent study has shown suppression of mitogen induced IL-1β, IL-1α and TNF production by PBM from volunteers following six week dietary supplementation with fish oil (Endres et al, 1989).
There is now a weight of circumstantial evidence to support an important role for IL-1 in the pathogenesis of RA. Most of the studies have been performed using bioassay which provides information on total IL-1 bioactivity. Very few studies have reported levels of the two subtypes IL-1α and IL-1β and little is known about their relative importance in rheumatoid inflammation. Two studies, one in RA synovial fluid (Bhardwaj et al., 1988) and the other in peripheral blood (Eastgate et al., 1988) have studied IL-1β only. IL-1β is the predominant secreted form in activated peripheral blood but a study at the mRNA level has suggested that IL-1α, which is functionally active when cell-associated, may play a specific role in rheumatoid inflammation. This study showed increased and prolonged production of IL-1α mRNA by RA synovial fluid cells (Buchan et al., 1988b). The importance of IL-1α is further supported by the recent report that this subtype accounts for a significant amount of the IL-1 bioactivity found in RA synovial fluid (Smith et al., 1989). The study of cell-associated IL-1 has been hampered by the lack of specific methods to identify IL-1α and IL-1β producing cells at the single cell level. This thesis discusses the development of a specific method for studying cell-associated IL-1 and the application of this method to study these two subtypes in RA peripheral blood.
DEVELOPMENT OF A METHOD FOR THE DETECTION OF CELL
ASSOCIATED IL-1α AND IL-1β.

2.1 Introduction

There are many limitations with the currently available assays for cytokines. IL-1 protein is most frequently assayed using a bioassay based on the proliferative response of mouse thymocytes to exogenous IL-1 (detected by tritiated thymidine incorporation). This has a major disadvantage that IL-6 is also active in this system (Van Damme et al, 1988). A more specific IL-1 bioassay using a T cell line, D10 (Kaye et al, 1984) has overcome this problem. Bioassay has the advantage that only biologically active forms are measured. There are however disadvantages of bioassay; assays of culture supernatants provide no information on cell-associated forms; bioactivity may be affected by the presence of naturally occurring inhibitors - these are known to exist for IL-1 (Larrick et al, 1989); and standard bioassay does not provide information about the two subtypes of IL-1 or the cells producing them. A radioimmunassay and ELISA are now available for IL-1β (Cistron, Pine Brook N.J, USA) based on reactivity with a polyclonal anti-IL-1β antibody. Immunoassays for IL-1α have proved difficult to develop, partly because these methods detect only secreted forms in serum/culture supernatants and significant amounts of IL-1α remain cell-associated. Extraction procedures are required for these methods and they
do not allow study of the cells producing the two types. The development of specific cDNA probes has allowed the measurement of mRNA for IL-1α and IL-1β in crude cell extracts (Demczuk et al, 1987; Buchan et al, 1988b). This provides information about the two subtypes but does not provide information on cells producing them. Also mRNA may not reflect protein levels as it may not be translated. This has been suggested to be the reason for the absence of IFNγ protein in the RA joint despite the presence of mRNA (Buchan et al 1988a). Methods for studying IL-1 production at the single cell level are limited to two previous studies demonstrating individual cells in suspension staining for IL-1β. In these experiments polyclonal antisera were used and IL-1α was not studied (Bayne et al, 1986, Singer et al, 1988). Specificity problems may be encountered with polyclonal antibodies as these often show non-specific binding and have cross reactions with other molecules. Monoclonal antibodies against IL-1α and IL-1β have been recently developed and the kind donation of these antibodies has allowed the development of this new and specific method for identifying cell-associated IL-1α and IL-1β at the single cell level.

2.2 **Aims**

1. To develop a simple and sensitive method for identifying cell-associated IL-1α and IL-1β in activated peripheral blood.

2. To determine the kinetics of cell-associated IL-1 protein in peripheral blood following mitogen stimulation.
3. To determine whether IL-1α and IL-1β are produced by the same cell or by distinct populations of cells in peripheral blood following mitogen stimulation.

4. To characterise the cells producing IL-1α and IL-1β in mitogen activated peripheral blood.

2.3 Characterisation of antibodies

Mouse monoclonal anti-human IL-1α (No 1190) was kindly donated by M. Okazaki of Dainippon Pharmaceuticals, Japan. Two mouse anti-human IL-1β monoclonal antibodies were used in this work. One was a kind gift from K. Matsushima, Laboratory of Molecular Immunoregulation, NCI, Frederick, MD. and the other (ANOC 205) was a kind gift from K. Hirai, Otsuka Pharmaceuticals, Japan. Both anti-IL-1β antibodies gave very similar results and identical patterns of staining. All antibodies were of the IgG1 sub-class. The stock antibody solutions had approximately 1mg/ml IgG1 and had been purified on protein A columns. The reactivity and specificity of these antibodies to rIL-1α and rIL-1β were determined both in an enzyme linked immunosorbent assay (ELISA) and by Western blotting.

Methods

ELISA

All incubations were carried out at 37°C. ELISA plates (Dynatech, Billingshurst, UK) were coated with 1μg/ml rIL-1α or 1μg/ml rIL-1β and incubated for 1 hour to allow the protein
to adhere to the plate. After washing with phosphate buffered saline (PBS), 2% casein was added to each well for 1 hour to reduce non-specific plastic binding. Following washing in PBS containing 1% Tween detergent (PBS/Tween), 50μl of appropriately diluted antibody (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600) was added to each well for 1 hour. After extensive washing with PBS-Tween, 50μl of 1:1000 alkaline phosphatase labelled goat anti-mouse IgG (Sigma Chemical Co, Poole, Dorset UK) diluted in PBS/Tween containing 0.5% casein was added for 1 hour. After washing the ELISA plate was finally incubated for 20 minutes with 50μl of substrate (Sigma 104 Phosphatase substrate tablets; 1 tablet dissolved in 5 ml of glycine buffer, pH 10.4). The optical densities were read at 405 nM on a Titertek Multiscan.

WESTERN BLOTTING

Gel electrophoresis: A vertical polyacrylamide gradient (5-15%) was poured using the solutions detailed in appendix 2. A stacking gel was poured above this gel with an 18 well spacing comb to produce separate wells for samples. 40μl of sample was applied to each well. Molecular weight standards (Pharmacia low molecular weight calibration kit, Sweden) were applied to the edge of the gel. The gel was electrophoresed in buffers containing sodium dodecyl sulphate (SDS) at 30mA, 300V for 5 hours.

Samples: Appropriate dilutions of rIL-1α and rIL-1β were boiled for 5 minutes in one volume of sample buffer (Laemmli, 1970) containing dithiothreitol (100mM) as a reducing agent. Cell lysates of peripheral blood mononuclear cells from 2
normal volunteers, unactivated and following 22 hour activation in vitro with PHA/PMA were also used (The methods for PBM separation and activation are outlined in the following section). The cell lysates were prepared by suspending cells at a concentration of $10^7$ per ml and sonicating for 10-15 seconds in the presence of 2mM of protease inhibitor, phenyl-methyl-sulphynil-fluoride (PMSF). The lysates were then boiled in sample buffer as for the recombinant IL-1.

**Immunoblotting:** Separated antigens were transferred from the gel on to nitrocellulose by electroblotting for 1 hour (Towbin et al, 1979). The edge of the nitrocellulose containing the molecular weight standards was removed. The remaining part of the blots were incubated with 2% casein in PBS for 1 hour to block non-specific binding. Monoclonal anti-IL-1α or anti-IL-β were diluted 1:100 in 0.5% casein in PBS and incubated with the blots for 1 hour. After several washes in PBS containing 1% Tween, the blots were incubated for 1 hour with horseradish peroxidase conjugated goat anti-mouse IgG (Sigma Chemical Co) diluted 1:1000 in 0.5% casein in PBS. The blots were washed again and incubated for 2 hours with diaminobenzidine (50mg) diluted in 100ml PBS containing 1% Cobalt/Nickel and H$_2$O$_2$ (as described by DeBlas and Cherwinski (1983). The reaction was stopped by washing in PBS. The molecular weight standards were developed using 0.1% amido black in 7% acetic acid. The standards were of molecular weights 14.4, 20.1, 30, 43, 67, 94 KD. By measuring the distance moved by these standards, the molecular weight (MW) of bands on the blot could be determined.
Results

ELISA

Figure 2.1 shows the optical density readings plotted against the dilutions of antibody used in the ELISA with (a) 1µg/ml rIL-1α as the antigen (b) 1µg/ml rIL-1β as the antigen. This shows typical dilution curves. The results show that the antibodies are specific; the monoclonal anti-IL-1α recognises rIL-1α with a typical dilution curve but does not react with rIL-1β (background OD for substrate alone=0.34) and the monoclonal anti-IL-1β recognises rIL-1β but not rIL-1α (background OD for substrate alone=0.21).

Western Blot

Figure 2.2 and 2.3 shows the results of immunoblotting. In figure 2.2 different concentrations of rIL-1α, rIL-1β and cell lysates have been run on a gel and immunoblotted with monoclonal anti-IL-1α. The band seen with rIL-1α has a calculated molecular weight of 19.2KD which is compatible with the recombinant protein. No band is seen with rIL-1β. The unactivated and activated cell lysates show several bands, the strongest of which has a calculated molecular weight of 32KD. There are faint higher MW bands and a faint band at 19KD. The appearances are very similar in activated and unactivated cells.

Figure 2.3 shows the samples immunoblotted with monoclonal anti-IL-1β. This shows similar results with no cross reactivity - the antibody recognising only IL-1β and not IL-1α. The band seen with rIL-1β has a calculated MW of 20 KD compatible with
Figure 2.1 (opposite) shows that monoclonal anti-IL-1α and anti-IL-1β react specifically with rIL-1α and rIL-1β respectively in an ELISA.
Figure 2.1

a) ELISA plate coated with rIL-1α

- Background OD 0.34
- anti-IL-1α
- anti-IL-1β

b) ELISA plate coated with rIL-1β

- Background OD 0.21
- anti-IL-1α
- anti-IL-1β
Figure 2.2 (opposite) shows a Western blot demonstrating that monoclonal anti-IL-1α reacts with both rIL-1α and native 19kD & 33kD forms of IL-1α (from activated PBM) but does not react with rIL-1β.
The position of the MW markers is indicated on the left.
Lane 1: rIL-1β (40ng); lanes 2–5: rIL-1α (0.6ng, 2.5ng, 10ng, 40ng);
lanes 6,7: Cell lysates (donor 1), 6=activated PBM, 7=unactivated PBM;
lanes 8,9: Cell lysates (donor 2), 8=activated PBM, 9=unactivated PBM.
Figure 2.3 (opposite) shows a Western blot demonstrating that monoclonal anti-IL-1β reacts with both rIL-1β and native 20kD & 33 kD forms of IL-1β (from activated PBM) but does not react with rIL-1α.
Figure 2.3

IMMUNOBLOT PROBED WITH ANTI-IL-1β

The position of the MW markers is indicated on the left.
Lanes 1–4: rIL-1β (40ng, 10ng, 2.5ng, 0.6ng); lane 5: rIL-1α (40ng);
lanes 6,7: Cell lysates (donor 1), 6=activated PBM, 7=unactivated PBM;
lanes 8,9: Cell lysates (donor 2), 8=activated PBM, 9=unactivated PBM.
the recombinant protein. No bands are seen with rIL-10. The activated and unactivated cell lysates show a band with a calculated MW of 33 KD and a faint band with MW 19KD.

Discussion

The ELISA and Western blot results show that both the anti-IL-1α and anti-IL-1β monoclonal antibodies are specific to their subtype and do not cross react with the other IL-1 type. The Western blot results show that the antibodies recognise both the recombinant 17KD protein and the naturally occurring 33KD precursor and 17KD processed form. In both blots there is very little difference between the unactivated and activated peripheral blood cell lysates. This is probably because Western blotting is predominantly a qualitative technique and will only detect fairly large differences in protein concentration. Monocytes account for only about 20% of PBM and the differences in IL-1 concentration in lysates of activated and unactivated PBM may not be sufficiently large to be seen on Western blotting. It may also be due to some stimulation of IL-1 production in unactivated PBM during the separation and lysis procedure. Higher molecular weight bands (approx 50, 70 and 90 KD) are present on both Western blots. It has been suggested that IL-1 may exist in high molecular forms within the cell and these higher molecular weight bands have been demonstrated previously, both by Western blot and immunoprecipitation (Bomford et al, 1987; Streck et al, 1988). Their significance is not understood. Western blotting can be seen to be a fairly sensitive method for identifying IL-1;
the blots show 0.6 ng of IL-1α and IL-1β can be detected by the appropriate antibody.

2.4 Study on in vitro activated blood

Materials and Methods

The method outlined in this section gave reproducible and optimal staining. Several titrations and modifications were made during the development to optimise staining. The critical points of the method are indicated. Appendix 3 shows details of tissue culture reagents used.

SEPARATION OF MONONUCLEAR CELL POPULATION FROM PERIPHERAL BLOOD

The method for separation of PBM and preparation of cytospins is summarised in figure 2.4. Peripheral venous blood was collected from 4 non-fasted healthy laboratory volunteers (3 female, 1 male; aged 20-36 years). The blood was placed immediately into sterile glass bottles containing preservative free heparin (10 U/ml of blood) (sodium heparin; Sigma Chemical Company, Poole, Dorset, UK). The blood was diluted 1/2 with sterile Dulbecco's balanced salt solution (DBSS) (Gibco Europe, Paisley, Scotland, UK). The diluted blood was laid on a density gradient (Ficoll-Paque, Pharmacia, Uppsala, Sweden) at a ratio of 2:1 respectively and centrifuged at 400 x g for 30 minutes at room temperature. Peripheral blood mononuclear
PREPARATION OF PBM FOR IMMUNOSTAINING

1. Fresh heparinised blood
2. Separate on Ficoll

PBM harvested from interface and pelleted

Wash x4

Activate with PHA/PMA (4, 16, 20, 24, 48 hours)

Unactivated cells

Cytospin

Fix in acetone/methanol at -70 C
Store at -20 C
cells (PBM), comprising monocytes, lymphocytes and dendritic cells were harvested from the Ficoll/plasma interface. The PBM were washed twice in DBSS containing 2% inactivated fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland, UK) and resuspended in RPMI 1640 medium (Flow laboratories). The number of viable cells was determined using a counting chamber and trypan blue dye exclusion (viable cells do not stain with trypan blue dye). The cells were then diluted to a density of $2.5 \times 10^5$/ml. The approximate yield of PBM from a healthy individual by this method was $1 \times 10^6$ cells/ml venous blood.

**IN VITRO CULTURE AND STIMULATION WITH MITOGEN**

PBM from the 4 normal individuals were stimulated to produce IL-1 by activation with mitogen *in vitro*. $10^6$ cells/ml were cultured with RPMI 1640 medium supplemented with 10% FCS (heat inactivated for 1 hour at 56°C) in the presence of 1μg/ml of phytohaemagglutinin (PHA) (purified PHA; Wellcome Diagnostics, Beckenham, UK) and 50 ng/ml of 12-myristate 13-acetate (PMA) (Sigma Chemical Company) in flat bottomed 24-well culture plates (Nunc, Gibco Europe). A combination of PHA/PMA is known to be a powerful activator of PBMs. Results obtained with a combination of these mitogens were superior to those when PMA was used alone. PHA is primarily an activator of lymphocytes and therefore almost no staining was seen when PHA was used alone. Incubations were carried out for 4, 16, 20, 24, 28 and 44 hours. Following incubation the adherent and non-adherent cells were removed by washing the culture wells vigorously with phosphate-buffered saline (PBS) pH 7.2. The cells were then washed x 3 with PBS, recounted by trypan blue dye exclusion and
resuspended in PBS at a density of $2.5 \times 10^5$/ml.

IMMUNOFLUORESCENCE

Cytocentrifuge preparations were made for immunofluorescent staining. 200μl of cell suspension ($5 \times 10^4$ cells) was centrifuged for 5 minutes at 500 rpm onto individual microscope slides using a cytocentrifuge (Cytospin, Shandon Southern Products, Runcorn, Cheshire, UK). The slides were then air-dried for 15 minutes. In order to permeabilise the cell membrane the slides were fixed for 10 minutes in a solution of 50% methanol and 50% acetone at -70°C (cooled in a solid CO$_2$ and methanol bath). Staining results were inferior if fixation was carried out at room temperature or -4°C, presumably due to some degradation of the antigen. Following fixation the slides were air-dried for 30 minutes and stored at -20°C. The slides could only be stored at -20°C for up to 6-8 weeks before losing reactivity. The immunofluorescent staining method is summarised in figure 2.5. Following removal from storage the slides were air-dried for 5 minutes and rehydrated in PBS for 10 minutes. All the following incubations were carried out at room temperature. Non-specific binding was blocked by incubating the slides in 1/5 normal goat serum (NGS) (Sigma Chemical Company) for 10 minutes. The primary antibody was applied to the cells for 1 hour. After washing three x 5 minute washes in PBS, goat anti-mouse IgG (whole molecule) conjugated to fluorescein isothiocyanate (FITC) (Sigma Chemical Company) diluted 1/50 in 2% NGS was applied to the cells for 30 minutes. This was deaggregated prior to use by centrifugation in a minifuge at 13000 RPM for 5 minutes. The incubation was
INDIRECT IMMUNOFLUORESCENCE METHOD

Slides stored frozen

10' Rehydrate with PBS

10' Block non-specific binding with normal goat serum

NGS

60' Incubate with primary MoAb (IL-1α, IL-1β, OX-1)

wash x3

30' Incubate with secondary antibody (Goat anti-mouse FITC)

wash x5

Mount in PPD

View under fluorescent microscope

Count +ve & −ve cells
carried out in the dark to reduce fading of the fluorochrome. Following a further three washes in PBS and two washes in distilled water the slides were mounted in 9:1 glycerol/PBS containing 1mg/ml p-phenylene diamine to retard fading.

**MONOCLONAL ANTIBODIES**
The mouse monoclonal antibodies to IL-1α and IL-1β discussed in section 2.3 were used. The antibodies were used at a 1/50 dilution in PBS. A control antibody OX-1 (supernatant) was kindly donated by D. Mason, Department of Cellular Immunology, William Dunn School of Pathology, Oxford. This mouse anti-rat-allotype was used undiluted in place of the primary antibody in immunofluorescent staining experiments to check for non-specific binding. All antibodies were of the IgG1 subclass.

**BLOCKING OF FLUORESCENT STAINING WITH EXCESS ANTIGEN**
To determine the specificity of the staining, the diluted antibody was preincubated overnight at 4°C (i) alone, (ii) in the presence of 10 μg/ml of the relevant recombinant IL-1 species, i.e., anti-IL-1α with rIL-1α, (iii) in the presence of 10μg/ml of the irrelevant IL-1 species, i.e., anti-IL-1α with rIL-1β. Recombinant IL-1α was kindly donated by P. Lomedico, Hoffmann-La Roche, Nutley, New Jersey and recombinant IL-1β by S. Gillis, Immunex, Seattle, Washington. The preincubated antibodies were then used for staining cell cytospins as above.

**MICROSCOPY AND QUANTITATION**
Slides were viewed using a Leitz Laborlux 12 fluorescent
microscope with a x 50 water immersion lens. Positive and negative cells were counted blind so that results could be expressed as % of total PBM showing positive staining. For each sample studied cytospins were stained on three different occasions and on each occasion at least 300 cells were counted and the mean calculated.

IDENTIFICATION OF CELL PHENOTYPES

In order to identify the phenotypes of the cells staining with anti-IL-1\(\alpha\) and anti-IL-1\(\beta\), the slides were also stained with monoclonal antibodies against T and B lymphocytes, monocytes and HLA-DR (Table 2.1). Attempts to perform double-labelling experiments, staining with both anti-IL-1\(\alpha\) or \(\beta\) and a cell marker on the same slide were unsuccessful because of the insensitivity of using directly conjugated antibodies for immunofluorescent staining viewed by microscopy; and binding of the anti-mouse conjugate to both primary antibodies in an indirect immunofluorescent method. The cells were therefore identified on the basis of their morphological characteristics when counterstained for 5 minutes with Mayer's Haematoxylin (Sigma Chemical Company). This histological stain does not interfere with immunofluorescent staining. The ability of the cells to phagocytose latex particles was also studied. Latex beads of 1.1\(\mu\)m size (LB11, Sigma Chemical Co) at a final concentration of 0.005% were added to the culture well for the last 30 minutes of a 20 and 24 hour incubation. The cells were then washed and cytospins made as described. Latex particles of this size are visible under light microscopy.
Table 2.1

Monoclonal antibodies used for cellular identification

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Leu 15</td>
<td>Monocytes</td>
<td>IgG2</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>(CD11)</td>
<td>NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Leu 4</td>
<td>T cells</td>
<td>IgG1</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>(CD3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD22</td>
<td>B cells</td>
<td>IgG1</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>L243</td>
<td>HLA-DR</td>
<td>IgG2</td>
<td>ATCC</td>
</tr>
</tbody>
</table>
Results

KINETICS OF CELL-ASSOCIATED IL-1α AND IL-1β FOLLOWING MITOGEN STIMULATION IN VITRO

The same pattern and time course of staining was seen in the cells from all four normal individuals studied. Table 2.2 and Figure 2.6 show the kinetics of IL-1α and IL-1β staining. No staining was seen with either antibody in unstimulated cells (T=0) or 4 hours post activation (Figure 2.7a, 2.8a). Many cells stained brightly with anti-IL-1α 16-24 hours after activation with a maximum intensity of staining seen at 24 hours post activation. The staining had a granular pattern, evenly distributed throughout the cell (Figure 2.7b). The stained cells were surrounded by clusters of unstained lymphocytes. The stained cells were of irregular dendritic shape and were larger (~ 2 x) than the surrounding lymphocytes. By 44 hours post activation the number of positive cells and the intensity of staining was much less (Figure 2.7c). Many cells also stained brightly with anti-IL-1β 16-24 hours after activation with a maximum intensity of staining seen at 20 hours post activation. The cells were of similar size to those staining for IL-1α but their morphology was more typical of a classical monocyte and the staining pattern was more homogeneous and evenly distributed throughout the cell (Figure 2.8b). By 44 hours post activation very few cells showed any positive staining (Figure 2.8c).

IDENTIFICATION OF 2 SEPARATE CELL POPULATIONS.

The cells producing IL-1α and IL-1β in normal PBM activated by
# TABLE 2.2  KINETICS OF IL-1α AND IL-1β STAINING

<table>
<thead>
<tr>
<th>% OF CELLS POSITIVE</th>
<th>Donor</th>
<th>anti-IL-1α alone</th>
<th>anti-IL-1β alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time post activation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td></td>
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<td></td>
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</tr>
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</tr>
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</table>
Figure 2.6 (opposite) shows the kinetics of IL-1α and IL-1β staining following *in vitro* stimulation of PBM with PHA/PMA. (The values plotted are the means of 4 individuals ±SEM).
Figure 2.6 Kinetics of IL-1α and IL-1β staining
Figure 2.7 (opposite) shows staining of normal PBM with anti-IL-1α. No staining is seen in unstimulated PBM (a). Bright IL-1α staining is seen in 3 irregularly shaped cells 24 hours post activation (b). Weak staining only is seen 44 hours after \textit{in vitro} activation (c).

(Original magnification $\sim$ x600)
Figure 2.7

INDIRECT IMMUNOFLUORESCENCE WITH MONOCLONAL ANTI-IL-1α ON PBM

a) Unstimulated

b) 24 hours post activation
c) 44 hours post activation
Figure 2.8 (opposite) shows staining of normal PBM with anti-IL-1β. As with IL-10 no staining is seen in unstimulated cells (a). 20 hours post activation there is bright staining of cells with monocytic morphology (b). By 44 hours post activation few cells show weak staining only (c).

(Original magnification ~ x600)
Figure 2.8

INDIRECT IMMUNOFLUORESCENCE WITH MONOCLONAL ANTI-IL-1β ON PBM

a) Unstimulated

b) 20 hours post activation  c) 44 hours post activation
mitogen in vitro had very different morphological characteristics. This suggested that the two IL-1 subtypes might be produced by distinct cell populations. At each time point, both antibodies were applied to the same slide as the primary layer. The percentage of cells staining was approximately equal to the sum of the percentage of cells staining for IL-1α or IL-1β alone assessed in separate experiments at the same time point (Table 2.3, Figure 2.9). Two populations of cells were identified; one population had the granular staining pattern and irregular morphology seen with anti-IL-1α alone and one population had the diffuse staining pattern and monocytic morphology seen with anti-IL-1β alone (Figure 2.10).

SPECIFICITY TESTS

Control antibody: No positive staining was seen at any time point post activation with OX-1 as the primary antibody or with FITC conjugated anti-mouse IgG alone.

Blocking experiments: Table 2.4 and Figure 2.11 show that the immunofluorescent staining could be inhibited by preincubation of the antibody with excess "antigen" (the appropriate IL-1 species). Figure 2.12 shows almost complete abolition of staining following preincubation of anti-IL-1α with 10μg/ml rIL-1α (b) but no inhibition following preincubation with 10μg/ml rIL-1β (c). Figure 2.13 shows a marked reduction of staining intensity and % of positive cells following preincubation of anti-IL-1β with 10μg/ml rIL-1β (b) but not with 10μg/ml rIL-1α (c).
<table>
<thead>
<tr>
<th>Time post activation</th>
<th>Donor</th>
<th>anti-IL-1α alone</th>
<th>anti-IL-1β alone</th>
<th>anti-IL-1α + anti-ILβ</th>
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</table>
Figure 2.9 (opposite) shows the kinetics of IL-1α and IL-1β staining following *in vitro* activation of normal PBM (Dotted and hatched lines respectively). The solid line shows the kinetics of staining when a mixture of anti-IL-1α and anti-IL-1β is used. This shows addition of the results for the antibodies alone suggesting that the two IL-1 molecules are produced by distinct population of cells. (The values plotted are the means of 4 individuals ±SEM).
Figure 2.9 Kinetics of IL-1α and IL-1β staining

% cells positive

0 2 4 6 8 10 12

Time post activation (hours)

0 10 20 30 40 50

- IL-1α alone
- IL-1β alone
- IL-1α + IL-1β
Figure 2.10 (opposite) shows IL-1α and IL-1β staining in normal PBM 22 hours post activation *in vitro*. (a) shows granular IL-1α staining, (b) shows IL-1β staining of cells with a monocytic morphology and (c) shows both these cell populations are stained when the two antibodies are used together.

(Original magnification ~ x600)
Figure 2.10

INDIRECT IMMUNOFLUORESCENCE ON PBM ACTIVATED FOR 22 HOURS IN VITRO

a) anti-IL-1α

b) anti-IL-1β

c) anti-IL-1α + anti-IL-1β
### Table 2.4 Inhibition of Staining with Excess Antigen

#### IL-1α Staining

<table>
<thead>
<tr>
<th>Time post activation</th>
<th>anti-IL-1α alone</th>
<th>MoAb pre-incubated with rIL-1α</th>
<th>rIL-1β</th>
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</thead>
<tbody>
<tr>
<td>16 hours</td>
<td>3.0</td>
<td>0</td>
<td>3.5</td>
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<tr>
<td>20 hours</td>
<td>4.2</td>
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<td>4.1</td>
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<td>24 hours</td>
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<td>0.5</td>
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#### IL-1β Staining

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<tr>
<th>Time post activation</th>
<th>anti-IL-1β alone</th>
<th>MoAb pre-incubated with rIL-1β</th>
<th>rIL-1α</th>
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<tbody>
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<td>16 hours</td>
<td>3.5</td>
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<td>3.0</td>
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<tr>
<td>20 hours</td>
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<tr>
<td>24 hours</td>
<td>2.0</td>
<td>0.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Figure 2.11 (opposite) shows inhibition of staining with excess antigen. The top graph shows that staining of PBM with monoclonal anti-IL-1α can be blocked by preincubating the antibody with rIL-1α but not with rIL-1β. The lower graph shows that staining with monoclonal anti-IL-1β can be blocked by preincubating the antibody with rIL-1β but not with rIL-1α. The values plotted are the means of the 4 individuals studied.
Blocking of staining with excess antigen

**ANTI-IL-1\(\alpha\)**

- \(\alpha\)IL-1\(\alpha\) alone
- \(\alpha\)IL-1\(\alpha\) + rIL-1\(\alpha\)
- \(\alpha\)IL-1\(\alpha\) + rIL-1\(\beta\)

**ANTI-IL-1\(\beta\)**

- \(\alpha\)IL-1\(\beta\) alone
- \(\alpha\)IL-1\(\beta\) + rIL-1\(\beta\)
- \(\alpha\)IL-1\(\beta\) + rIL-1\(\alpha\)
Figure 2.12 (opposite) (a) shows typical granular IL-1α staining of PBM activated for 24 hours $\textit{in vitro}$. (b) shows complete abolition of this staining when the anti-IL-1α antibody is preincubated overnight with 10μg/ml rIL-1. The IL-1α staining is unaffected by preincubating the antibody with 10μg/ml rIL-1β (c).

(Original magnification ~ x600)
Figure 2.12

INDIRECT IMMUNOFLUORESCENCE ON PBM ACTIVATED FOR 24 HOURS IN VITRO

a) anti-IL-1α alone

b) anti-IL-1α + rIL-1α

c) anti-IL-1α + rIL-1β
Figure 2.13 (opposite) (a) shows typical IL-1β staining of monocytes 20 hours post activation in vitro. (b) shows that this staining is markedly attenuated by preincubating the antibody with 10 μg/ml rIL-1β but is unaffected by preincubation with 10μg/ml rIL-1α (c).

(Original magnification ~ x600)
Figure 2.13

INDIRECT IMMUNOFLUORESCENCE ON PBM ACTIVATED IN VITRO FOR 20 HOURS

a) anti-IL-1β alone

b) anti-IL-1β + rIL-1β
c) anti-IL-1β + rIL-1α
CELLULAR PHENOTYPES

IL-1α staining cells with dendritic morphology were HLA-DR positive but CD11b, CD22 and CD3 negative. IL-1β staining cells had monocytic morphology and were HLA-DR and CD11b positive, CD22 and CD3 negative (Figure 2.14). The unstained cells surrounding the IL-1 containing cells were CD3 positive. Both types of cells actively phagocytosed latex particles (Figure 2.15). The IL-1α stained cells contained significantly more latex particles (mean of 10 cells = 7 latex beads per cell) than the IL-1β stained cells (mean of 10 cells = 2 latex beads per cell).

Discussion

These experiments were designed to develop a method to study cell-associated IL-1α and IL-1β at the single cell level in activated peripheral blood. The specificity of the antibodies for one IL-1 type only and the reactivity with recombinant and naturally occurring precursor and processed forms are demonstrated by the ELISA and Western blot results. The experiments on PBM activated in vitro show that using these monoclonal antibodies and an indirect immunofluorescent method IL-1α and IL-1β can be demonstrated within individual PBM following stimulation with PHA/PMA. IL-1α has not been demonstrated at the single cell level before and IL-1β has not been demonstrated previously at the single cell level using monoclonal antibodies. This method is specific as the observed immunofluorescence can be blocked by preincubation of
Figure 2.14 (opposite) shows HLA-DR staining of in vitro activated PBM with similar morphology to those cells which stain for IL-1α (a) and those which stain for IL-1β (b).

(Original magnification ~ x600)
Figure 2.14

HLA-DR STAINING OF NORMAL PBM ACTIVATED IN VITRO FOR 22 HOURS

a) Cells with IL-1α morphology

b) Cells with IL-1β morphology
Figure 2.15 (opposite) shows that both IL-1α positive cells and IL-1β positive cells in activated PBM are able to phagocytose latex particles. IL-1α positive cells (a) contain significantly more latex particles than IL-1β positive cells (b).

(Original magnification ~ x600)
Figure 2.15

LATEX PARTICLE INGESTION BY NORMAL PBM ACTIVATED IN VITRO FOR 22 HOURS

a) IL-1α positive cells

b) IL-1β positive cells
the antibody with the relevant recombinant IL-1; is not present in unactivated cells; and follows a time course compatible with data on IL-1mRNA expression and IL-1 production measured by bioassay following *in vitro* activation (Demczuk et al, 1987).

The kinetics of IL-1α and IL-1β production at the single cell level have not been demonstrated before. The results from these experiments show that IL-1α production reaches a peak approximately 4 hours after that for IL-1β and remains cell-associated for longer. This is in keeping with the suggestion that IL-1α is the cell-associated form, whereas IL-1β is the secreted form.

It has not been reported whether IL-1α and IL-1β are produced by the same cell or by different cells in peripheral blood activated *in vitro* as studies have been done on crude cell extracts or culture supernatants. The different morphology of the cells producing the two subtypes in these experiments suggest that they might be produced by two separate cell populations. The cells staining for IL-1α have an irregular dendritic shape with granular staining whereas the IL-1β cells have a classical monocytic morphology. It has not been possible to demonstrate production of the two IL-1 types by separate cell populations with double staining methods because of technical difficulties. However the summation of the percentage of cells staining when both anti-IL-1α and anti-IL-1β are used together (Table 2.3) is indicative of the two forms being produced by distinct cell populations. This is further confirmed by the morphological and phenotypic
differences between IL-1α and IL-1β staining cells. The different pattern of staining for the two IL-1 species supports different cellular localisations. IL-1α staining is granular and may represent membrane-associated IL-1 whereas IL-1β staining appears cytoplasmic. This is supported by the observation that the IL-1α staining was unaffected by omitting the fixation step whereas the IL-1β staining was markedly attenuated.

Characterisation of the cells producing the two subtypes has proved technically difficult. Attempts to double stain with antibodies directly conjugated to a fluorochrome have been unsuccessful. Indirect immunofluorescence results in higher fluorescent intensities than direct immunofluorescence due to the second layer producing amplification of the signal. Directly conjugated antibodies are therefore often unsuitable for microscopy unless the antigen is present in great amounts. Double staining using polyclonal antibodies against IL-1α and IL-1β has not been possible because of high background fluorescence due to non specific binding with these reagents. Double staining with the monoclonal anti-IL-1α and anti-IL-1β antibodies and directly conjugated antibodies to cell surface antigens visualised by FACS scan (Fluorescein Activated Cell Sorter) has not been successful because of high backgrounds with the indirect immunofluorescent conjugate. Sufficient quantities of anti-IL-1α and anti-IL-1β are not available to directly conjugate them for this use. Characterisation is therefore based on single immunofluorescent staining and identification of the cells on the basis of their morphological
differences. The irregular shaped IL-1α producing cells are strongly HLA-DR +ve but lack monocyte or lymphocyte markers. The IL-1β producing cells are HLA-DR and monocyte marker +ve, lymphocyte marker negative. Both cell types are able to phagocytose latex particles. The phenotype of the IL-1β staining cells is that of classical monocytes. The IL-1α staining cells are strongly HLA-DR positive but do not bear monocyte antigens. This phenotype would be compatible with blood dendritic cells which are potent antigen presenting cells. However dendritic cells are non-phagocytic and since the IL-1α staining cells are able to phagocytose latex particles they must represent a subpopulation of monocytes. The absence of monocyte markers is surprising although monocytes can lose characteristic surface antigens on activation (Jayaram et al, 1989). A cell with similar phenotype has been demonstrated in human peripheral blood by another group: a non-B cell, non-monocytic, HLA-DR positive accessory cell, distinct from dendritic cells and able to produce IFNγ (Chehimi et al, 1989). There is also some evidence that different subsets of human peripheral blood monocytes secrete different cytokines. In a study by Hermann et al (1989), when PBMC were separated on the basis of their density, IL-1β producing cells resided in the most dense fraction and TNFα secretion was most pronounced in the less dense fraction. It is known that cells become less dense on activation and it remains unclear whether these subpopulations represent truly distinct cell populations or a different stage of differentiation or activation of the same population.
2.5 Conclusions

This section outlines the development of a new and specific method for detecting cell-associated IL-1α and IL-1β at the single cell level. It demonstrates the kinetics of IL-1α and IL-1β production by PBM activated with mitogen \textit{in vitro}. Use of this method has allowed the demonstration that IL-1α and IL-1β are produced by distinct populations of mononuclear cells in activated human peripheral blood. This has not been previously reported and may be important in our understanding of the fine regulation of the immune response by cells and their mediators in health and disease. It might also explain the existence of the two forms of IL-1, both with identical biological activities but each with different localisation (cell-associated/secreted) and each produced by different sub-populations of cells. This method will be useful for the study of IL-1α and IL-1β at the single cell level but it has some important limitations; it is time consuming and not suitable for studying large numbers of samples. It is also not quantitative, so that the relative amounts of IL-1 present cannot be measured. These problems and the technical problems associated with double staining could be overcome by FACS analysis when sufficient quantities of anti-IL-1α and anti-IL-1β monoclonal antibodies are available for direct conjugation to fluorochromes.
CHAPTER 3

STUDY OF CELL-ASSOCIATED IL-1α AND IL-1β IN THE PERIPHERAL BLOOD OF PATIENTS WITH RA

3.1 Introduction

Interleukin 1 has the potential to mediate many of the local and systemic features of RA. Despite the systemic nature of RA, studies on peripheral blood are limited. There have been no studies on cell-associated IL-1 in RA blood despite suggestions that IL-1α, the cell-associated form, may play an important role in rheumatoid inflammation. This chapter outlines the application of the method described in chapter 2 to study cell-associated IL-1α and IL-1β in freshly isolated unactivated blood from patients with RA. Interpretation of immunological abnormalities found in RA, particularly abnormalities in the cytokine network, is often hampered by the lack of suitable controls. Abnormalities may simply be sequelae of any arthritic or inflammatory process and may not play any major pathogenic role in RA. Therefore the study of normal volunteers as a control may be useful initially but is inadequate for full interpretation of data. Patients with osteoarthritis (OA) serve as a good non-autoimmune arthritis control but differences between this disease and RA may simply be explained by the lack of any inflammatory process in most patients with OA. Ankylosing spondylitis is a suitable group for comparison, with an inflammatory arthritis of different aetiology. Reactive arthritis patients are also a suitable
group for comparison because they have a marked inflammatory
synovitis which is usually self limiting and does not progress
to joint destruction as in RA. Unfortunately samples are rare
in this latter group. Samples from a non-arthritic
inflammatory control group should be included in studies on
immunological abnormalities in RA. Ideally this should be a
disease characterised by chronic immune activation with
mononuclear cell infiltration without a prediliction for the
joints. These controls are difficult to obtain and are often
not included in studies. Dr Geraint James, Royal Free
Hospital, London has kindly allowed me to collect peripheral
blood from 5 of his patients with sarcoidosis, a disease
characterised by chronic immune activation and mononuclear cell
infiltration.

3.2 Aims

To study cell-associated IL-1α and IL-1β in freshly isolated
peripheral blood from patients with RA compared to normal
volunteers and patients with OA, ankylosing spondylitis (AS)
and sarcoidosis.

3.3 Materials and methods

VOLUNTEERS AND PATIENTS

Blood samples were collected from healthy laboratory and non
laboratory volunteers and from patients attending Charing Cross
Hospital Rheumatology Unit. All patients in this study gave informed consent. Blood was taken at the same time as the patients were undergoing routine venepuncture for diagnosis or monitoring of their disease or treatment. Ethical approval was obtained. Blood was obtained from 10 normal volunteers. The median age of this control group was 40 (range:25-71) and comprised 3 men and 7 women (Table 3.2). 28 RA patients fulfilling the ARA criteria for classical or definite disease selected at random from RA patients attending the rheumatology outpatient clinic. The median age was 63 (range:34-82) and comprised 5 men and 23 women (Table 3.1). 25 had a positive rheumatoid factor. The duration of the disease at the time the patients donated blood ranged from 3 months - 40 years. 2 patients were receiving no medication, 7 were receiving NSAIDS alone and the remaining 19 were receiving disease modifying drugs (DMDs). 8 of these patients were receiving parenteral gold therapy. Blood was also collected from 10 patients with OA and 5 patients with AS attending the rheumatology outpatient clinic. (Table 3.2). Blood was taken from 5 patients selected at random from those attending the sarcoidosis clinic at the Royal Free Hospital, London (Table 3.3).

COLLECTION AND SEPARATION OF PBM, CYTOSPINS AND IMMUNOSTAINING
Peripheral venous blood was collected as outlined in chapter 2. Blood samples were kept at room temperature until separation of the PBM which was carried out as soon as possible after vesection (maximum delay=2 hours). A maximum of 4 blood samples could be processed together. On most occasions blood
Table 3.1  Clinical details of RA patients

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>AGE</th>
<th>SEX</th>
<th>DISEASE DURATION</th>
<th>RHEUMATOID FACTOR</th>
<th>TREATMENT</th>
<th>C-REACTIVE PROTEIN</th>
<th>%IL-1α +VE CELLS</th>
<th>%IL-1β +VE CELLS</th>
</tr>
</thead>
<tbody>
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<td>3 years</td>
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<td>18</td>
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<td></td>
</tr>
<tr>
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<td>34</td>
<td>F</td>
<td>7 years</td>
<td>-ve</td>
<td>None</td>
<td>12</td>
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<td></td>
</tr>
<tr>
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<td>69</td>
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<td>+ve</td>
<td>NSAID</td>
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<tr>
<td>4</td>
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<tr>
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<td>F</td>
<td>3 months</td>
<td>+ve</td>
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<td></td>
</tr>
<tr>
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<td>7 years</td>
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<td>NSAID</td>
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<tr>
<td>11</td>
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<td>+ve</td>
<td>D-P</td>
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<td>+ve</td>
<td>D-P</td>
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<tr>
<td>14</td>
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<td>M</td>
<td>40 years</td>
<td>+ve</td>
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<td>2.0</td>
<td>e</td>
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<td>15</td>
<td>54</td>
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<td>+ve</td>
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<td>D-P</td>
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<td>g</td>
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<td>a</td>
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<td>+ve</td>
<td>AZA</td>
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<td>F</td>
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<td>+ve</td>
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<td>t</td>
</tr>
<tr>
<td>22</td>
<td>65</td>
<td>F</td>
<td>19 years</td>
<td>+ve</td>
<td>Gold</td>
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</tr>
<tr>
<td>23</td>
<td>44</td>
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<td>3 years</td>
<td>+ve</td>
<td>Gold/Salazop</td>
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<td>0.3</td>
<td>i</td>
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<td>+ve</td>
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<td>+ve</td>
<td>Gold</td>
<td>50</td>
<td>1.8</td>
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</tbody>
</table>

NSAID = nonsteroidal antinflammatory drugs; D-P = D-penicillamine; MTX = Methotrexate; Pred = Prednisolone; Salazop = Salazopyrine; AZA = Azathioprine.
Table 3.2  Details of non-RA controls

<table>
<thead>
<tr>
<th>Normals</th>
<th>AGE</th>
<th>SEX</th>
<th>TREATMENT</th>
<th>IL-1α +VE CELLS</th>
<th>IL-1β +VE CELLS</th>
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<td>0.0</td>
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<td>0.1</td>
<td>l</td>
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<tr>
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<td>40</td>
<td>M</td>
<td>None</td>
<td>0.0</td>
<td>l</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>F</td>
<td>None</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>F</td>
<td>None</td>
<td>0.0</td>
<td></td>
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<tr>
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<td>71</td>
<td>F</td>
<td>None</td>
<td>1.4</td>
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<tr>
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<td>65</td>
<td>F</td>
<td>Paracetamol</td>
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<td>60</td>
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<table>
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<th>TREATMENT</th>
<th>IL-1α +VE CELLS</th>
<th>IL-1β +VE CELLS</th>
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</thead>
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<td>2</td>
<td>79</td>
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<td>None</td>
<td>0.4</td>
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<td>75</td>
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<td>56</td>
<td>M</td>
<td>NSAID</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>52</td>
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<td>NSAID</td>
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<td>a</td>
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<tr>
<td>6</td>
<td>52</td>
<td>M</td>
<td>NSAID</td>
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<td></td>
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<tr>
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<td>0.1</td>
<td>t</td>
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<tr>
<td>8</td>
<td>53</td>
<td>F</td>
<td>NSAID</td>
<td>0.1</td>
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<tr>
<td>9</td>
<td>50</td>
<td>F</td>
<td>None</td>
<td>0.4</td>
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</tr>
<tr>
<td>10</td>
<td>46</td>
<td>F</td>
<td>NSAID</td>
<td>0.9</td>
<td>n</td>
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<th>Ank Spond</th>
<th>AGE</th>
<th>SEX</th>
<th>TREATMENT</th>
<th>IL-1α +VE CELLS</th>
<th>IL-1β +VE CELLS</th>
</tr>
</thead>
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</tr>
<tr>
<td>2</td>
<td>45</td>
<td>M</td>
<td>NSAID</td>
<td>1.0</td>
<td>v</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>M</td>
<td>NSAID</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>M</td>
<td>Salazopyrine</td>
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<td>5</td>
<td>30</td>
<td>F</td>
<td>Salazopyrine</td>
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Table 3.3 \ Details of sarcoidosis patients

<table>
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<tr>
<th>AGE</th>
<th>SEX</th>
<th>DISEASE DURATION</th>
<th>DISEASE STATE</th>
<th>TREATMENT</th>
<th>%IL-1α +ve CELLS</th>
<th>%IL-1β +ve CELLS</th>
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</thead>
<tbody>
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<td>1</td>
<td>47</td>
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<td>active lupus pernio</td>
<td>Hydroxychloroquine</td>
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<td>2.6</td>
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<tr>
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<td>inactive pulmonary sarcoid</td>
<td>Prednisolone</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>6 years</td>
<td>moderately active pulmonary sarcoid</td>
<td>Prednisolone</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>6 years</td>
<td>active pulmonary sarcoid</td>
<td>Prednisolone</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>7 years</td>
<td>inactive pulmonary sarcoid</td>
<td>None</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
from RA patients and blood from control groups were processed
together ensuring that differences between the RA patients
and the controls were not due to differences in processing on
different days.
Separation of PBM, preparation of cytospins and immunostaining
was carried out as outlined in the previous chapter. Inhibition
of positive staining with preincubation of the antibodies with
excess antigen was performed to check the specificity of any
staining. The method for this is described in the previous
chapter. In the staining experiments on unactivated PBM a
positive control slide was included to ensure that the method
and antibodies were working. This positive control was normal
PBM activated in vitro with PHA/PMA for 22 hours. The method
for activation of PBM is described in chapter 2. This duration
of activation (22 hours) was chosen as it represents the time
point between maximum staining for IL-1α and IL-1β determined
in chapter 2, allowing it to be used as a positive control for
both IL-1α and IL-1β staining (Figure 2.9).

3.3 Results

IL-1α POSITIVE CELLS IN UNSTIMULATED RA BLOOD
In unactivated blood from the 28 RA patients significantly more
cells showed bright IL-1α staining when compared to unactivated
blood from the 10 normal controls (RA: median=2.7%, range=0-
7.5%; Normals: median=0.1%, range=0-2.3%, p<0.01) (Figure 3.1).
The IL-1α stained cells in unactivated RA blood had a similar
dendritic morphology and granular pattern of staining to the
Figure 3.1

PERCENTAGE OF IL-1α POSITIVE CELLS IN FRESHLY ISOLATED PBM FROM NORMAL VOLUNTEERS AND PATIENTS WITH RA

% cells positive

median value

Normals RA
IL-1α stained cells seen following *in vitro* activation of normal blood. The cells in RA blood varied considerably in size and shape and in some individuals the cells appeared rather disintegrated (Figure 3.2 shows IL-1α stained cells from 6 different RA patients). No staining was seen with anti-IL-1β in any RA patient or any normal studied although staining was seen in the positive control.

**EFFECT OF THERAPY**

Early in the study it was noted that one of the RA patients on gold therapy had no positive IL-1α stained cells despite a raised C-reactive protein (CRP) at the time the blood was studied. This suggested that these IL-1α stained cells might be modified by gold therapy. Figure 3.3 shows that this initial observation was confirmed as the study progressed with the 8 patients receiving parenteral gold therapy having significantly fewer IL-1α positive cells (median=0.7%, range 0-1.8%) than the 20 RA patients receiving other DMDs or NSAIDs alone (median=4.3, range=1.2-7.5%, p<0.01). This is discussed in section 3.5. The morphology of the cells that did stain in the RA patients on gold had the same appearance as those in RA patients not on gold (Figure 3.4). Analysis of the differential counts obtained from the routine haematology results showed that there was no significant difference in the % monocyte count from RA patients on and off gold therapy, (RA patients on gold: 17.7% PBM = monocytes, range 4-36%; RA patients not on gold; 17.1% PBM = monocytes, range 9-30%).
Figure 3.2 (opposite) shows examples of IL-1α positive cells in freshly isolated PBM from 4 patients with RA (a)-(d).

(Original magnification ~ x600)
Figure 3.2

INDIRECT IMMUNOFLUORESCENCE WITH ANTI-IL-1α ON UNACTIVATED RA PBM

a) Patient 2  
b) Patient 6  
c) Patient 10  
d) Patient 13
Figure 3.3

PERCENTAGE OF IL-1α POSITIVE CELLS IN FRESHLY ISOLATED PBM FROM RA PATIENTS RECEIVING GOLD THERAPY AND RA PATIENTS RECEIVING OTHER MEDICATION

median value

% cells positive

RA patients not on gold therapy

RA patients on gold therapy
Figure 3.4 (opposite) shows typical granular IL-1α staining in freshly isolated PBM from a RA patient receiving gold therapy. Few of these cells only are seen in RA patients receiving gold.
Figure 3.4

INDIRECT IMMUNOFLUORESCENCE WITH ANTI-IL-1α ON UNACTIVATED PBM FROM RA PATIENT ON GOLD

Patient 22
RELATION WITH BLOOD MARKERS OF DISEASE ACTIVITY

Figure 3.5 a) shows the % of IL-1α positive cells plotted against the CRP for all 28 RA patients studied. The correlation between these was poor (r=0.21). However Figure 3.5 b) shows that when the 20 patients not receiving parenteral gold therapy were considered separately there was a significant correlation (r=0.65, p<0.01). Figure 3.5 c) shows a different relationship between the % of IL-1α staining cells and the CRP in the 8 patients on gold therapy (r=0.61; however this group is small and this correlation does not reach conventional levels of statistical significance, p=0.1). The results suggest that with respect to IL-1α positive cells patients on gold therapy and patients on other treatments represent two distinct populations. This trend is also seen when other blood markers of disease activity are studied. Figure 3.6 and 3.7 show this trend for ESR and Hb.

DISEASE SPECIFICITY

Figure 3.8 shows the percentage of IL-1α staining cells in the unactivated blood of patients with non-rheumatoid rheumatic diseases. Patients with OA had very few IL-1α positive cells in their peripheral blood (median=0.4%, range=0.1-0.7%). The 5 patients with AS also showed very few IL-1α positive cells (median=0.4%, range=0.3-1.0%). No IL-1β staining was seen in these patients.

The patients with sarcoidosis showed few IL-1α staining cells (median=0.8%, range=0.5-1.0%, figure 3.9). In contrast to the other disease groups, the PBM of some sarcoidosis patients also showed IL-1β staining (Figure 3.9) The 3 patients in whom this
Figure 3.5

CORRELATION BETWEEN IL-1α POSITIVE CELLS AND CRP

a) ALL RA PATIENTS

\[ r = 0.20 \]
\[ (p > 0.1) \]

b) RA PATIENTS NOT ON GOLD

\[ r = 0.65 \]
\[ p < 0.01 \]

c) RA PATIENTS ON GOLD

\[ r = 0.61 \]
\[ (p = 0.1) \]
Figure 3.6

CORRELATION BETWEEN IL-1α POSITIVE CELLS AND ESR

a) ALL RA PATIENTS

\[ r = 0.3 \]
\[ (p > 0.1) \]

b) RA PATIENTS NOT ON GOLD

\[ r = 0.44 \]
\[ p = 0.05 \]

c) RA PATIENTS ON GOLD

\[ r = 0.55 \]
\[ (p > 0.1) \]
Figure 3.7

CORRELATION BETWEEN IL-1α POSITIVE CELLS AND Hb

a) ALL RA PATIENTS

\[ r = 0.04 \]
\[ (p > 0.1) \]

b) RA PATIENTS NOT ON GOLD

\[ r = -0.37 \]
\[ (p = 0.1) \]

c) RA PATIENTS ON GOLD

\[ r = -0.54 \]
\[ (p = 0.1) \]
Figure 3.8

PERCENTAGE IL-1α POSITIVE CELLS IN FRESHLY ISOLATED PBM FROM RA PATIENTS AND CONTROLS
Figure 3.9

PERCENTAGE OF IL-1α AND IL-1β POSITIVE CELLS IN FRESHLY ISOLATED PBM FROM RA PATIENTS AND SARCOIDOSIS PATIENTS

anti IL-1α

anti IL-1β

% cells positive

RA  Sarcoid

RA  Sarcoid
was seen had evidence of disease activity with the most active sarcoid patient (1) showing the highest percentage of IL-1β positive PBM (Table 3.3).

SPECIFICITY TESTS

**Positive control:** This was included in all staining experiments. Staining with both anti-IL-1α and anti-IL-1β was seen in normal cells activated *in vitro* with PHA/PMA for 22 hours. The patterns of staining and percentages of positive cells seen were similar to the results obtained in chapter 2. Including the positive control ensured that the method and antibodies were working in the staining experiments.

**Negative control:** No staining was seen with OX-1 used in place of the primary IL-1 monoclonal antibody.

**Blocking with excess antigen:** The IL-1α staining of unactivated PBM from RA patients was completely abolished by preincubation of the anti-IL-1α monoclonal antibody with 10μg/ml rIL-1α.

3.5 Discussion

This study demonstrates cell-associated IL-1α in the peripheral blood of patients with RA compared to controls. This has not been previously described. Previous studies on RA blood have been limited and the interpretation of experiments on *in vitro* cytokine production is difficult as it may not reflect the situation *in vivo*. Reports of increased spontaneous production of IL-1 by cultured monocytes from blood, synovial fluid and
synovial membrane of RA patients (Danis et al, 1987; Miyasaki et al, 1988; Bhardwaj et al, 1988) may not reflect the situation in vivo and may occur as a result of culture (eg. due to the presence of lipopolysaccharide or due to plastic adherence). The finding of increased levels of IL-1β in freshly obtained plasma from RA patients does suggest that IL-1 levels are raised in vivo (Eastgate et al, 1988). However it is not known whether the IL-1 measured in this study was biologically active as the IL-1β was extracted from binding proteins and assayed by an immunoassay. In that study, secreted material was assayed and IL-1α was not studied. A further study by this group has demonstrated raised levels of IL-1α in the serum of patients with RA compared to normals (Symons et al, 1989). In this latter study IL-1α was assayed by radioimmunoassay and cell-associated IL-1 was not studied. IL-1α is of interest as this form, which is active when cell-associated, plays an important role in T cell activation (Hurme, 1987) and has been suggested to play a major role in rheumatoid inflammation. IL-1α mRNA has been found to be expressed in increased amounts in mononuclear cells from RA joints at levels equal or greater than IL-1β (Buchan et al, 1988b). The method developed in chapter 2 has enabled this study of cell-associated IL-1 in the peripheral blood of RA patients and controls. The cell-associated IL-1α is seen in unactivated freshly isolated PBM from RA patients and is not seen in PBM from normal blood processed in the same way. The IL-1α is therefore not simply induced by the processing of the samples and is likely to have occurred in vivo.
The cells showing IL-1α staining had similar morphology and granular pattern of staining to those seen in normal blood activated in vitro with mitogen. The granular staining may represent surface IL-1α as it was unaffected by omitting the fixation step from the processing. The positive cells had the same phenotype as the IL-1α positive phagocytic mononuclear cells seen in activated normal blood; HLA-DR positive but negative for a monocyte marker (CD11b) or T & B lymphocyte markers (CD3/CD22). The results suggest that IL-1α positive cells circulating in the peripheral blood of patients with RA may represent a sub-population of activated monocytes. The absence of CD11 is surprising but there is evidence that monocyte markers may be lost on activation under certain conditions (Jayaram et al, 1989). The morphological appearances may be due to activation. Technical difficulties with double staining do not allow definitive identification of the cells. This is discussed in the previous chapter.

The percentage of IL-1α positive cells in RA blood ranged from 0-7.5% of PBM. In some patients the percentage of these cells was similar to the percentage found in normal blood maximally activated with mitogen in vitro. The RA patients on parenteral gold therapy had significantly fewer IL-1α staining cells than the RA patients on other therapies. The mechanism of action of gold as a disease modifying agent in RA is not understood although it has been suggested to act via an effect on IL-1 production as it is known to inhibit IL-1 production in vitro (Remvig et al, 1988). The results of this study would support this occurring in vivo also. The mechanism by which
this may occur is considered in more detail in the next chapter. The percentage of IL-1α positive cells appears to correlate with blood markers of disease activity (positive correlation with CRP and ESR, negative correlation with Hb) but only when those RA patients not on gold and those on gold therapy are considered separately. This is surprising but suggests that these these IL-1α staining cells may be modified by parenteral gold therapy in vivo. However the patients on gold therapy have similar disease activity markers as the patients on other therapies but very low levels of IL-1α positive cells. One interpretation of this is that cell-associated IL-1α in peripheral blood has no role in disease pathogenesis in RA and gold does not exert its effect via modification of this IL-1α. Another interpretation is that the IL-1α is important in RA but that CRP, ESR and Hb are not a good indicator of the chronic inflammatory and destructive process in the joint which may be modified by gold therapy. It is also possible that changes in CRP, ESR and Hb may occur over a different timecourse than changes in IL-1α positive cells. Certainly one patient on gold therapy had an ESR of 72 on the day blood was processed for cell-associated IL-1 and 3 days later had an ESR of 16. A longitudinal study with assessment of disease progression in RA patients on different therapies would be needed to investigate this further.

No IL-1β staining was seen in unactivated blood from RA patients or normals. This is in contrast to the reported finding of increased levels of IL-1β in RA plasma correlating with disease activity (Eastgate et al, 1988). This discrepancy
can be explained either by the IL-1β produced in vivo not remaining cell-associated during the separation procedure or that the IL-1β detected by Eastgate and colleagues is produced by cells in the joint rather than cells in the blood and levels detected represent "overspill" from the joint. The first explanation is unlikely as cell-associated IL-1β is detected in the blood from patients with sarcoidosis. This blood was processed in the same way as RA blood so that the absence of cell-associated IL-1β in RA blood cannot be explained by loss/secretion during processing. The IL-1β detected by Eastgate is therefore likely to be produced locally in the joint. The IL-1α positive cells demonstrated in this study might also represent "overspill" from the joint. However if they are a population of monocytes they would not represent overspill as blood monocytes arise from bone marrow, circulate through blood and pass to tissues where they develop into fixed tissue macrophages. They are not thought to recirculate into the blood pool.

Immunological abnormalities found in RA, particularly of the cytokine network, must be interpreted in the context of control groups. Patients with OA, ankylosing spondylitis and sarcoidosis had very few IL-1α staining cells in their blood suggesting that compared to these diseases the presence of these cells is specific to RA. IL-1β positive cells were not present in the blood of patients with RA, OA, AS or normals. However IL-1β positive cells were seen in the blood of patients with sarcoidosis. IL-1 has been suggested to play a role in sarcoidosis and increased IL-1 production by alveolar
macrophages from patients with sarcoidosis has been reported (Barth et al, 1989). These IL-1β positive cells in sarcoidosis have not been previously reported. Although in this study it was only possible to study a small group of sarcoidosis patients and to assess disease activity clinically the percentage of these cells appeared to correlate with disease activity. The sarcoidosis group of patients was used as a "control" for RA but this interesting preliminary observation is worth pursuing by those interested in the role of IL-1 in sarcoidosis.

The mechanisms by which these IL-1α positive cells may be induced in RA and the modification by gold therapy is considered in more detail in the next chapter.

3.6 Conclusions

This is the first demonstration of IL-1α positive cells in the peripheral blood of patients with RA compared to controls. The percentage of positive cells correlates with disease activity and is modified by gold therapy. These IL-1α positive cells may play an important role in the pathogenesis of rheumatoid inflammation. They may mediate some of the systemic features and following migration to the joint may be involved in presentation of self antigens, T cell activation and cartilage and bone destruction. The modification of the percentage of these IL-1α positive cells by gold therapy is consistent with the mechanism of action of gold being via an effect on IL-1.
CHAPTER 4

IN VITRO STUDIES OF IL-1 INDUCTION BY GM-CSF AND IMMUNE COMPLEXES & MODIFICATION BY GOLD

4.1 Introduction

The previous chapter describes bright IL-1α staining cells in the peripheral blood of patients with RA compared to controls and patients with RA on gold therapy. This cell-associated IL-1α may play an important role in rheumatoid inflammation. This chapter describes in vitro experiments to investigate some potential inducers of IL-1α in vivo and the effect of gold on the induction of IL-1 production.

Interleukin 1 production and release is triggered by a wide variety of different stimuli (Table 4.1). IL-1 production can be induced by contact between accessory cells and T cells, either by cell contact itself (Weaver et al, 1986; Mizel, 1987) or by cytokines released from activated T cells. IL-2 (Numerof et al, 1988), IFNγ (Boraschi et al, 1984) and colony stimulating factors (Sisson et al, 1988) are able to induce IL-1 production. Monokines are also able to induce IL-1 production; IL-1 and TNFα participate in self augmentation induction mechanisms. Recombinant human IL-1 and TNF are capable of inducing the production of their respective molecules as well as each other (Dinarello et al, 1986; Philip et al, 1986; Ikejima, 1987). Complement component C5a is able to induce IL-1 production (Goodman et al, 1982) and a recent
TABLE 4.1 STIMULI OF IL-1 PRODUCTION

IMMUNOLOGICAL:

activated T cells - cell contact (class II restricted)
- IL-2, IFNγ
- colony stimulating factors

monokines - IL-1, TNFα

other cytokines - IFNγ, IFNβ, TGFβ

immune complexes

C5a

MICROBIAL:

gram -ve bacteria - endotoxin

gram +ve bacteria - cell walls
exotoxins

yeast - cell walls

virus - haemaglutinins
- double stranded RNA

OTHER:

silica crystals

urate crystals

asbestos

phorbol myristate acetate
report showing that immune complexes are able to trigger IL-1 production from human monocytes (Chantry et al, 1989) suggests this as a mechanism of immune complex mediated damage which may be important in RA.

Various microbial agents are able to induce IL-1 including viruses (Scala et al, 1984; Ensoli et al, 1989), bacteria (Dinarello et al, 1978; Wallis et al, 1986), spirochaetes (Habicht et al, 1985) and yeasts (Lombardi et al, 1984). These agents may play an important role in IL-1 production in the normal response to infections. Bacterial lipopolysaccharide (LPS) is a very potent inducer of IL-1 production. It is able to stimulate the release of IL-1 by human monocytes at concentrations as low as 10pg/ml (Arend et al, 1985). It is therefore important in all experiments on induction of IL-1 to ensure that reagents are LPS free.

Other inducers of IL-1 include silica (Krakauer et al, 1983), urate crystals (Di Giovine et al, 1987) and asbestos (Hartmann et al, 1984). These are thought to induce IL-1 via an action on the cell membrane as inhibition of phagocytic uptake does not inhibit their stimulatory capacity. PMA, a mitogen which acts via protein kinase-C, stimulates IL-1 production by monocytes and macrophage cell lines (Mizel et al, 1978) and is frequently used as a means of inducing IL-1 \textit{in vitro}.

There is now considerable interest in the role of granulocyte-macrophage colony stimulating factor (GM-CSF) in RA. This factor is a 22 kD product of activated T cells (Moore et al, 1980) although it can also be produced by a wide variety of cells including fibroblasts, keratinocytes, endothelial cells
and smooth muscle cells (Reviewed by Metcalf, 1985). It is able to stimulate the formation of granulocytes, macrophages and eosinophils from haemopoetic progenitor cells (Metcalf, et al 1986). More recent studies have shown that GM-CSF is more than a haemopoetic growth-inducing molecule. It has stimulatory effects on polymorphonuclear cells, it is involved in the maturation of dendritic cells into potent immuno-stimulatory cells (Heufler et al, 1988) and it produces a striking enhancement of antigen presenting capacity of accessory cells (Morrissey, 1987; Witmer-Pack et al, 1987). The interest in this cytokine in relation to rheumatoid inflammation arises from recent reports that GM-CSF is present in RA synovial effusions (Xu et al, 1989; Haworth et al, personal communication) and has the potential to stimulate two important pro-inflammatory features seen in RA. It is able to stimulate IL-1 production, both in vitro (Sisson et al, 1988) and in vivo; GM-CSF transgenic mice show elevated levels of IL-1 in serum, peritoneal and pleural cavities (Gearing et al, 1989) and it is able to induce HLA-DR and HLA-DQ expression on human monocytes (Alvaro-Gracia JM et al, 1989; Chantry et al, 1990). A role for GM-CSF in rheumatoid inflammation is supported by the recent report that treatment of granulocytopenia in patients with Felty’s syndrome with GM-CSF results in a flare of the arthritis (Hazenberg et al, 1989).

Many studies on induction of IL-1 have been based on bioassay and there is little available information on the relative induction of the two subtypes of IL-1. This chapter describes experiments on cell-associated IL-1α and IL-1β induction by
rGM-CSF and rheumatoid factor immune complexes in vitro and the effect of gold. Both GM-CSF and immune complexes occur in RA. A substance which is able to induce cell-associated IL-1α in normal PBM in vitro which is inhibited by gold might suggest that it is also a potential in vivo inducer of the cell-associated IL-1α seen in the blood of patients with RA.

4.2 Aims

1. To reproduce selective IL-1α staining as demonstrated in RA blood in vitro by incubating normal PBM with various stimuli.

2. To inhibit this induction with sodium aurothiomalate in vitro

4.3 Materials and methods

REAGENTS

Details of the mitogens used (50 ng/ml PMA & 1μg/ml PHA) are outlined in chapter 2. IgG/IgM immune complexes were kindly donated by Dr C Winearls, Hammersmith Hospital, London, UK. The complexes were derived from a patient with type II essential cryoglobulinaemia with a monoclonal IgM rheumatoid factor. The LPS content of the immune complexes was <0.03 ng/ml as determined by the limulus amoebocyte assay (Sigma Chemical Co). The immune complexes were used in culture at a
concentration of 100µg/ml. Recombinant GM-CSF (1.0 x 10^8U/mg, diluted to a concentration of 1µg/ml; LPS content <0.03 ng/ml) was a kind gift from Dr A Galazka, Glaxo Institute for Molecular Biology, Geneva, Switzerland. rGM-CSF was used at concentrations of 500, 1000, 5000, 10000 U/ml in culture. Sodium aurothiomalate (Myocrisin, May and Baker) was used at a concentration of 50µg/ml. This concentration is equivalent to gold levels found in the serum/tissues of patients receiving chrysotherapy (Lipsky et al, 1977)

COLLECTION AND SEPARATION OF PBM
Peripheral blood was collected from normal volunteers and separated on a Ficoll gradient as outlined in chapter 2. Cytospins of uncultured cells were made and stored as in chapter 2.

IN VITRO CELL CULTURE
10^6 PBM/ml were cultured in flat bottomed 24-well culture plates (Nunc, Gibco Europe) with RPMI 1640 medium supplemented with 10% heat inactivated FCS in the presence of different inducers. Following incubation for 22 hours the supernatants were removed for bioassay of secreted IL-1. The adherent and non-adherent cells were removed from the culture wells by vigorous pipetting and were then washed in PBS x3. Following washing, the cells were recounted by trypan blue dye exclusion and an assessment of viability made. Freeze-thaw lysates of cells for bioassay of cell-associated IL-1 were made by snap freezing cells at a concentration of 10^6/ml at -60°C followed by thawing at 37°C three times. Cytospins of intact cells were
made, fixed and stored as outlined in chapter 2. Cells were cultured either with medium to assess the effect of culture on IL-1 production or with mitogen, immune complexes or rGM-CSF. These incubations were carried out either in the presence or absence of 50μg/ml of sodium aurothiomalate in the culture well.

IMMUNOFLUORESCENCE

Immunofluorescent staining of the cytospins was carried out using the monoclonal antibodies to IL-1α and IL-1β as outlined in chapters 1 & 2. Positive and negative cells were counted as before (blinded).

IL-1 BIOASSAY

IL-1 was measured in culture supernatants and freeze-thaw lysates using a standard thymocyte co-mitogenic assay. This assay was carried out with the help of Dr C Hawrylowicz, Charing Cross Sunley Research Centre. Briefly, freshly obtained mouse thymocytes from 3 week old mice were washed and resuspended at a concentration of 1 x 10⁷ cells/ml in RPMI 1640 supplemented with 20% FCS (heat inactivated). 1μg/ml of PHA was added to this suspension. 100μl volumes of rIL-1 and test supernatants were plated out in 96 well flat bottomed microtitre plates. Each sample was assayed in triplicate. 100μl of thymocytes + PHA suspension was added to each well and incubated at 37°C for 3 days. The samples were pulsed with 1μCi of ³H-thymidine per well for 4-6 hours on day 3 of the culture. Incorporated ³H-thymidine was counted using a scintillation counter.
4.4 Results

IMMUNOFLUORESCENCE

Figure 4.1 a) shows the percentage of IL-1α positive cells after 22 hours of culture in the presence of various compounds. About 1% of the cells showed IL-1α staining after culture with medium alone. This represents induction of IL-1 by the culture conditions alone, e.g. by plastic adherence (Van der Meer et al, 1988) and thus "background" induction. As expected cell-associated IL-1α was induced by PHA/PMA in approximately 5% of PBM. The characteristic granular pattern of staining was seen. Immune complexes also induced cell-associated IL-1α, in approximately 6% of the cells. The staining was not as bright as with PHA/PMA and had a more homogenous distribution. rGM-CSF induced cell-associated IL-1α in a dose dependant fashion with more than 8% of the PBM showing staining with the highest dose of GM-CSF used (10,000U/ml). The staining was bright, considerably brighter than that seen with immune complexes and had the characteristic granular pattern in most cells (Figure 4.2). It can be seen that in vitro gold had a marked inhibitory effect on IL-1α production induced by rGM-CSF and immune complexes. The inhibitory effect on PHA/PMA induced IL-1α is less pronounced. Table 4.2 shows that the inhibitory effect of gold is not due to a cytotoxic effect as there is no significant difference between the viability of the cells after culture with or without gold.

Figure 4.1 b) shows the percentage of cells showing positive
Figure 4.1 (opposite) shows the percentage of IL-1α (a) and IL-1β (b) positive cells (detected by indirect immunofluorescence) in normal PBM after 22 hour culture with different stimuli in the presence and absence of 50µg/ml sodium aurothiomalate.

These induction experiments have been performed using three different donors and similar results are obtained. The results shown are from a typical experiment.
Figure 4.1

a) IL-1α +ve cells

b) IL-1β +ve cells
Figure 4.2 (opposite) shows a cell with granular IL-10 staining after culture of normal PBM for 22 hours in the presence of 5000U/ml rGM-CSF
IL-1α STAINING OF PBM CULTURED IN VITRO WITH rGM-CSF FOR 22 HOURS
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>99%</td>
</tr>
<tr>
<td>Medium + gold</td>
<td>97%</td>
</tr>
<tr>
<td>PHA/PMA</td>
<td>81%</td>
</tr>
<tr>
<td>PHA/PMA + gold</td>
<td>79%</td>
</tr>
<tr>
<td>Immune complexes</td>
<td>92%</td>
</tr>
<tr>
<td>Immune complexes + gold</td>
<td>96%</td>
</tr>
<tr>
<td>GM-CSF 500U/ml</td>
<td>95%</td>
</tr>
<tr>
<td>GM-CSF 500U/ml + gold</td>
<td>93%</td>
</tr>
<tr>
<td>GM-CSF 1000U/ml</td>
<td>93%</td>
</tr>
<tr>
<td>GM-CSF 1000U/ml + gold</td>
<td>92%</td>
</tr>
<tr>
<td>GM-CSF 5000U/ml</td>
<td>100%</td>
</tr>
<tr>
<td>GM-CSF 5000U/ml + gold</td>
<td>100%</td>
</tr>
<tr>
<td>GM-CSF 10000U/ml</td>
<td>98%</td>
</tr>
<tr>
<td>GM-CSF 10000U/ml + gold</td>
<td>100%</td>
</tr>
</tbody>
</table>
IL-1β staining following culture with the same compounds. Less than 1% of cells stained after culture with medium alone. Some induction of IL-1β was seen with PHA/PMA. The most marked induction was seen with immune complexes with approximately 5% of the PBM showing very bright IL-1β staining. A similar number of cells showed IL-1β staining following incubation with rGM-CSF but the staining was fairly dull. Gold did not inhibit IL-1β induction by these stimuli.

BIOASSAY

Figure 4.3 a) shows the results of IL-1 bioassay on supernatants from the 22 hour culture. This assay will measure any IL-1 secreted into the medium during culture. It can be seen that PHA/PMA induces large amounts of IL-1 secretion and gold has a small inhibitory effect. Although less than PHA/PMA, immune complexes also stimulate marked IL-1 secretion which is almost unaffected by gold. GM-CSF does not stimulate significant IL-1 secretion after 22 hours of culture.

Figure 4.3 b) shows the bioassay results on freeze-thawed cell lysates. This assay will detect cell-associated IL-1 bioactivity. Activity is seen in the cells stimulated with PHA/PMA with inhibition by gold. Activity is also seen following incubation with immune complexes but with no inhibitory effect of gold. GM-CSF induces cell-associated IL-1 activity with a marked inhibition by gold in the 1000 and 10,000U/ml doses. (The bioactivity of the cells treated with 500U & 5000U/ml GM-CSF in the presence of gold showed rather high variability in this experiment between the triplicate
Figure 4.3 (opposite) shows the IL-1 bioactivity in
a) supernatants and b) freeze-thaw lysates of PBM following 22
hour culture with different stimuli in the presence and absence
of 50μg/ml sodium aurothiomalate. These results are from the
same experiment as those in figure 4.1
Figure 4.3

a) Culture supernatants

b) Cell lysates
samples so that interpretation of the effect of gold in these 2 samples is difficult. Marked inhibition with these doses was seen in other experiments).

4.5 Discussion

These experiments were carried out to investigate whether immune complexes or GM-CSF are potential in vivo mediators of the cell-associated IL-1α identified in the blood of patients with RA. It is not known whether different stimuli induce different relative production of the two subtypes IL-1α and IL-1β. In the peripheral blood from patients with RA only cell-associated IL-1α was demonstrated suggesting in RA blood there is preferential induction of IL-1α or IL-1β production is selectively inhibited. Any mediator playing a significant role in this induction in vivo is likely to have a preferential effect on IL-1α which should be demonstrable in vitro.

The results of these experiments confirm previous studies that IL-1 production by mononuclear cells can be induced by both immune complexes and GM-CSF in vitro (Chantry et al, 1989; Sisson et al, 1988). The results also demonstrate that the relative production of the two subtypes of IL-1 may be determined by the stimulus. PHA/PMA induced the production of both IL-1α and IL-1β. Bright cellular IL-1α and IL-1β staining was seen following culture in the presence of PHA/PMA. Although the intensity of fluorescence reflects the amount of IL-1
present, this is very subjective and the bioassay results provide more quantitative data on total IL-1 production. However interpretation of the bioassay results alone may be difficult as IL-6 is also active in this system. IL-1β is known to be predominantly secreted in contrast to IL-1α which may remain cell-associated and active in this form (Hazuda et al, 1988). Therefore the finding of most IL-1-like bioactivity in the culture supernatant following PHA/PMA stimulation is compatible with previous reports that significantly more IL-1β than IL-1α (ratio 10:1) is produced by PBM following stimulation with mitogen.

Immune complexes induced bright IL-1β staining and weak IL-1α staining. IL-1 bioactivity was seen in both culture supernatants and cell lysates. This was in contrast to the staining seen following incubation of the PBM with different concentrations of rGMCSF. rGM-CSF induced bright granular IL-1α staining in many PBM, with the percentage of cells showing positive staining following a dose dependent increase. Weak IL-1β staining was seen. The bioactivity was almost entirely confined to the cell lysates, compatible with the induction of IL-1α, the cell-associated form. Therefore the pattern of staining and preferential induction of IL-1α by GM-CSF mirrors the finding in RA blood and suggests a possible mechanism for the in vivo induction of the cell-associated IL-1α found in RA.

Gold salts are known to inhibit the production of total IL-1 bioactivity in vitro. Gold salts have also been shown to
inhibit the development of macrophage and granulocyte colonies in vitro and in vivo at concentrations as low as $10^7-10^8\text{M}$ suggesting an effect on GM-CSF activity (Hamilton et al, 1985). The results from the fluorescent experiments show that gold has a profound inhibitory effect on the production of IL-1α, particularly following induction by GM-CSF, but very little effect on the production of IL-1β. The cell lysate IL-1 bioactivity following GM-CSF induction is also markedly inhibited by gold. This inhibitory action of gold appears to be greater for production of the IL-1α subtype. This has not been previously reported and may be important in our further understanding of the mechanism by which gold exerts in therapeutic effect in RA.

4.6 Conclusions

The preferential induction of IL-1α by GM-CSF, the pattern of staining and the marked inhibition by gold are consistent with the findings in RA blood. The results suggest a possible mechanism by which the cell-associated IL-1α demonstrated in RA blood may be induced in vivo and inhibited by gold therapy. The hypothesis that GM-CSF induces cell-associated IL-1 in RA blood is discussed in the next chapter. Obviously the situation in vivo may be considerably more complicated with synergy amongst many different cytokines eg IFNγ, TNFα and a detailed in vitro study of other mediators and combinations would be required to address this fully. However if a cytokine
can be identified which plays a major role in the induction of pathogenic features, it may be possible to inhibit the action of this cytokine (eg anti GM-CSF) and downregulate the damaging inflammatory process occurring in RA.
CHAPTER 5

CONCLUSIONS

5.1 Discussion of results

Cytokines play a major role in inflammation, probably both in the normal immune response to foreign antigens and in the pathological immune reaction seen in diseases such as RA. Cytokines are small, often unstable proteins and the study of these molecules has been hampered by difficulties with assays and the problems of interpreting results following in vitro culture. This thesis outlines the development of a new method to identify both cell-associated IL-1α and IL-1β at the single cell level; the application of this method to study normal PBM activated in vitro and freshly isolated PBM from normals, RA, OA, AS and sarcoidosis patients; the study of possible in vivo inducers of cell-associated IL-1α; and the action of gold in RA.

The studies on normal PBM activated in vitro demonstrate the protein kinetics of cell-associated IL-1α and IL-1β. These are as expected from mRNA data and bioassay data on secreted IL-1. The most interesting observation in normal PBM activated with mitogen in vitro is the finding that IL-1α and IL-1β are produced by two distinct cell populations. This has not been previously reported. The two populations are both class II positive mononuclear phagocytes. They are unlikely to be polymorphonuclear cells as the cells did not express CD11b
(present on granulocytes) and did not show the typical morphology. Also polymorphs die rapidly in culture under normal conditions and should be removed by Ficoll separation. The IL-1α positive cells phagocytosed significantly more latex beads than the IL-1β positive cells and this may reflect a greater degree of activation. The IL-1α positive cells do not bear the CD11b marker whereas the IL-1β positive cells do. This is surprising as it is likely that the two populations are subsets of blood monocytes. It is known that under certain circumstances blood monocytes can lose characteristic surface markers on activation (Jayaram et al, 1989) and the IL-1α positive cells may have lost CD11. However this is speculative only and full phenotyping of the cells with double staining and FACS analysis (when sufficient quantities of monoclonal antibodies become available for direct conjugation) will clarify the cells types. The secretion of specific cytokines by different sub-populations of monocytes has also been suggested recently by another group with the finding that TNFα and IL-1β are produced by different populations from PBM separated on Percoll gradients (Hermann et al, 1989). It remains unclear why the two forms, IL-1α and IL-1β have been conserved as they have identical biological activities. The findings in normal blood activated in vitro would support the hypothesis that IL-1α and IL-1β are produced by distinct subsets of monocytes with specialised functions in the immune response and that the two molecules have different cellular localisations for specific functions i.e. surface IL-1α for T cell activation and secreted IL-1β for effects on non-immune cells.
Cell-associated IL-1α in RA peripheral blood has not been previously demonstrated and therefore was studied in detail. RA is a systemic disease, although the joints are the major site of damage. Immune abnormalities are therefore likely to be present in the peripheral blood and may play a pathogenic role. The cells showing bright IL-1α staining have the same phenotypic characteristics as the IL-1α staining cells seen in normal peripheral blood following activation in vitro. These may represent a specialised sub-population of monocytes which produce IL-1α rather than IL-1β. Membrane associated IL-1α is thought to be important in T cell activation (Hurme et al, 1987) and these cells may play an important role in this. The reduced numbers of these IL-1α staining cells in RA patients on gold suggest that IL-1α production may be modified by gold in vivo. This is of particular interest as the therapeutic action of gold salts has been suggested to occur via the inhibition of IL-1 production which is known to occur in vitro (Remvig et al, 1988). Very few IL-1α staining cells were found in unactivated normal blood or blood from patients with OA, AS and sarcoidosis. No IL-1β staining was seen in normals, RA, OA, or AS. However some IL-1β staining was seen in the blood of patients with active sarcoidosis. This may play a role in the sarcoid disease process and should be investigated further.

If IL-1α and IL-1β are produced by separate cell populations with specific functions, the two subtypes may be regulated by different molecules. The finding of cell-associated IL-1α only in the blood of patients with RA suggests that this may be under the control of a mediator with a preferential effect on
IL-1α production. The results of the *in vitro* experiments in chapter 4 suggest that IL-1α and IL-1β may be preferentially produced in response to different stimuli. Immune complexes appear to induce both IL-1α and IL-1β but with a preferential induction of IL-1β and little inhibition by gold. rGM-CSF preferentially induces cell-associated IL-1α which is markedly inhibited by gold at a concentration similar to that found *in vivo* in patients receiving chrysotherapy for RA. Although many other molecules could be potential inducers of the cell-associated IL-1α demonstrated in the blood of RA patients, GM-CSF has been suggested to play an important role in RA (Alvaro-Gracia et al., 1989) and the preferential induction of granular IL-1α staining identical to that seen in RA blood with marked inhibition by gold would support this. Gold has been reported to inhibit granulocyte-myeloid colony formation at concentrations as low as $10^{-8} \text{M}$ (Hamilton et al., 1985) and this may also be via inhibition of GM-CSF (or M-CSF). Induction of cell-associated IL-1α in RA may occur in the bone marrow or in the peripheral blood. Bioassays and immunoassays for GM-CSF are becoming available and studies of GM-CSF levels in blood, bone marrow and synovial fluid of patients with RA on and off gold therapy will help to elucidate the role of GM-CSF. The *in vitro* results show that gold has a more profound effect on the production of IL-1α than IL-1β. This has not been previously reported. If gold exerts its therapeutic effect in RA via downregulation of IL-1 production this appears to be specific to the IL-1α subtype. This and the finding of cell-associated IL-1α in RA blood may have important implications for the development of new therapies. Compounds able to inhibit
synthesis or activity of the IL-1α subtype may be potential disease modifying agents in RA.

5.2 Hypothesis

Figure 5.1 shows a hypothesis for the involvement of IL-1 in the disease perpetuation and joint destruction in RA and a possible site of therapeutic action of gold salts. Following exposure to an antigen (eg. a virus), a normal immune response occurs with processing and presentation of this antigen to T cells. These T cells produce lymphokines, eg GM-CSF which "overspill" into the blood. These lymphokines (GM-CSF) act either at the bone marrow or peripheral blood level to stimulate a sub-population of monocytes to produce IL-1α which remains membrane bound. These cells then pass to the tissues (eg. joint) where they may behave as highly efficient antigen presenting cells. In individuals with the predisposing DR sequence these cells bind selected peptides which have homology with the original exogenous agent (eg. a connective tissue component explaining the predilection for the joint). Presentation of these self antigens and T cell activation occur which results in further GM-CSF production. GM-CSF in the peripheral blood, either from "overspill" from the joint or from blood lymphocytes (GM-CSF production can be directly stimulated by IL-1 (Hermann et al, 1988)) induces further cell-associated IL-1α production by bone marrow and blood monocytes and also upregulates DR and DQ expression which further
Figure 5.1

Hypothesis for disease perpetuation in RA

Bone marrow

IL-1α

Gold

MEDIATOR
eg GM-CSF

Peripheral blood

IFR/DQ expression

"overspill" into blood

IL-1β

TNFα

GM-CSF

Activ T cells

RhF — B cells

IL-1β

Fibroblasts
Collagenase

Cartilage damage
enhances the antigen presenting capabilities of the cells. IL-1β production (and TNFα) is stimulated in the joint by the cell-associated IL-1α (IL-1 is known to stimulate its own production (Dinarello et al, 1987) although this is concentration dependent (Manson et al, 1989), by other cytokines and by Rh Factor immune complexes produced by B cells in response to lymphokines eg IL-6. This IL-1β has a direct effect on connective tissue and results in cartilage and bone degradation. Thus a vicious cycle is set up which results in immune activation, systemic effects, synovitis and joint destruction. Interference with a critical part of this cycle may downregulate the destructive inflammatory response. RA patients on gold therapy have significantly fewer IL-1α staining cells in their peripheral blood and gold salts appear to inhibit GM-CSF induced IL-1α production in vitro. This suggests that gold may exert its therapeutic via inhibition of GM-CSF induced cell-associated IL-1α. This hypothesis could be tested by studies of GM-CSF levels in joint, blood and bone marrow from RA patients on and off gold therapy, by longitudinal studies of IL-1 and GM-CSF in patients before and during gold therapy and by the study of GM-CSF in animal models. Unfortunately time does not permit these to be done for this thesis. If this hypothesis is correct it suggests many novel approaches to therapy in RA to interfere with different parts of this cycle. eg anti-GM-CSF, soluble IL-1 receptor, anti-IL-1α, joint targeted anti-IL-1β, anti-DQ or perhaps ideally a combination of sub-optimal doses of different inhibitors which might synergise to interfere with this disease cycle but not with the normal immune response.
5.3 Final Conclusions

This thesis describes the development and application of a new method for studying IL-1. Progress in immunopathology is dependent on new methods for studying the complex abnormalities in disease states and this method allows the study of cell-associated IL-1α and IL-1β. Using this method this thesis demonstrates cell-associated IL-1α and IL-1β in normal peripheral blood activated in vitro with mitogens and shows that the two subtypes are produced by distinct cell populations. This finding may be important in our understanding of the fine regulation of the immune system and the imbalances which occur in disease. Bright cell-associated IL-1α is demonstrated in the blood of RA patients, correlating with disease activity and modified by gold therapy. This IL-1α may play an important role in disease pathogenesis. It may be a critical mediator in a vicious cycle leading to pathological inflammation in the rheumatoid joint. GM-CSF may induce this IL-1α and this is a possible site of action of gold in vivo.

The cytokine network is very complicated and finely controlled. It is not fully understood in health and it is even less understood in disease states. This thesis barely scratches at the surface of this problem and like all studies leads to many more questions and experiments for the future. It does however describe a new method for the study of cell-associated IL-1α and IL-1β and may shed a minute piece of light on the complicated world of interleukin 1 in RA suggesting possible avenues for therapeutic developments in this crippling disease.
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BARKLEY, D., FELDMANN, M. & MAINI, RN. (In preparation) Sodium aurothiomalate inhibits GM-CSF induced IL-1 production - possible mechanism of therapeutic action in rheumatoid arthritis.

Abstracts (Presentations):


BARKLEY, D., FELDMANN, M. & MAINI, R.N. (1989) Cells with dendritic morphology and bright IL-1α staining circulate in the peripheral blood of patients with rheumatoid arthritis.
The detection by immunofluorescence of distinct cell populations producing interleukin-1α and interleukin-1β in activated human peripheral blood

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An immunofluorescent staining method using specific monoclonal antibodies was used to detect IL-1α and IL-1β in individual cells in stimulated human peripheral blood. No staining was seen in unstimulated cells but intense, maximal staining of approximately 5% of the cells was seen 20–24 h after activation with PHA/PMA. The large irregularly shaped stained cells were surrounded by smaller unstained cells with lymphocyte-like morphology. By 44 h post activation a few cells only showed weak staining. The staining pattern was different for the two molecules studied, with a granular pattern for IL-1α staining and diffuse cytoplasmic staining for IL-1β. Staining post-activation could be abolished by preincubation of the monoclonal antibody with the appropriate recombinant IL-1, but not by pre-incubation with the other IL-1 type.

When both anti-IL-1α and anti-IL-1β were used together two populations of cells were identified; one had the granular staining as seen with anti-IL-1α alone and the other had the diffuse staining pattern as seen with anti-IL-1β. The percentage of cells showing bright staining with anti-IL-1α and anti-IL-1β together was approximately equal to the sum of the percentage of cells staining for IL-1α or IL-1β alone. This study demonstrates a method for the detection of individual IL-1α- and IL-1β- producing cells and suggests that in activated human peripheral blood IL-1α and IL-1β are produced by separate populations of cells.

Key words: Immunofluorescence; Monoclonal antibody; Interleukin-1

Introduction

Interleukin-1 (IL-1) has multiple biological activities and appears to be a factor of major importance in the regulation of both immune and non-immune responses (Durum et al., 1985). The major sources of IL-1 are thought to be macrophages and monocytes although it can be produced by almost all cell types including T and B lymphocytes, fibroblasts, natural killer cells, skin keratinocytes, brain astrocytes, epithelial cells, mesangial cells and vascular tissues (Scala et al., 1984; Dinarello, 1986; Libby et al., 1986; Tartakovsky et al., 1986; Bell et al., 1987). Monocyte-derived IL-1 plays a key role in T cell activation and in the triggering of B cell activities (Gery and Waksman, 1972; Giri et al., 1984).
At least two monocyte-derived IL-1 species have been identified and sequenced both in man and mouse (March et al., 1985; Oppenheim et al., 1986). These molecules, termed IL-1α and IL-1β have 28% amino acid homology. They appear to have the same biological activities and bind to the same receptor (Kilian et al., 1986; Rupp et al., 1986). It is not yet clear why these two distinct types of IL-1 have been conserved although it is known that certain cells produce more of one type than another, e.g., human umbilical vein endothelial cells produce mostly IL-1α mRNA following LPS stimulation (Stern et al., 1985). There has been considerable interest in the production and action of IL-1. It is not known whether IL-1α and IL-1β are produced by the same cell following activation of peripheral blood as there has been a lack of specific methods to identify IL-1α- and IL-1β-producing cells at the single cell level. The production of IL-1α and β by activated peripheral blood cells has been studied at the mRNA level using crude cell extracts (Demczuk et al., 1987) and studies at the protein level have focused on bioassays and activity in culture supernatants, (Matsushima et al., 1986; Demczuk et al., 1987; Goto et al., 1987). These methods do not give any information about the cells producing the two IL-1 subtypes. Two previous studies have demonstrated individual cells in suspension staining for IL-1β but in these experiments polyclonal antisera were used which may present specificity problems, and IL-1α was not studied (Bayne et al., 1986; Singer et al., 1988). The recent production and characterisation of monoclonal antibodies against both distinct IL-1 molecules (e.g., Kasahara et al., 1987; Kenney et al., 1987) has enabled us to demonstrate individual cells separately producing IL-1α or IL-1β in activated peripheral blood mononuclear cells by an indirect immunofluorescent staining method.

Materials and methods

Separation of peripheral blood mononuclear cells (PBMs)

Peripheral blood was taken from four healthy volunteers into sterile bottles containing preservative-free heparin (10 U/ml of blood) and diluted 1/2 with Hanks' balanced salts solution (HBSS) (Gibco Europe, Paisley, Scotland, U.K.). The diluted blood was laid on a density gradient (Ficoll-Paque, Pharmacia, Uppsala, Sweden) at a ratio of 2:1 respectively and centrifuged at 400 x g for 30 min. PBMs were harvested from the interface, washed twice in Dulbecco's balanced salt solution (DBSS) containing 2% heat inactivated fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland, U.K.) and resuspended in RPMI 1640 medium (Flow Laboratories). The number of viable cells was determined by trypan blue dye exclusion.

Activation of cells

PBMs (10⁶/ml) were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS in the presence of 1 µg/ml of phytohaemagglutinin (PHA) (Wellcome Laboratories, Beckenham, U.K.) and 50 ng/ml of 12-myristate 13-acetate (PMA) (Sigma Chemical Company, Poole, Dorset, U.K.) in flat-bottomed 24-well culture plates (Nunc, Gibco Europe). Incubations were carried out for 4, 16, 20, 24, 28, and 44 h. At the end of the incubation, cells were washed three times in phosphate-buffered saline pH 7.2, recounted by trypan blue exclusion and resuspended in phosphate-buffered saline (PBS) at a density of 2.5 x 10⁵/ml.

Cytocentrifuge preparations

200 µl of cell suspension (5 x 10⁴ cells) was centrifuged for 5 min at 500 rpm onto individual microscope slides using a cytocentrifuge (CytoSpin, Shandon Southern Products, Runcorn, Cheshire, U.K.). The slides were then air-dried for 15 min, fixed for 10 min in a solution of 50% methanol and 50% acetone at −70°C (cooled in a solid CO₂ and methanol bath). Following fixation the slides were air-dried for 30 min and stored at −20°C.

Immunofluorescent staining:

Following removal from storage, the slides were air-dried for 5 min and rehydrated in PBS for 10 min. Non-specific binding was blocked by incubating the slides in 1/5 normal goat serum (NGS) (Sigma Chemical Company). The primary antibody was applied to the cells for 1 h. After washing three times in PBS, goat anti-mouse IgG
(whole molecule) conjugated to fluorescein isothiocyanate (FITC) (Sigma Chemical Company) diluted 1/50 in 2% NGS was applied to the cells for 30 min. Following a further three washes in PBS and two washes in distilled water the slides were mounted in 9:1 glycerol/PBS containing 1 mg/ml p-phenylene diamine to retard fading.

**Antibodies used**

Mouse monoclonal anti-IL-1α was a kind gift from M. Okazaki of Dainippon Pharmaceuticals, Japan. Mouse monoclonal anti-IL-1β was a kind gift from K. Matsushima, Laboratory of Molecular Immunoregulation, N.C.I., Frederick, MD 21701. Both antibodies were used at a 1/50 dilution in PBS. A control antibody OX-1 was kindly donated by D. Mason, Department of Cellular Immunology, William Dunn School of Pathology, Oxford. This mouse anti-rat allotype was used undiluted in place of the primary antibody at each time point to check for non-specific binding. All antibodies used were of the IgG1 subclass.

**Blocking of fluorescent staining with excess antigen**

To determine the specificity of the staining, the diluted antibody was preincubated overnight at 4°C (i) alone, (ii) in the presence of 10 μg/ml of the relevant recombinant IL-1 species, i.e., anti-IL-1α with rIL-1α, (iii) in the presence of 10 μg/ml of the irrelevant IL-1 species, i.e., anti-IL-1α with rIL-1β. Recombinant IL-1α was kindly donated by P. Lomedico, Hoffmann-La Roche, Nutley, New Jersey and recombinant IL-1β by S. Gillis, Immunex, Seattle, Washington.

**Microscopy and quantitation**

Slides were viewed using a Leitz Laborlux 12 fluorescent microscope. Positive and negative cells were counted using a ×50 water immersion lens. 200 cells were counted three times for each slide and the mean calculated.

**Results**

The same pattern and time course of staining was seen in the cells from all four normal individuals studied.

**Cytoplasmic staining for IL-1α** (Table I, Fig. 1)

Many brightly stained cells were seen 16–24 h after activation with PHA/PMA, with a maximum intensity of staining seen at 24 h post-activation. The staining had a granular pattern, evenly distributed throughout the cell (Fig. 2a). The stained cells were surrounded by clusters of unstained lymphocytes. The stained cells were of irregular shape and were larger (~2X) than the surrounding lymphocytes. Cells with this morphology also stained with HLA-DR antibodies (L243) but did not stain with a monocyte/macrophage marker (CD11b) or T and B cell markers (CD3, CD21) (data not shown). No staining was seen in unstimulated cells or in cells 4 h post-activation. By 44 h post-activation the number of positive cells and the intensity of staining was much less.

**TABLE I**

**KINETICS OF IL-1α AND IL-1β STAINING**

<table>
<thead>
<tr>
<th>Time post-activation</th>
<th>% of cells positive</th>
<th>Donor Anti-IL-1α alone</th>
<th>Anti-IL-1β alone</th>
<th>Anti-IL-1α + anti-IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
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<td>1</td>
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<td>4.0</td>
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<td>7.4</td>
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<td>2.5</td>
<td>3.0</td>
<td>5.7</td>
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<td>3.0</td>
<td>4.0</td>
<td>6.9</td>
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Kinetics of IL-1α and IL-1β staining

Fig. 1. Fig. 1 shows the kinetics of IL-1α and IL-1β staining of PBMs following activation with PHA/PMA. The values plotted are the means of four individuals—see Table 1 for individual data. It shows that the percentage of cells stained with anti-IL-1α and anti-IL-1β together is approximately equal to the sum of the percentage of cells stained with either antibody alone at each time point.

Cytoplasmic staining for IL-1β (Table I, Fig. 1)

Many brightly stained cells were seen 16–24 h after activation with a maximum intensity of staining seen at 20 h. The cells were of similar size to those staining for IL-1α but the pattern of staining was more homogeneous and evenly distributed throughout the cytoplasm of the cells (Fig. 2b). As with the IL-1α staining the cells were surrounded by lymphocytes. The cells had monocytic morphology and cells with this morphology were HLA-DR and CD11b positive (data not shown). No staining was seen in unstimulated cells or in cells 4 h post-activation. By 44 h post-activation very few cells showed any positive staining.

Staining with anti-IL-1α and IL-1β combined (Table I, Fig. 1)

At each time point, both antibodies were applied to the same slide as the primary layer. The percentage of cells staining was approximately equal to the sum of the percentage of cells staining for IL-1α or IL-1β alone assessed in separate experiments at the same time point. Two populations of cells were identified; one population had the granular staining seen with anti-IL-1α alone and one population had the diffuse staining pattern seen with anti-IL-1β alone.

Specificity tests

(a) Control monoclonal antibody. No positive staining was seen at any time point post-activation with OX-1 as the primary antibody or with FITC conjugated anti-mouse IgG alone.

(b) Blocking experiments (Fig. 3). Almost complete abolition of staining was seen following incubation of anti-IL-1α with 10 μg/ml rIL-1α but no inhibition was seen following preincuba-

Fig. 2. Fig. 2 shows IL-1α and IL-1β staining cells in peripheral blood 24 h post-activation with PHA/PMA. a: Granular IL-1α staining of large irregularly shaped cells. b: IL-1β staining of cells with monocytic morphology. (In both cases the original magnification is ×600.)
Blocking of staining with excess antigen

**Fig. 3.** Fig. 3 shows the inhibition of staining with excess antigen. It shows that staining of PBMs with monoclonal anti-IL-1α could be blocked by pre-incubating the antibody with rIL-1α but not with rIL-1β. Staining with monoclonal anti-IL-1β could be blocked by pre-incubating the antibody with rIL-1β but not with rIL-1α.

Discussion

This study shows that using specific monoclonal antibodies and an indirect immunofluorescent method IL-1α and IL-1β can be demonstrated within individual PBMs following stimulation with PHA/PMA. The observed immunofluorescent staining is specific as it can be blocked by preincubation of the antibody with the relevant recombinant IL-1, is not present in unstimulated cells, and follows a time course anticipated from data on IL-1 mRNA expression following activation and IL-1 production based on bioassay (Demczuk et al., 1987).

This is the first report in which monoclonal antibodies against IL-1α and -β have been used to identify individual cells containing these molecules. This method offers advantages with regard to specificity compared with methods based on polyclonal antisera (Bayne et al., 1986). Previous experiments on IL-1α and -β production have not determined whether the molecules are produced by the same cell or by different cells as they have studied crude cell extracts or culture supernatants. Our results suggest that in activated normal peripheral blood IL-1α and IL-1β are produced by separate populations of cells. The cells staining for IL-1β appeared morphologically to be monocytes, which are CD11b and HLA-DR positive. The cells staining for IL-1α were irregularly shaped, possibly due to disruption during centrifugation. Cells with this morphology were also HLA-DR positive but did not stain with CD11b or with T or B cell markers. These IL-1α staining cells might represent blood dendritic cells.

The different patterns of staining for the two IL-1 molecules raises the question whether these molecules may be exported from the cell in different ways. It remains unclear how these molecules are exported. It is known that IL-1α and IL-1β are produced as 31 kDa inactive precursors which are then cleaved by undefined mechanisms to the active 18 kDa C-terminal fragments (March et al., 1985). However the apparent lack of a hydrophobic leader sequence within the precursor molecule suggests that IL-1 may not be exported from cells in a manner typical for secretory proteins (Furutani et al., 1985). The processing of IL-1 precursors appears to occur in conjunction with externalisation since only the 31 kDa precursors are found intracellularly whereas the mature 18 kDa form is found extracellularly. The granular staining pattern seen with anti-IL-1α staining might be compatible with passage through the Golgi apparatus or might represent surface stain-
ing – it is known that IL-1α may be observed on the cell surface (Conlon et al., 1987). The diffuse pattern seen with anti-IL-1β is in keeping with the observation that IL-1β is localised in the cytoplasmic ground substance but that the pathway of IL-1β secretion does not involve passage through the Golgi apparatus and ER (Singer et al., 1988). Previous data have suggested that IL-1α and IL-1β show similar kinetics of production in response to stimulation (Demczuk et al., 1987). This study suggests that IL-1β production may reach a peak earlier than IL-1α. IL-1 plays an important role in immune regulation and the immunocytochemical detection of IL-1α and IL-1β by this method may allow the detection of these molecules in different cell types and allow simultaneous surface phenotyping of these cells. It may also be used for the in situ localisation of IL-1-producing cells in diseased cells and tissue. The observation that IL-1α and IL-1β are not produced by the same cell but are produced by two different cell populations in normal peripheral blood is important for our understanding of the fine regulation of the immune response by cells and their mediators in health and disease.

Acknowledgements

We wish to thank Christina Brown and Dr. Christine Plater-Zyberk for help and advice. DB is an Arthritis and Rheumatism Council research fellow.

We would like to thank the Arthritis and Rheumatism Council for their support.

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Cells with dendritic morphology and bright interleukin-1α staining circulate in the blood of patients with rheumatoid arthritis


(accepted for publication 31 October 1989)

SUMMARY

Freshly isolated peripheral blood mononuclear cells (PBMC) from 10 healthy volunteers, 28 patients with rheumatoid arthritis (RA), eight patients with osteoarthritis, and five patients with ankylosing spondylitis were examined for interleukin-1α (IL-1α) and interleukin-1β (IL-1β) production using monoclonal antibodies and an indirect immunofluorescent method. In freshly isolated PBMC from healthy controls very few cells were stained for either IL-1 type. All 20 RA patients who were not receiving parenteral gold therapy had PBMC staining for IL-1α. In these patients, up to 7.5% of PBMC showed bright IL-1α staining (range 1.2–7.5%). No IL-1β staining was seen. These IL-1α-staining cells had a dendritic morphology and the percentage of cells staining correlated well with levels of C-reactive protein, an index of disease activity in these RA patients. Significantly fewer IL-1α-staining cells were present in the peripheral blood of RA patients receiving gold therapy and in the blood of patients with osteoarthritis and ankylosing spondylitis. These IL-1α-containing cells, circulating in the blood of RA patients and correlating with disease activity have not been previously described. These results support the idea that IL-1α plays an important role in the pathogenesis of rheumatoid inflammation.

Keywords: rheumatoid arthritis, interleukin-1α, indirect immunofluorescence, dendritic cells, gold therapy

INTRODUCTION

Rheumatoid arthritis (RA) is a disorder of unknown aetiology with autoimmune features. It is a systemic disease characterized by hypercellularity of the synovial tissue and degradation of cartilage and bone in the inflamed joint. The biological activities of the pro-inflammatory cytokine interleukin-1 (IL-1) suggest that it may mediate many of the systemic effects and destruction of the joints seen in RA (reviewed by Miller & Dinarello, 1987). There is accumulating evidence for the involvement of IL-1 in RA synovitis with raised levels of IL-1 found in peripheral blood, synovial fluids and synovial membrane culture supernatants from patients with RA compared with controls (Wood et al., 1983; Danis et al., 1987; Goto et al., 1987; Bhardwaj et al., 1988). IL-1 is known to exist in two molecular forms, IL-1α and IL-1β. It remains unclear why these two distinct subtypes exist as despite only 26% amino acid homology they act on the same receptor and have identical biological activities (Rupp et al., 1986). Recent evidence suggests that IL-1α may be functionally active in its cell associated 33 kD form as well as in its secreted 17 kD form, whereas IL-1β exerts its effects only in the secreted and processed 17 kD form (Conlon et al., 1987). IL-1α has not been studied in detail in RA because of a lack of methods for studying cell associated IL-1 and because in most assays of blood or monocytic cells IL-1β is the dominant form. Many studies have measured total secreted IL-1 by bioassay (e.g. Danis et al., 1987; Hopkins, Humphreys & Jayson, 1988) or have used an immunoassay specific for the predominant form, IL-1β (Eastgate et al., 1988). Our group has shown high IL-1α mRNA in cultured mononuclear cells from RA joints, suggesting that this cell associated form may play an important role in rheumatoid inflammation (Buchan et al., 1988). In order to study this we have developed an immunocytochemical method using specific monoclonal antibodies for identifying cell-associated IL-1α and IL-1β. With this method, IL-1-staining cells were only detectable in low numbers in freshly isolated peripheral blood mononuclear cells (PBMC) from healthy individuals but staining for both IL-1α and IL-1β was seen following activation with mitogen in vitro (Barkley, Feldmann & Maini, 1989). We have now used this method to study cell-associated IL-1α and IL-1β in freshly isolated PBMC from patients with RA, osteoarthritis (OA), ankylosing spondylitis (AS) and healthy volunteers.

Correspondence: Dr D. E. H. Barkley, Department of Clinical Immunology, Kennedy Institute of Rheumatology, 6 Bute Gardens, London W6 7DW, England.
Table 1. Clinical features of patients with rheumatoid arthritis

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<th>Treatment</th>
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<th>Rheumatoid factor</th>
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<td>2 y</td>
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<td>11 y</td>
<td>Gold</td>
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NSAID, non-steroidal anti-inflammatory drugs; D-P, D-penicillamine; MTX, methotrexate; Pred., prednisolone; Salazop., Salazopyrine; Aza., Azathioprine.

PATIENTS AND METHODS

Controls
Peripheral blood was taken from 10 healthy volunteers (seven women, three men). The median age was 40 years (range 25-71).

Patients
Rheumatoid arthritis. Peripheral blood was taken from 28 patients with classical RA (23 women, five men). These were randomly selected from RA patients attending a Rheumatology Outpatient Clinic (Table 1). Two were receiving no medication, seven were receiving NSAIDs alone and 11 were receiving various disease-modifying drugs (DMDs) including penicillamine, salazopyrine, methotrexate, azathioprine and prednisolone. Eight patients were receiving parenteral gold therapy. Median age was 63 years (range 34-82); 25/28 had a positive rheumatoid factor.

Osteoarthritis. Peripheral blood was taken from eight patients with OA attending the Outpatients Clinic (five women, three men; two with localized OA, six with generalized OA). Four were receiving no medication and four were receiving NSAIDs. Median age was 57 years (range 41-79).

Ankylosing spondylitis. Peripheral blood was taken from five patients with AS attending the Clinic (four men, one woman). Median age was 45 years (range 30-50).

Separation of PBMC and preparation of cytospins
Peripheral venous blood was collected from patients and volunteers into preservative-free heparin tubes (10 U/ml). Following the method previously described (Barkley et al., 1989), PBMC were separated from freshly obtained blood and cytospins of unactivated cells made as quickly as possible. Briefly, after separation on a Ficoll–Paque density gradient (Pharmacia, Uppsala, Sweden) PBMC were washed, the number of viable cells was determined by trypan-blue dye exclusion and the cells were resuspended in phosphate-buffered saline (PBS). Cells (5 x 10⁴) were centrifuged onto individual microscope slides using a cytocentrifuge (Cytospin, Shandon Southern Products, Runcorn, UK). The cells were fixed for 10 min in 50% methanol and 50% acetone at −70°C and the slides were stored at −20°C.

Immunofluorescent staining
Following removal from storage the cytospins were stained using monoclonal antibodies against the two IL-1 subtypes, IL-1α and IL-1β and an indirect immunofluorescent method described previously (Barkley et al., 1989).

Antibodies used
Mouse anti-IL-1α (No. 1190) was a kind gift from M. Okasaki of Dainippon Pharmaceuticals, Japan. Two mouse monoclonal antibodies against IL-1β were used and gave identical staining
patterns. One was a kind gift from K. Matsushima, Laboratory of Molecular Immunoregulation, N.C.I., MD. The other (ANOC 205) was a kind gift from Y. Hirai of Otsuka Pharmaceuticals, Japan. Both anti IL-1α and anti IL-1β antibodies were used at a 1/50 dilution in PBS. A control antibody OX-1 was kindly donated by D. Mason, Department of Cellular Immunology, William Dunn School of Pathology, Oxford. This mouse anti-rat allotypic monoclonal antibody (supernatant) was used undiluted in place of the primary antibody to check for non-specific binding. All antibodies were of the IgG1 subclass.

Quantification

Cells were viewed under a Leitz Laborlux 12 fluorescent microscope with a ×50 water-immersion lens. Positive and negative cells were counted blind so that results could be expressed as % of total PBMC showing positive staining. For each individual studied, the cells were stained on three different occasions and on each occasion at least 300 cells were counted and the mean calculated.

Specificity tests

Positive control. PBMC from healthy individuals were cultured in the presence of 1 μg/ml of phytohaemagglutinin (PHA) (Wellcome Laboratories, Beckenham, UK) and 50 ng/ml of 12-myristate 13-acetate (PMA) (Sigma Chemical Company, Poole, UK) for 22 h as described (Barkley et al., 1989). At the end of the incubation cells were washed three times in PBS, counted by trypan-blue dye exclusion and cytocin preparations made as above.

Negative control. The antibody OX-1 was used as described above on all cells studied.

Blocking with excess antigen. Anti-IL-1α and anti-IL-1β, diluted to their working dilutions were incubated overnight at 4°C either alone or in the presence of either recombinant IL-1α or recombinant IL-1β. The antibody was then used for immunofluorescent staining as above. Recombinant IL-1α was kindly donated by P. Lomedico, Hoffmann-La Roche, Nutley, NJ, and recombinant IL-1β by S. Gillis, Immunex, Seattle, Washington.

Statistical analysis

The Mann–Whitney two-sample rank test was used.

RESULTS

Freshly isolated PBMC

Unactivated PBMC from 10 healthy controls showed few cells only staining with anti IL-1α (median 0.1%; range 0–2.3%; Fig. 1).

In unactivated PBMC from 28 RA patients significantly more cells showed bright IL-1α staining when compared with controls (median 2.7%; range 0–7.5%; P < 0.01, Fig. 1). The stained cells had a similar dendritic morphology and granular pattern of staining to the IL-1α-staining cells in activated normal PBMC (Figs 2 and 3). No staining of RA PBMC was seen with anti-IL-1β. Figure 4 shows that the eight RA patients receiving parenteral gold therapy had significantly fewer IL-1α-positive cells (median 0.7%; range 0–1.6%) than the 20 RA patients receiving other DMDs or NSAIDs alone (median 4.3; range 1.2–7.5%; P < 0.01). In the latter group of RA patients the
showed a granular pattern of staining. The cells staining with anti IL-1\(\beta\) had a monocytic morphology (Fig. 2).

**Negative control.** No staining was seen with OX-1, an irrelevant antibody of the same IgG subclass (IgG1) used in place of the primary IL-1 monoclonal antibody.

**Blocking with excess antigen.** IL-1\(\alpha\) staining of unactivated cells from RA patients and in vitro activated cells from healthy volunteers was completely abolished by pre-incubation of the antibody overnight with 10 \(\mu\)g/ml of recombinant IL-1\(\alpha\). IL-1\(\alpha\) staining was unaffected by pre-incubation of the antibody overnight alone or with 10 \(\mu\)g/ml of recombinant IL-1\(\beta\). Pre-incubation of anti IL-1\(\beta\) with 10 \(\mu\)g/ml of recombinant IL-1\(\beta\) resulted in marked reduction of IL-1\(\beta\) staining intensity and % of positive cells (in vitro activated cells from healthy volunteers). IL-1\(\beta\) staining was unaffected by pre-incubation of the antibody with 10 \(\mu\)g/ml IL-1\(\alpha\).

**DISCUSSION**

IL-1 is thought to play a key role in RA. This is because IL-1 levels are known to be increased in the synovial fluid of patients with RA (Nour, Panayi & Goodman, 1984; Miyasaka et al., 1986; Bhardwaj et al., 1988) and animal studies suggest that IL-1 mediates cartilage and bone destruction (Henderson & Pettipher, 1989). RA is known to be a systemic disease; however, studies on IL-1 in the peripheral blood of patients with RA have been limited. Raised IL-1\(\beta\) levels correlating with disease activity have been found using an immunoassay on RA plasma and a phenol extraction procedure to separate IL-1 from its binding proteins (Eastgate et al., 1988). In another study, using a bioassay, total IL-1 production by peripheral blood monocytes from RA patients was found to be increased when compared with healthy individuals or with patients with osteoarthritis or patients with RA receiving gold therapy (Danis et al., 1987).

Peripheral blood IL-1\(\alpha\) has not been studied in RA patients.
Fig. 3. Immunofluorescence staining of unactivated peripheral blood mononuclear cells (PBMC) from a rheumatoid arthritis patient with anti-IL-1α. Granular IL-1α staining of a cell with dendritic morphology. Magnification x 390.

Fig. 4. Percentage of IL-1α-staining cells in freshly isolated peripheral blood mononuclear cells (PBMC) from eight rheumatoid arthritis (RA) patients receiving parental gold therapy and 20 RA patients receiving other disease-modifying drugs, NSAIDs, or no therapy.

is disputed in the mouse (Koide & Steinman, 1987). Further studies on IL-1 production by dendritic cells have shown that synovial dendritic cells do not produce IL-1β (Bhardwaj et al., 1988) suggesting that the previously noted IL-1 bioactivity may be due to IL-1α or even possibly interleukin-6 (IL-6) which has similar effects. Our studies on normal blood stimulated by mitogens suggest that the source of IL-1β is blood monocytes. The lack of IL-1β staining in RA PBMC in this study despite its finding in other studies by immunoassay on plasma samples may be explained by IL-1β being the secreted form of IL-1 which does not remain cell-associated during the Ficoll PBMC separation procedure. It is also possible that IL-1β detected in peripheral blood in RA is derived from its site of production in joints.

The RA patients receiving parenteral gold therapy had significantly fewer IL-1α-stained cells. The mechanism of action
of gold as a DMD in RA is not understood. It is known to inhibit IL-1 production \textit{in vitro} (Remvig, Enk & Bligaard, 1988) and our observation in this study would support this occurring \textit{in vivo}. It has been suggested that the inhibition of IL-1 production may explain the therapeutic mechanism of gold and further understanding of this may assist in the development of novel DMDs for use in RA.

This study demonstrates cell-associated IL-1α but not IL-1β in the peripheral blood of patients with RA compared with controls. This has not been previously described and may be important in our understanding of the pathogenesis of rheumatoid inflammation. These IL-1α-staining cells in the circulation of RA patients could mediate some of the systemic features of the disease. They may be blood dendritic cells or a subpopulation of monocytes that migrate to the synovial membrane. In the joint they may be involved in self antigen presentation and the cartilage and bone destruction which characterise RA. The finding of these cells in RA, modified by parenteral gold therapy and correlating with disease activity is consistent with the involvement of IL-1, especially IL-1α in the pathogenesis of this disease.

ACKNOWLEDGMENTS

D.E.H.B. is an Arthritis & Rheumatism Council research fellow. We would like to thank the Arthritis and Rheumatism Council for their support.

REFERENCES


IL-1α staining cells in rheumatoid arthritis


APPENDIX 1
INCREASED EXPRESSION OF HLA-DQ ANTIGENS BY INTERSTITIAL CELLS AND ENDOTHELIUM IN THE SYNOVIAL MEMBRANE OF RHEUMATOID ARTHRITIS PATIENTS COMPARED WITH REACTIVE ARTHRITIS PATIENTS

DIANA BARKLEY, SIMON ALLARD, MARC FELDMANN, and RAVINDER N. MAINI

We investigated cellular phenotypes and expression of class II major histocompatibility complex antigens on endothelium and cellular infiltrates in synovium from patients with rheumatoid arthritis (RA) or reactive arthritis, using an indirect immunoperoxidase technique. The RA specimens showed synovial lining layer hypertrophy and several focal accumulations of lymphocytes, both of which were absent in the reactive arthritis synovium. The percentage of cells expressing monocyte/macrophage markers was significantly higher in RA specimens. The percentages of cells expressing B and T cell markers were similar in both diseases. There was no significant difference in the expression of HLA-DR or DP by endothelium in the 2 diseases, but a marked increase in expression of HLA-DQ by endothelium was observed in the RA synovium versus that from patients with reactive arthritis. This overexpression of HLA-DQ was also seen in the interstitial cells of RA patients compared with reactive arthritis patients. In the reactive arthritis synovium, a significant population of cells (30%) was noted to be HLA-DR positive, and negative for macrophage and lymphocyte markers. Some of these cells had a dendritic morphology. The coexpression of HLA-DQ and HLA-DR may play an important role in antigen presentation and disease chronicity in RA.

Rheumatoid arthritis (RA) and reactive arthritis are both inflammatory diseases characterized by synovitis. In RA the synovitis tends to be chronic, following a remitting/relapsing course and progressing to a destructive arthritis, whereas in reactive arthritis the synovitis tends to be self-limiting and does not progress to joint destruction. It is often not possible to differentiate between RA and reactive arthritis using conventional histologic techniques, which is surprising in view of the very different courses of the 2 diseases (1).

Several investigators have studied the immunohistology of RA in an attempt to find characteristics that may lead to clues about the chronic immune activation and cartilage destruction that are features of this disease (2-6). Increased HLA-DR expression is a feature of RA synovium and has been noted to be present on cells that constitutively express these molecules, e.g., macrophages (2,7,8), but it is also found on vascular endothelium (5,9,10), cells with a dendritic morphology (10-12), and on T cells (3). However, these results are not specific to RA: Similar findings in other forms of synovitis (13,14) and in normal synovium (15) have been reported. The immunohistology of reactive arthritis has not been reported in detail previously, although small numbers of reactive arthritis patients have been included as controls in immunohistologic studies of RA (13,14).

In this study, we applied a panel of monoclonal
antibodies, using immunohistochemical techniques, to
de fine differences between RA and reactive arthritis in
the expression of HLA-DR, DP, and DQ antigens and
cellular phenotypes. Elucidation of such differences
might provide important clues about the immunologic
mechanisms of the persistence of inflammation in RA.

PATIENTS AND METHODS

RA patients. Synovial biopsy specimens from 5 pa-
tients (3 male, 2 female) with classic RA (16), taken at
arthroscopy of the knee joint, were randomly chosen from
specimens collected over an 18-month period from 25 pa-
tients with classic RA. Four of the 5 patients were sero-
positive. The median age of the patients at the time of arthro-
scopy was 54 years (range 30-70), and the median duration of
disease was 4 months (range 3 months-14 years). All patients
were receiving nonsteroidal antiinflammatory drugs
(NSAIDs), and 3 were receiving disease-modifying antirheu-
matic drugs (DMARDs). Followup data were available for
18-20 months post-arthroscopy. None of the patients had a
complete remission of the disease, although 4 had experi-
enced transient suppression of their knee symptoms. Three
of these 4 had a relapse of these symptoms within the
followup period; the fourth has remained symptom free.
The fifth patient experienced persistent symptoms throughout
the followup period, despite introduction of DMARD therapy.

Reactive arthritis patients. Synovial biopsy samples
were also obtained from all 5 patients with reactive arthritis
who underwent arthroscopy of the knee joint at our insti-
tution over the same 18-month period. The group included 4
men and 1 woman. The median age at the time of arthro-
scopy was 25 years (range 19-29), and the median duration of
disease was 4 months (range 3 weeks-13 years). Three
patients had antecedent nonspecific urethritis, and the other
2 had histories of diarrhea and foreign travel. Chlamydia
was definitely isolated in 1 patient only. Three patients were
receiving NSAIDs, and 1 of these had received predni-
solone. At followup (2-40 months), 4 patients were com-
pletely asymptomatic, with no evidence of disease activity
and receiving no drug therapy. The remaining patient had
disease activity in the distal interphalangeal joints, but the
knee synovitis had resolved.

In both the RA and the reactive arthritis patients, the
criterion for arthroscopic biopsy was clinical evidence of
acute knee synovitis. In all cases, several biopsy specimens
(2-8 specimens) were taken from macroscopically inflamed
areas under direct vision. For each patient, 50% of the
specimens were processed for routine histologic study and
the remainder were frozen for cryostat sectioning. Charac-
teristics of each of the patients are shown in Table 1.

Preparation of samples. After removal, the biopsy
specimens were mounted on cork, coated in OCT compound
(Miles Scientific, Naperville, IL) snap frozen in liquid nitro-
gen, and stored at -70°C. Tissue was also processed for
routine histologic study. Cryostat sections (5μ-thick) were
cut at -25°C and placed on glass slides coated with poly-
L-lysine for better retention of the tissue. These were then
air dried at room temperature for 15 minutes and stored
frozen at -20°C.

Staining of sections. After removal from storage, the
sections were air dried and fixed in acetone at room tem-
perature for 10 minutes. Nonspecific binding was blocked by
incubating the sections in fetal calf serum diluted 1:5 for 10
minutes. The primary antibody, appropriately diluted, was
applied to the tissue section and incubated for 4 hours at
room temperature. The excess antibody was removed by 3
washes in phosphate buffered saline (PBS).

The secondary antibody, peroxidase-conjugated goat
anti-mouse immunoglobulin (Sigma, Poole, UK) was diluted
1:50 in 2% normal goat serum and applied to the sections for
1 hour (goat anti-rabbit secondary antibody was used on the
section incubated with α1-antichymotrypsin, a polyclonal
antibody). After 3 further washes in PBS, the peroxidase
substrate 3'-3-diaminobenzidine. 0.5 mg/ml with 0.01% H2O2,
was added for 10 minutes. After washing in tap water,
the sections were briefly counterstained with Mayer's he-
malum, washed, dehydrated in alcohol, and mounted on
coverslips in Eukitt (BDH, Poole, UK).

The monoclonal antibodies used in this study are
listed in Table 2. Controls using diluted normal mouse serum
or PBS alone in place of the primary antibody were included
for all antibodies studied.

Determination of positive cells. Sections were counted
under high-power magnification. Since the number of cells in
the infiltrate tends to be higher in RA patients than in
reactive arthritis patients, the results are expressed as the
percentage of cells that were positive. This was calculated
by counting both positive and negative cells; for each
section, a minimum of 3 graticule fields was counted and the
average taken. In each field, at least 300 cells were counted
(mean number of cells counted per field: RA 628, reactive
arthritis 412). No assessment was made of the relative
intensity of staining, since this was deemed too subjective.
The initial counting was performed by an observer who was
not blinded, but the observations were validated by a sec-
ond, blinded, independent observer.

Each of the sections was also processed routinely
using conventional histologic stains.

RESULTS

Findings of conventional histologic studies. All of
the RA and reactive arthritis synovial membrane bi-
opsy specimens showed active synovitis with venous
congestion, mononuclear cell infiltrates, fibrin exuda-
tion, and plasma cells. Three of the 5 RA specimens
and 4 of the 5 reactive arthritis specimens showed
scattered polymorphonuclear cells, most commonly
seen in vessels and in the fibrinous exudate adjacent to
the lining layer. In the RA specimens, there was
marked villous hyperplasia, lining layer hyperplasia,
and focal accumulations of lymphocytes. These fea-
tures were absent in the reactive arthritis samples.
Table 1. Clinical features of the patients studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>HLA type</th>
<th>Disease duration</th>
<th>History of infection</th>
<th>Treatment at time of arthroscopy</th>
<th>Months of followup</th>
<th>Followup findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Classic RA. RF+</td>
<td>A2:B14.52: Cw2, w8: DR3.4: DRw52.53</td>
<td>3 months</td>
<td>None</td>
<td>NSAIDs</td>
<td>20</td>
<td>Well, taking SSA</td>
</tr>
<tr>
<td>2</td>
<td>Classic RA. RF+</td>
<td>A2:B7: Cw7: DR2: DQw1</td>
<td>10 months</td>
<td>None</td>
<td>DP, NSAIDs</td>
<td>20</td>
<td>Relapse of disease activity in knees, taking DP and NSAIDs</td>
</tr>
<tr>
<td>3</td>
<td>Classic RA. RF-</td>
<td>A1.3:B38.44: Cw5: DR4.5</td>
<td>3 months</td>
<td>Flu-like illness</td>
<td>Pred., NSAIDs</td>
<td>18</td>
<td>Continuous disease activity, taking HCQ and NSAIDs</td>
</tr>
<tr>
<td>4</td>
<td>Classic RA. RF+</td>
<td>A3.25:B18.35: Cw4: DR4.5</td>
<td>4 months</td>
<td>None</td>
<td>NSAIDs</td>
<td>19</td>
<td>Relapse of disease activity in knees, taking DP</td>
</tr>
<tr>
<td>5</td>
<td>Classic RA. RF-</td>
<td>Not done</td>
<td>14 years</td>
<td>None</td>
<td>SSA, NSAIDs</td>
<td>18</td>
<td>Relapse of disease activity in knees, taking AZA</td>
</tr>
<tr>
<td>6</td>
<td>Reactive arthritis</td>
<td>Not done</td>
<td>7 months</td>
<td>Urethritis, <em>Chlamydia</em> isolated</td>
<td>None</td>
<td>2</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>7</td>
<td>Reactive arthritis</td>
<td>B27+</td>
<td>3 weeks</td>
<td>Infective diarrhea</td>
<td>None</td>
<td>33</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>8</td>
<td>Reactive arthritis</td>
<td>Not done</td>
<td>4 months</td>
<td>Nonspecific urethritis</td>
<td>NSAIDs</td>
<td>26</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>9</td>
<td>Reactive arthritis</td>
<td>B27-</td>
<td>2 months</td>
<td>Circinate balanitis</td>
<td>NSAIDs</td>
<td>40</td>
<td>Knees normal, continued involvement of DIP joints</td>
</tr>
<tr>
<td>10</td>
<td>Reactive arthritis</td>
<td>B27-</td>
<td>13 years</td>
<td>Diarrhea</td>
<td>Pred., NSAIDs</td>
<td>34</td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>

* RA = rheumatoid arthritis; RF = rheumatoid factor; NSAIDs = nonsteroidal antiinflammatory drugs; SSA = sulfasalazine; DP = D-penicillamine; Pred. = prednisolone; HCQ = hydroxychloroquine; AZA = azathioprine; DIP = distal interphalangeal.

Expression of class II major histocompatibility complex (MHC) molecules. In both the RA and the reactive arthritis specimens, the synovial lining cells showed intense staining of all 3 class II molecules studied, i.e., HLA-DR, DP, and DQ. In the reactive arthritis sections, the lining layer was of normal thickness (1–3 cell layers thick), whereas in the RA sections, there was evidence of hypercellularity (3–5 cell layers thick).

Figure 1 shows the percentages of DR, DP, and DQ positive interstitial cells and vessels in the synovial membrane of the RA and reactive arthritis pa-

<table>
<thead>
<tr>
<th>Table 2. Antibodies used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody</strong></td>
</tr>
<tr>
<td>L243</td>
</tr>
<tr>
<td>B7/21</td>
</tr>
<tr>
<td>TU22</td>
</tr>
<tr>
<td>Leu-4</td>
</tr>
<tr>
<td>Leu-3a</td>
</tr>
<tr>
<td>UCHT4</td>
</tr>
<tr>
<td>Anti-CD22</td>
</tr>
<tr>
<td>OKM1</td>
</tr>
<tr>
<td>63D3</td>
</tr>
<tr>
<td>Polyclonal antibody</td>
</tr>
</tbody>
</table>

*ATCC = American Type Culture Collection; Trowbridge = Prof. Trowbridge, Oxford; Tubingen = Tubingen, West Germany; Becton Dickinson = Becton Dickinson, NJ; Unipath = Unipath, Oxford; Dakopatts = Dakopatts, Denmark; Rabbit = Dakopatts.*
patients. More than 80% of the vessels in both the RA specimens and the reactive arthritis specimens stained for DR. Only about 20% of the vessels were positive for DP, with no significant difference between the RA and the reactive arthritis specimens. However, there was a marked difference between the RA patients and the reactive arthritis patients in the number of vessels expressing DQ. Over 60% of the vessels from RA patients showed staining for DQ, compared with ~10% of the vessels from reactive arthritis patients.

Differences in the expression of class II molecules were also seen in the interstitial cell staining. More than 80% of the cells from RA patients expressed HLA-DR, DP, and DQ. In the reactive arthritis specimens, in contrast, many of the cells expressed DR (50%), fewer cells expressed DP, and very few expressed DQ (6%) (Figures 1–3).

Cellular composition. Table 3 shows the cellular composition of synovium from patients in the 2 disease groups. It can be seen that in the reactive arthritis synovium, there were no T cell foci and fewer cells staining for monocyte/macrophage markers. In the RA synovium, there were many large, dense accumulations of T cells. These foci contained ~50% T cells, with the predominant phenotype being CD4. In the areas between these lymphocytic foci, there were more T cells in the infiltrates of the RA patients than the reactive arthritis patients, but there were approximately equal numbers of CD4-positive and CD8-positive cells in the 2 disease groups. There was a very small number of cells expressing the CD22 marker present on B cells (but not on plasma cells) in RA and reactive arthritis synovium.

It was noted that in the reactive arthritis synovial membrane, there was a significant population of cells that were strongly positive for DR but negative for macrophage and lymphocyte markers. This population accounted for nearly 30% of the cells in reactive
Figure 2. Immunoperoxidase localization of HLA class II antigens in synovium from a patient with rheumatoid arthritis. A. Staining for HLA-DR, showing positive findings in the majority of interstitial cells and vessels. B. Staining for HLA-DP, showing interstitial staining and many unstained vessels. C. Staining for HLA-DQ, showing staining of the majority of interstitial cells and vessels. (Original magnification × 300.)

Figure 3. Immunoperoxidase localization of HLA class II antigens in synovium from a patient with reactive arthritis. A. Staining for HLA-DR, showing positive findings in vessels and many interstitial cells. B. Staining for HLA-DP, showing unstained vessels (open arrowheads) and scattered positive interstitial cells (closed arrowheads). C. Staining for HLA-DQ, showing unstained vessels and very few positive interstitial cells. (Original magnification × 300.)
Table 3. Cellular composition of synovium from patients with rheumatoid arthritis (RA) and patients with reactive arthritis

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RA</th>
<th>Reactive arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells (CD3)</td>
<td>15 (12-19)</td>
<td>8 (1-28)</td>
</tr>
<tr>
<td>CD4</td>
<td>5 (2-11)</td>
<td>6 (2-14)</td>
</tr>
<tr>
<td>CD8</td>
<td>5 (2-8)</td>
<td>3 (1-6)</td>
</tr>
<tr>
<td>B cells (CD22)</td>
<td>4 (2-8)</td>
<td>1 (0.1-4)</td>
</tr>
<tr>
<td>Monocytes/ macrophages</td>
<td>51 (46-57)</td>
<td>9 (6-17)</td>
</tr>
<tr>
<td>Lymphocytic foci (&gt;50 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>43 (38-47)</td>
<td>None</td>
</tr>
<tr>
<td>CD4</td>
<td>41 (36-48)</td>
<td>None</td>
</tr>
<tr>
<td>CD8</td>
<td>3 (2-33)</td>
<td>None</td>
</tr>
</tbody>
</table>

Arthritis samples, compared with only 9% of the cells in the RA samples. Most of these cells were oval-shaped, but some appeared to have dendritic processes, which were highlighted by DR staining. The interpretation of cellular morphology on cryostat sections is difficult, however.

**DISCUSSION**

The mechanism underlying the persistence of immune activation in RA and other autoimmune diseases remains obscure. Excessive HLA-DR expression is found on the target tissue in most human autoimmune diseases (17,18), and it has been suggested that aberrant expression of these class II molecules plays an important role in the perpetuation of the chronic inflammation (17).

Results of several immunohistochemical studies would support the involvement of class II molecules in RA synovitis. Evidence for this involvement includes intense HLA-DR expression by the majority of synovial cells, activated lymphocytes, B cells, and endothelial cells (4,7,8,10) and by cells with a dendritic morphology (2,3,8,10,14). Interpretation of these findings in terms of disease pathogenesis is difficult, however, since there are few suitable controls. Results of the few immunohistologic studies of synovium from patients with other types of synovitis and from normal controls have indicated that these findings are not specific for RA. It is now evident that HLA-DR is present on the endothelium, dendritic cells, and lymphocytes of synovial membrane from normal subjects and osteoarthritis patients (2,3,15) and that patterns of staining in all forms of chronic synovitis are similar to that found in RA (14). It is therefore unlikely that the overexpression of HLA-DR alone is important for the chronic, persistent inflammation seen in RA.

There is now considerable interest in the importance of differential expression of the products of the 3 subregions, HLA-DR, DP, and DQ, in the initiation and perpetuation of chronic inflammation (19). It has been suggested that the antigen-presenting function of adherent cells resides in the HLA-DQ positive population (20), and a study on rejection following cardiac transplantation has suggested that the expression of HLA-DQ antigens may play an important role in the persistent inflammation seen during chronic rejection (21).

Previous studies on differential expression of class II MHC antigens in RA synovium have been limited, and results have been somewhat conflicting. Some studies have shown significantly less HLA-DP and DQ staining compared with DR staining in RA synovium (22) and RA pannus (9). In contrast, investigations at both the messenger RNA level and the protein level have shown that all 3 HLA-D region products were highly expressed in RA synovium (23). These diverse findings may be due to differences in disease activity and/or therapy of the patients selected: a longitudinal study of RA patients showed that HLA-DP and DQ expression in synovial membrane decreased following gold and penicillamine therapy, whereas DR expression was unaffected (24).

The cellularity and expression of class II antigens in reactive arthritis synovium have not been studied in detail before. Very small numbers of patients have been included in mixed control groups in other studies, and the synovial membrane from these patients has shown staining patterns for cell phenotypes and DR expression similar to those seen in RA, apart from the absence of lymphoid aggregates (13,14). Our results have shown striking differences between these 2 diseases in class II antigen staining. We found similar expression of HLA-DR and DP antigens in RA and reactive arthritis synovium, but a marked overexpression of HLA-DQ antigens by both endothelium and interstitial cells of RA patients compared with reactive arthritis patients.

This excessive expression of HLA-DQ in RA may be important in the presentation of self antigens to T cells, possibly in association with HLA-DR. The interstitial cell expression of HLA-DQ might be explained by the differences in cellularity, particularly since the percentage of macrophages present was significantly greater in the RA specimens. However,
the expression of HLA-DQ by >60% of the vessels in RA patients but by very few vessels in reactive arthritis patients suggests that these cells may be induced to express these antigens in RA. This might result from the relative amounts of mediators present or the presence of different mediators in RA. Low levels of γ-interferon (γ-IFN) are known to induce HLA-DR expression, with higher levels required to induce HLA-DP or DQ (25). It is also known that a mediator produced by human leukocytes, but different from γ-IFN, induces fibroblasts to express both HLA-DR and DQ (26).

A further significant difference between the 2 diseases was the finding of a higher percentage of cells expressing HLA-DR antigens, but no monocyte/macrophage or lymphocyte markers, in the reactive arthritis synovium compared with the RA synovium. Some of these cells appeared to have a dendritic morphology, although the interpretation of this in cryostat sections is difficult. There has been considerable interest in the role of dendritic cells as antigen-presenting cells involved in the triggering and subsequent perpetuation of the immune response in RA (11,27,28). These cells have potent accessory function and have been reported to be increased in RA synovium compared with synovium from normal subjects or patients with degenerative arthritis (8,10,12, 14,27,28).

Findings of most studies suggest that dendritic cells express all 3 class II MHC antigens (29), although the results of 1 study of RA patients suggest that they express HLA-DR but have undetectable levels of DQ (23). These conflicting results may arise from differences in the definition of dendritic cells; for example, in some studies they are defined simply as being strongly HLA-DR positive but monocyte/macrophage marker negative, while in other studies they are defined by their binding of monoclonal antibody RFD1 (30) or by their semiaherence to plastic (29). Differences in the processing of cells may affect apparent expression of antigens (31), but this is not usually a problem with immunohistologic studies.

The population of cells we have identified in reactive arthritis synovial membrane is HLA-DR positive, DQ negative, and monocyte/macrophage and lymphocyte marker negative. In the absence of specific markers, it is not possible to confirm whether these cells represent classic dendritic cells or fibroblasts with induced DR expression. If they are classic dendritic cells, it is surprising that they do not express DQ antigens, although this could be explained by down-regulation by negative feedback, as part of the regulation of normal antigen presentation. If these cells do represent dendritic cells, our results suggest that these are present in greater numbers in a self-limiting synovitis than in RA. Previous researchers have found differing numbers of dendritic cells in RA synovium: Some found up to 4% of infiltrating cells with this phenotype (7), whereas others found 10–20% or less (6). There has been very little research on the presence of dendritic cells in other forms of arthritis; 1 study showed these cells accounting for 5% of the infiltrating cells in patients with spondylarthropathies (32).

Reactive arthritis serves as a good control for RA since, at the onset of reactive arthritis, there is a marked inflammatory synovitis that is usually self-limiting and does not progress to joint destruction. In reactive arthritis, extrinsic antigen is thought to be presented within the joint. Chlamydial antigen (33) and yersinial antigen (Toivanen A: personal communication) have been found in the synovium or synovial fluid of some patients with reactive arthritis, whereas in RA, no extrinsic antigen has been identified. Presentation of self antigens is thought to occur within the joint. The study of these 2 diseases therefore allows comparison of changes in the synovium that occur during presentation and elimination of extrinsic antigen (reactive arthritis) and during chronic presentation of autoantigen (RA).

Although reactive arthritis serves as a good control for studies of factors contributing to the chronicity in RA, patients with RA often have a much longer disease duration, so differences could be attributed simply to the duration of inflammation. In our study, however, the 2 groups had the same median disease duration (4 months). This makes it unlikely that the findings were due to the duration of the synovitis. Followup showed continuing disease activity in 4 of 5 RA patients: 1 had uninterrupted disease activity and 3 had the typical remitting/relapsing course of RA. In the reactive arthritis patients, the synovitis resolved during the followup period, suggesting that the course was characteristic for this disease. Studies on intraarticular variation in synovitis suggest that biopsy under direct vision of macroscopically inflamed areas, as done in this investigation, may show more florid changes than samples from uninflamed areas, but they also suggest that a single specimen is representative of the whole synovial membrane, because there is considerable histologic homogeneity within an individual joint (13,34).
Although the numbers of RA and reactive arthritis patients in the present study were small, the differences between the 2 groups in the cellular composition and expression of the HLA class II antigens in synovium were large, and in addition to achieving conventional levels of statistical significance, observations were consistent among all patients within each group, allowing confidence in the observed differences. These findings may contribute to elucidation of the mechanism of chronic, persistent inflammation in RA synovitis.

REFERENCES


Appendix 2

SOLUTIONS USED IN WESTERN BLOTTING

RESOLVING GEL (2 GELS)  
5%  
20%  
30% Acrylamide 5.0mls 20.0mls  
Distilled water 17.5mls -  
Separating buffer pH 8.8 7.5mls 7.5mls  
Glycerol - 2.5mls  
10% Ammonium persulphate 160μl 40μl  
10% Tetramethylethylenediamine 160μl 40μl

STACKING GEL (2 GELS)  
30% Acrylamide 4.0mls  
Distilled water 14.0mls  
Stacking buffer pH 6.8 6.0mls  
10% Ammonium persulphate 200μl  
10% Tetramethylene diamine 200μl

SEPARATING BUFFER  pH 8.8 with 1M HCL  
90gms Tris base  
2gms sodium dodecyl sodium  
~500mls distilled water (final volume 500mls)

STACKING BUFFER  pH 6.8 with 1M HCL  
30gms Tris base  
2 gms sodium dodecyl sodium  
~500mls distilled water (final volume 500mls)

ELECTROPHORESIS BUFFER  
144 gms glycine  
30 gms Tris base  
5 gms sodium dodecyl sulphate  
~ 5 litres distilled water (final volume 5 litres)

BLOTTING BUFFER  
72 gms glycine  
15 gms Tris base  
1 litre technical methanol  
~ 4 litres distilled water (final volume 5 litres)

SAMPLE BUFFER  (2x)  
30 gms Tris base  
20 gms sodium dodecyl sulphate  
100 mls glycerol  
200mM dithiothreitol  
~ 400 mls distilled water (final volume 500mls)
Appendix 3

SOLUTIONS USED IN BLOOD SEPARATION & TISSUE CULTURE

DULBECCO PBS

50mls PBS (10 x) w/o calcium and magnesium (GIBCO)
450mls double distilled water
2.5 mls 0.5% phenol red
50 mg streptomycin sulphate
30 mg benzyl penicillin
4 mls 6% sodium bicarbonate

RPMI CULTURE MEDIUM

500 mls RPMI 1640 medium (GIBCO)
5 mls glutamine
50 mg streptomycin sulphate
30 mg benzyl penicillin
5 mls L-glutamine (200mM solution)
10 mls sodium pyruvate (100mM solution)
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