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**STUDIES ON CHEMOATTRACTANT ACTIVITY OF
RHEUMATOID SYNOVIAL FLUID FOR HUMAN
LYMPHOCYTES IN VITRO**

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Thesis submitted to the faculty of Medicine, University of
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ABBREVIATIONS USED IN THE TEXT

α	Anti-
Ab	Antibody
AS	Ankylosing spondylitis
APAAP	Alkaline phosphatase anti-alkaline phosphatase
CD	Cluster determinant
CD45R	Restricted CD45 gene exon usage
Con A	Concanvalin A
C.R.P	C-reactive protein
C.S.A	Cyclosporin A
DM	Dexamethasone
DMARDs	Disease modifying antirheumatic drugs
ELISA	Enzyme linked immunosorbent assay
S. E.M	Standard error of the mean
E.S.R	Erythrocyte sedimentation rate
EXPT	Experiment
FACS	Fluorescence-activated cell sorter
F (ab') ₂	Antigen-binding fragment of an Ig following pepsin digestion
FITC	Fluorescein isothiocyanate
Fc	Crystallisable fragment of immunoglobulins
FCS	Foetal bovine calf serum

g	Gram; or acceleration due to gravity
HBSS	Hanks' balanced salt solution
HEPES	N-2-[hydroxyethyl] piperazine-N-2[2-ethanesulphonic acid]
HEV	High endothelial venule
Hrs	Hours
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL-	Interleukin
IL-2	Interleukin 2
IL-8	Interleukin 8
IL-15	Interleukin 15
Kd	kilodalton
L	liter
LFA	Lymphocyte function-associated antigen
MIP-1 α	Macrophage inflammatory protein alpha
MCP-1	Macrophage chemotactic protein
mg	milligram
ml	millilitre
mm	millimeter
mM	Milimolar

M	Molar
MLR	Mixed lymphocyte culture
MNC	Mononuclear cells
Min	Minutes
MOPs	3-[N-morpholino] propanesulphonic acid
ng	Nanogram
NK	Natural killer cells
NSAIDs	Non-steroidal anti-inflammatory drugs
nM	Nanomolar
OA	Osteoarthritis
PBS	phosphate buffer saline
PE	Phycoerythrin
pH	Negative logarithm of hydrogen ion concentration
PHA	Phytohaemagglutinin
PMR	Polymyalgia rheumatica
PRN	Prednisone
PRL	Prednisolone
PSA	Psoriatic arthritis
PWM	Pokweed mitogen
PPD	Purified protein derivative of Mycobacterium tuberculosis
RANTES	Regulated upon activation , normal T expressed and presumably secreted.

RA	Rheumatoid arthritis
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute, medium number 1640
Tris	Tris (hydroxymethyl) methylamine
U	units
VCAM	Vascular cell adhesion molecule
VLA	very late antigen
Vol	volume
V/V	volume/volume
W/V	weight/ volume
°C	Degrees centigrade
>	Greater than
<	Less than
&	and
%	Percent
+	Positive
-	Negative
±	with or without
:	Colon

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DECLARATION

Part of the work described in this thesis included in the following publications

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AL-Mughales, J., Blyth.T.H., Hunter.J & Wilkinson, P.C (1995) Chemoattractant activity of rheumatoid synovial fluid for human lymphocytes. Submitted for publication.

McInnes. I.B. AL-Mughales, J. Field, M. Leung, B.P. F-P.Hang. Dixon, R. Sturrock, R.D. Wilkinson, P. C. Liew, F (1996). The role of interleukin-15 in T cell migration and activation in rheumatoid arthritis. Nature Medicine. Volume 2.

SUMMARY

The present research was carried out to investigate the chemoattraction activity of rheumatoid and non rheumatoid synovial fluids for human lymphocytes separated from peripheral blood, synovial tissue and synovial fluid. The phenotyping of locomotor cells in response to these fluids was also studied. Established procedures were used to separate lymphocytes from blood, synovial tissue and synovial fluid. The level of the chemotactic factors in the synovial fluid was measured by commercial and in-house developed methods. The inhibitory effect of anti-inflammatory drugs on the lymphocyte locomotion was also studied.

The chemoattractant activity of synovial fluid for human lymphocytes was investigated using the following methods: i. A Polarization assay which measures the shape change from spherical or round to a polarized shape (i.e from immotile to a motile shape) following stimulation with chemoattractants. ii. Collagen gel invasion, which measures the migration of lymphocytes into collagen gels-containing chemoattractants. Two methods were used for phenotyping the cells responding to the synovial fluids (I) APAAP which allowed the detection of lymphocyte surface markers in stained cytospin preparations, (ii) FACS which allowed the detection of cell surface markers of lymphocytes recovered from collagen gels after collagenase digestion. In addition methods used to measure the levels of chemotactic factors in the synovial fluid, were (I) Commercial single

antibody sandwich ELISA kits (R&D) which measured IL-2, IL-8, MIP-1 α and MCP-1, (ii) In-house developed multiple antibody sandwich ELISA which measured IL-15 in the fluids.

The ability of synovial fluids from patients with rheumatoid (n=35) and other arthritides (n=18) to attract lymphocytes from peripheral blood of normal subjects, from rheumatoid synovia, and from joint fluids, was studied. The majority of synovial fluids from 29 rheumatoid arthritis patients were strongly attractive for blood lymphocytes which had been cultured overnight. Three out of five fluids from OA also attracted lymphocytes but to a lesser extent than RA fluids. In addition four of seven fluids from other inflammatory arthritides also gave high responses

Rheumatoid synovial tissue lymphocytes responded to synovial fluids without a requirement for a period of culture. In contrast lymphocytes derived from rheumatoid and other synovial fluids were completely unresponsive to locomotor stimulants. Most of the responding cells from blood mononuclear cell fractions were T lymphocytes and the CD45RO isotype was attracted preferentially. Rheumatoid synovial fluids contained IL-8, IL-15, MIP-1a and MCP-1 at levels in the nanogram range, sufficient to attract lymphocytes, but levels of IL-2 were too low to exert a chemoattractant effect. In contrast the levels of chemotactic factors in OA fluids were low and these fluids also showed less activity in attracting lymphocytes. The activity of the fluids could not be abolished by

treatment with antibodies to IL-8, IL-2, MIP-1a, MCP-1 or IL-15 tested individually, but combinations of these antibodies inhibited most of the activity, suggesting that attraction of lymphocytes by the fluids is due to a combination of attractants. The accumulation of lymphocytes within the synovial fluids was not correlated with any single chemotactic factor mentioned above, suggesting that such accumulation is due to combined chemoattractants.

In the present study it was also observed that neutrophils separated from normal blood gave a strong chemotactic response to the synovial fluids. In contrast neutrophils separated from the synovial fluid were immotile, suggesting that these cells had an intrinsic defect or that their locomotion was selectively blocked by synovial fluid chemotactic inhibitors. Moreover there was no correlation between IL-8 or levels of any other single cytokine and the accumulation of these cells in the fluids, indicating the possibility of multiple chemotactic factor involvement.

The manipulation of the locomotion activity of lymphocytes in vitro in response to synovial fluid was studied using anti-inflammatory drugs. It was demonstrated that NSAIDs (including Aspirin, Ibuprofen and indomethacin), DMARDs (including gold, D-penicillamine and primaquine) and cytotoxic drugs including rapamycin and cyclophosphamide had no inhibitory effect on lymphocyte locomotion. On the other hand cyclosporin A and Glucocorticosteroids (including dexamethasone, prednisone and prednisolone) showed a significant inhibitory effect.

CHAPTER:1 INTRODUCTION

Section 1: Lymphocyte Locomotion in Inflammation

1.1 General Introduction

The inflammatory response protects the body from physical and chemical agents and infectious pathogens. Inflammation is defined as the response of living tissue to injury and it may be divided into acute and chronic responses (Cotran *et al.*, 1988). The acute response is dominated by infiltration of tissues with neutrophils associated with the exudation of fluid and plasma protein. The chronic response is usually of longer duration and characterised by the presence of macrophages and lymphocytes and by the activation and proliferation of connective tissue (Cotran *et al.*, 1988). This chapter will discuss first, the current knowledge about joint diseases (rheumatoid arthritis and other inflammatory arthritides) which represent the best example of undesirable reaction of a complex inflammation (acute and chronic) in the synovium. Second the properties, **adhesion, locomotion and chemotaxis** of the above mentioned inflammatory cells will be examined. Particular attention will be paid to the pathophysiology of T lymphocytes which are reported to be the most predominant cells in the rheumatoid synovium. Finally the drugs that have been reported to control the acute and inflammatory immune response in the joints will be discussed in detail.

1.2 Disease associated with acute and chronic inflammation

1.2.1 Rheumatoid arthritis

1.2.1 A. Definitions of disease and criteria

Rheumatoid arthritis (RA) is an inflammatory disease which primarily affects the joints, with formation of chronic synovitis leading to bone and cartilage damage. It also involves the skin, lymph node, heart, lungs, blood and nerve system. Three times as many women as men are affected. As the clinical diagnosis is difficult (due to overlapping of signs and symptoms with other rheumatoid diseases) most rheumatologists use the clinical criteria of the American College of Rheumatology (Ropes *et al.*, 1956) to differentiate RA from other arthritides. These criteria include clinical, serological, radiological and histological features (Arnett *et al.*,1988) and have been widely used in clinical diagnosis of RA and in epidemiological studies.

1.2.1 B. Aetiology

Despite many years of investigation of metabolic, nutritional and environmental factors the aetiology of RA is still unknown. As will be discussed below most current studies have focused on various immune factors, including autoimmune reactions, immunogenetics, and the initiation of acute and chronic inflammatory immune responses in rheumatoid arthritis and other inflammatory arthritides.

1.2.1.B1. Autoimmunity and autoimmune disease

i. Background

To discriminate between self and non self is an essential feature of the normal immune system . When something occurs to destroy the ability of discrimination (such as endogenous or exogenous factors) an immune response to self may develop. This immune response is called autoimmunity or an autoimmune response. Autoimmunity may be organ specific, localized or systemic. The consequence of this reaction may be minimal or catastrophic damage depending on the extent to which the integrity of self tolerance has been affected. The distinction should be made between an autoimmune reaction and autoimmune disease in which autoimmunity evokes pathologic consequences with the possible involvement of antibody, complement, immune complexes and cell mediated immunity . In 1963 Burnet, defined autoimmune disease to include multiple conditions in which structural or functional damage is produced by the immunologically competent cells or antibodies against normal body components. The presence of autoimmune reactions was confirmed by the presence of autoantibodies and of lesions in the tissue against which the antibodies were directed (Burnet, 1972).

According to the site of autoimmune reaction, type of antibody production, and clinical manifestations, the autoimmune diseases can be classified into three groups. The first group is characterised by specific organ damage and the

presence of specific antibody against antigens in that organ, for example Graves disease where thyroid gland hyperfunction is driven by an autoantibody which reacts with and stimulates the thyroid stimulating hormone (TSH) receptor (Strakosch *et al.*, 1982). The second group is characterized by specific organ damage but the antibody is non specific for that organ such as primary biliary cirrhosis in which the bile ducts within the liver are destroyed causing cirrhosis and portal hypertension. In this group the autoimmune antibody reacts with the mitochondria in all cells (Taal *et al.*, 1983, Frazer *et al.*, 1985). The third group is characterized by damage in different organs of the body (joints, nervous system, heart, kidney, lungs, and skin) including rheumatoid arthritis and other inflammatory diseases.

ii. Arthritis and Autoimmunity

The concept of autoimmunity in rheumatoid arthritis was introduced in the 1940s by Waaler when he described the abnormality of connective tissue metabolism in this disease. He found high levels of an autoantibody called rheumatoid factor (RF). This observation gave an evidence that the immune system might be involved in the disease. As mentioned above autoimmune diseases may be a consequence of the generation of self reactive antibodies and the significant relationship between rheumatoid factor and rheumatoid arthritis provided evidence that autoimmunity is involved in RA. In 1970 Steffen, proposed a hypothesis in which he suggested that RA may be a collagen autoimmune disease, and animals models of collagen type II-induced arthritis have confirmed his theory (Stuart, *et*

al., 1982; Klareskog, *et al.*, 1983, Cremer, 1988; Terato., 1985, 1990; Holmdahl. *et al.*, 1986; Myres, *et al.*, 1989; Watson and Townes 1985., Watson, *et al.*, 1986). Several autoantibodies including RF and anti-collagen antibodies are produced by synovial plasma cells suggesting that local antigen may be involved in both immune complex formation and lymphocyte activation in this tissue (Munthe and Natvig 1972., Hoffman. *et al.*, 1982, 1990; Wernick. *et al.*, 1985). However despite many reports of a variety of autoantibodies in RA, rheumatoid factors are still considered as the unique serological indicator in RA diagnosis.

iii. Rheumatoid Factors

The rheumatoid factors are a group of antibodies directed against the Fc portion of the immunoglobulin G (IgG) molecule. Waaler in 1939 and in 1940 and Rose and Co-workers in 1948 reported that the sera from patients with rheumatoid arthritis agglutinated sheep erythrocytes coated with rabbit anti-sheep erythrocyte antibody. The serum factor responsible for the agglutination was a high molecular weight immunoglobulin of the IgM Class. In the first years of RF research the factors were detected in serum but later several experimental studies reported RF production in the synovial tissue and synovial fluids of RA patients (Mellors *et al.*, 1959., Munthe and Natvig 1971, 1972a, 1972b). Although they are present in 70-80% in patients with RA during the course of the diseases (Carson *et al.*, 1981) , their occurrence in many connective tissue diseases , chronic infections and healthy individuals without clinical manifestation of arthritis raised

doubts about how specific a role they played in the pathogenesis of rheumatoid arthritis (Johnson and Faulk., 1976; Mannik., 1979). A possible harmful effect of RF is suggested by clinical data showing a correlation between RF and disease activity, particularly extra-articular manifestations (Allen, *et al.*, 1981; Masi *et al.*, 1976; Feigenbaum *et al.*, 1978). Further interesting observations of IgG oligosaccharides have shown that IgG in active RA disease lacks the terminal galactose in the Fc region of IgG at position 297 (Mullinax *et al.*, 1976; Young, *et al.*, 1991). This structural abnormality alter the IgG antigenicity and may facilitate immune complex formation and effective binding of IgG to RFs (Soltys *et al.*, 1994). Plasma cells in the synovial membrane produce RF of five immunoglobulin classes (Muthel and Natvig 1972; Soltys., *et al.*, 1994; Randen *et al.*, 1993).

1.2.1.B2. Immunogenetic and susceptibility to rheumatoid arthritis

During the past four decades it has been described that there is an association between RA and certain alleles of human leukocyte antigens (HLA), particularly HLA class II. The human class II locus has at least 14 different genes on the sixth chromosome, most of which are found in three major subregions, designated as DP, DQ and DR (Ollier and Symmons 1992). The first evidence of a genetic linkage was reported by Astorga and Williams (1969) who noticed that the peripheral blood lymphocytes from unrelated patients with RA were non stimulatory in mixed leukocyte cultures. Subsequently it has been reported that

Caucasians with seropositive RA have an increased frequency of expression of the cell surface human leukocyte antigen DR4 (Stastny 1976; Grennan *et al.*, 1983; Del Junco *et al.*, 1984; Walker *et al.*, 1987). In addition it has been reported that the RA association with HLA is localized to the third hypervariable region (HVRIII) of the HLA-DR4 β chain. This region is a critical component in the formation of the groove which binds antigenic peptide for their presentation to the T cell receptor of responding cells (Panayi, 1995). DR4 are expressed by 70% of white seropositive RA compared to 28 % of normal controls (Stastny, 1978). It has been observed also that an individual heterozygotic for DR4 has an eight fold higher risk than normal for developing RA, while DR4 homozygotes have a 36 fold risk factor (Legrand *et al.*, 1984; Nepom *et al.*, 1984). Further evidence of an influence of HLA on the severity of disease has come from studies of the Felty's syndrome (another variant of RA characterized by splenomegaly and leg ulcers associated with polyarticular RA) (Lanchbury, *et al.*, 1991; Wordsworth, *et al.*, 1-992; Van Zebend *et al.*, 1991) in which 95% of patients with this disease were found to be HLA-DR+ (Bowman 1995 *et al.*, 1995). Many patients with most severe RA are DR4 homozygotes (Brackeretz and Wernet 1980). Other studies reported that RA was linked to co-expression of certain DQ alleles in association with DR4 (Wallin *et al.*, 1988; McCusker *et al.*, 1991).

Recent developments in understanding antigen presentation or the interaction between MHC and T cells (Babbit *et al.*, 1985; Guiltt *et al.*, 1987) have led to the suggestion of several molecular mechanisms concerned the role of MHC in RA.

These are (i) The expression of specific D molecules may allow the presentation of foreign antigen that results in a cross-reactivity of certain T-cells with self antigen causing an autoimmune reaction. (ii) Class II MHC molecules may themselves resemble an exogenous epitope. Exposure of an individual with a haplotype to the exogenous epitope may trigger T-cells that cross react with cells bearing MHC class II molecules. Despite the importance of immunological mechanisms mentioned above, the critical question now relates to the type of antigen which initiates the inflammatory immune response in human rheumatoid arthritis and to the molecular events involved in the formation of peptide-MHC complex and towards understanding how, when and where MHC-I or MHC-II molecules are associated with this unknown antigen. Several studies have suggested that heat shock proteins (hsps) may play a significant role in arthritis aetiology (see below).

1.2.1. B3. Heat shock proteins and rheumatoid arthritis

i. Background

Heat is a major clinical sign of inflammation. It may be either systemic (fever) or localized to the sites of inflammation including rheumatoid synovium. It can modulate the inflammatory immune response and induces the synthesis of specific proteins. This phenomenon was firstly described by Ritossa in 1962, where he observed that exposure to heat induced a physiological response in host cells called heat shock response (Polla B.S 1988) which causes chromosomal puffing associated with synthesis of proteins termed heat shock proteins (hsps). These were classified according to their molecular weights, e.g high molecular weight hsps (65, 68, 72, 83, 92, and 110 Kda) and low molecular weight hsps (20 Kda) (Polla, 1988).

ii. Pathophysiology of hsps in rheumatoid arthritis

Heat shock proteins are induced not only by high fever but by many other factors including, heavy metals, ethanol, viral and bacterial infections. It has been reported that hsps play a complex role in the interaction between host and pathogens. In human rheumatoid arthritis it has been observed that the 65 Kd heat shock proteins found in the joints is similar to that found in *Mycobacterium leprae* and *Mycobacterium tuberculosis*. This molecular mimicry could initiate an autoimmune reaction in the rheumatoid joints, because the immune system

recognizes the mimicking microbe as a self antigen. Such reactions have been produced in animal models when certain strains of rats have been injected intradermally with an emulsion of killed *Mycobacterium tuberculosis* in mineral oil (complete Freund's adjuvant) which caused a rheumatoid-like disease (Pearson and Wood 1959). Also this disease can be transferred from affected to healthy rats through T cell lines or clones that recognize a mycobacterial antigen and that cross react with a self antigen in joint cartilage (Van Eden *et al.*, 1985). It has been clearly established that the 65 KD hsp found in the synovial fluid (Fisher *et al.*, 1991) induces proliferative responses in synovial fluid T cells from rheumatoid arthritis patients (Res *et al.*, 1988) and also 65 KD hsp induced adjuvant arthritis (AA) in rats by a T cell clone specific for amino acids 180-188 of the 65 KD HSP (Gaston *et al.*, 1990). However one senior rheumatologist criticises the role of hsps in RA (Panayi 1995) because of their lack of tissue specificity and the low frequency of T cells responding to the 65KD protein which is the most predominant hsp in the synovial fluid.

1.2.1 C. Histology of Synovial compartments in rheumatoid arthritis

1.2.1.C1. Synovium

Several reports have been described the histological features of the synovium during early and late stages of the rheumatoid disease (Schumacher and Kitridou 1972; Schumacher 1975). During early stage of the disease there is congestion, erythrocyte extravasation and luminal obliteration. Fibrin deposition occurs throughout the synovium. Endothelial cells are swollen and exhibit vascular activity. Neutrophils appear in the superficial synovium and lymphocytes are rare. Plasma cells and germinal follicles are uncommon or absent (Schumacher and Kitridou 1972; Schumacher 1975; Kontinen *et al.*, 1986). **In contrast** the histopathologic observations of the synovium during chronic inflammation have shown that the synovium contains swollen synovial cells (Lewis and Ziff 1966) and massive hyperplasia with 100 to 1000 fold weight increase (Smiley *et al.*, 1985). It has been observed also that the hyperplastic synovial tissue responsible for the joint destruction has an extensive vascular network. (Koch *et al.*, 1986). In addition it has been demonstrated that a subpopulation of macrophages is responsible for neovascularization probably by elaboration of IL-1, IL-6 and TNF- α cytokines that have the ability to promote the growth of blood-vessels. As will be discussed below numerous investigations have shown that the most predominant cells in the chronic stage of synovitis are lymphocytes. Most of these cells are T cells bearing surface markers that reflect *in vivo* activation

(Pearson and Wood 1964; Cush and Lipsky 1988; John *et al.*, 1975; Kurosaka and Ziff, 1983; Panayi 1992).

1.2.1.C2. Synovial fluid

Synovial fluid contains a large variety of inflammatory cells including neutrophils, lymphocytes (prominently T and B cells, and NK cells) and monocytes. Eosinophils and basophils are uncommon. Many cytokines (e.g IL-1, IL-6, IL-8, IL-15, MCP-1, MIP-1 α , TNF- α) are present. Also synovial fluid contains enzymes (e.g collagenase), proteins (e.g C-RP; Albumin; lubricin), carbohydrates, hyaluronic acid (which gives the fluid the consistency of egg white or glare and viscosity) and a variety of fibrillar materials (fibrin, collagen) and crystals (Urate, Calcium pyrophosphate dehydrate; Cholesterol crystals) (Kitridou *et al.*, 1969; Kitridou *et al.*, 1969; Zuckner *et al.*, 1964). Synovial fluid is important for the diagnosis and evaluation of many patients with rheumatic diseases particularly rheumatoid arthritis. In monoarticular arthritis, septic arthritis must be distinguished from other possible causes. Also synovial fluid cell count and volume are useful approximate measures of the intensity of the inflammation. For instance it has been observed (Al-Mughales unpublished observations) that total white cell count (WBCs) in most active RA synovial fluids were very high (range from 6.4-65.5 x 10⁶/ml) compared with other diseases such as osteoarthritis (range from 0.1-3.3 x 10⁶/ml). Also the synovial fluid volume

collected from most active RA was high (20-40ml/patient) compared with osteoarthritis (5-7ml/patient).

1.2.1. D. Lymphocyte and macrophage infiltrate in the synovium.

1.2.1.D1. Background

Over the past two decades a considerable amount of evidence has accumulated to implicate the role of lymphocytes and macrophages in the pathogenesis of this disease. As mentioned above, the first evidence is the hyperplasia and hypertrophy of the synovium with infiltration of mononuclear cells mainly lymphocytes, macrophages and plasma cells in the sublining layer of the synovium. The importance of lymphocytes was shown by improvement of patients with severe RA after depletion of lymphocytes through thoracic duct fistula drainage (Wegelius *et al.*, 1970; Paulus *et al.*, 1977) and total lymphoid irradiation (Panayi *et al.*, 1982). Further evidence for the role of lymphocytes is the discovery of rheumatoid factor (RF), immune complexes in joint fluid with depressed complement and deposition of immune complexes in the synovial membrane, which attract the attention to the inflamed synovial tissue (Kunkel and Tan 1964; Harnstedt, 1967). However recent opinion suggests that synovitis is no longer seen as an antibody-mediated process involving RF and immune complexes, but rather as mediated by T cells (Panayi *et al.*, 1992) and

macrophages (Firestein et al., 1990) which are reported to play an important role in the pathophysiology of RA.

1.2.1.D2. Cells: phenotype and distribution.

i. T lymphocytes

As mentioned above, the majority of lymphocytes in synovial tissue and synovial fluid of RA patients are activated T lymphocytes as phenotyped using monoclonal antibodies (MoAb) directed against T cell activation molecules such as human leukocytes antigens (HLA-DR) or the interleukin-2 receptor α chain (IL-2R; CD25) (Panayi 1994). The majority of these cells are primed and express surface antigens characteristic of mature memory cells, such as CD4⁺ CD45RO⁺ (Pitzalis *et al.*, 1987; Cush *et al.*, 1988; Burmester *et al.*, 1987). In addition to CD45RO⁺ and HLA-DR antigens, they express very late antigens (VLA-1-VLA-4) and the integrin LFA-1 with low expression of IL-2 (Iannoni *et al.*, 1994) and γ -IFN. It was recently observed that synovial fluid T cells express CD69 an early activation marker (Ionnone *et al.*, 1994). CD69 is a member of the c-type animal lectin superfamily and is the earliest inducible antigen expressed on the surface of T cells after activation with various stimuli (Fernandez-Gutierrez *et al.*, 1995).

ii. B lymphocytes

B cells constitute about 1-5% of synovial cells fraction. As mentioned above these cells contribute to the inflammation of the joint through a number of physiological processes. First production of antibodies such as IgM, IgG, and IgA. These antibodies especially IgM form immune complexes (ICs) in situ leading to a subsequent antigen processing and presentation to immune reactive T cells (Munthe and Natvig 1977; ; Van Snick *et al.*, 1978; Aho *et al.*, 1987). However other studies have concluded that during RA there is a preferential clonal expansion of RF B cells which lead to the production of high circulating RFs. As mentioned above the RFs are not purely pathogenic, but their concentration is significantly correlated with the disease activity (Smiley *et al.* , 1968; Munthe and Natvige 1972; Tuomi 1988).

iii. Natural Killer cells (NK cells)

The NK cells constitute approximately 1% of mononuclear cells in the synovial membrane (Fox *et al.*, 1984; Dobloug *et al.*, 1982). NK cells play several functions in the immune response including, target cell destruction, autologous mixed leukocyte reactions and suppression of other immune functions (Shah *et al.*, 1985). Their absence in RA may contribute to the chronicity of the disease.

iv. Macrophages

Although the RA synovial tissue is characterized by the predominant infiltration of T lymphocytes, macrophages also participate in the inflammatory immune response in the joints. Most of these cells express CD14 and CD11 and high levels of activation markers including, HLA-DR which are involved in antigen presentation. It has been observed that synovial macrophages produce a variety of inflammatory mediators , including prostaglandins, collagenase , elastase and cytokines such as IL-1, TNF- α , IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) (Dayer *et al.*, 1986; Arned and Dayer 1990). It has been also reported that most of the IL-8 found in the synovial fluid is produced by macrophages in the synovial tissue (Koch *et al.*,1991).

In conclusion, the critical question now relates to which cells mediate the complex inflammation in the synovium. Firestein and Zvaifler (1990) assign primacy to the macrophage in the immunopathogenesis of RA. Their argument is supported by strong evidence (mentioned above) including cytokine and enzyme production, and expression of activation markers. Panayi and his colleagues (1992) proposed that both cells are important in RA pathogenesis, but suggested that the T cell is the conductor of the orchestra. Their argument was supported by several immunological studies including involvement of T cells in most immunological aspects of RA aetiology including, predominant infiltration of T cells in the synovium with their cytokine production and numerous activation markers (more

than six hundred studies reported the immunological activity of T cell in the rheumatoid synovium) their important role in antigen presentation, their association with MHC in RA, evidence of animal models such as adjuvant arthritis in which this disease can be transferred from ill mice to healthy mice by T cell clones, beneficial effect of immunotherapy directed against T cells such as thoracic duct drainage, total lymphoid irradiation, beneficial effect of cyclosporin A and monoclonal antibodies.

However I do suggest that both macrophages and lymphocytes mediate the complex inflammation in RA (direct or indirect, either through antigen presentation or production of enzymes). In addition, the locomotion studies in the present research produced further important evidence that the T cells play a role in RA pathogenesis.

1.2.1 E. Pathophysiology of cytokines in the synovium

Several studies reported that the cytokines produced in the synovial tissue and synovial fluid serve as important mediators of inflammation and joint destruction in RA. This was shown by numerous investigators who found that macrophages, fibroblasts or T cells in RA synovial tissue release a variety of cytokines which can be detected in the synovial fluid. These are first, IL-1 (Miyasaka, *et al.*, 1988; Wood *et al.*, 198; Nouri *et al.*, 1984; Hopkins, *et al.*, 1988., Dalton *et al.*, 1989) TNF- α (Chu, *et al.*, 1991; Yocum *et al.*, 1989; Brenann, *et al.*, 1992). Both IL-1 and TNF-alpha stimulate chondrocytes and synovial fibroblasts in the synovial tissue to release collagenase and elastase, and neutral protease which have been reported to degrade proteoglycans and collagen resulting in cartilage destruction. TNF also has been documented in vivo to stimulate the neovascularization and leukocyte infiltration (Frater-Schroder *et al.*, 1987). However animal studies have shown that the inflammatory activity of TNF- α might be secondary to IL-1. Injection of TNF-alpha into the rabbit knee induced a weak inflammatory response but injection of both IL-1 and TNF produced strong inflammatory activity (Arned *et al.*, 1990). IL-6 is also detected in the synovial tissue and synovial fluid and is produced by macrophages, fibroblasts (Field, *et al.*, 1991; Bhardawi, *et al.*, 1989; Guerne, *et al.*, 1989;), T lymphocytes and chondrocytes (Guerne, *et al.*, 1989; Wong *et al.*, 1988). IL-6 is reported to be an RF B cell stimulating factor (Yasukawa, *et al.*, 1987) and also stimulates lymphocyte proliferation of the CD8+ subset.

Third GM-CSF has reported to be detected in the rheumatoid synovium (Leizer *et al.*, 1990; Alvaro-Gracia *et al.*, 1991; Haworth *et al.*, 1991) and plays an important role in the HLA-DR and HLA-DQ expression by synovial tissue macrophages and fibroblasts (Dayer *et al.*, 1990). Finally various chemotactic factors have reported in the synovial tissue and synovial fluid including IL-8 (Brennan *et al.*, 1990; Sietz, *et al.*, 1991; Sietz *et al.*, 1992; Koch, *et al.*, 1991; Hirota *et al.*, 1992), IL-15 (MacInness *et al.* 1995), and MIP-1 alpha. As will be discussed below these chemoattractant factors induce polarization and locomotion of lymphocytes in vitro (Wilkinson and Newman 1992; Wilkinson and Newman 1994; Wilkinson and Liew 1995;) and recently MCP-1 was reported to cause polarization of T cells (Wilkinson 1995 unpublished observations). IL-2 is a potent chemotactic factor for lymphocytes but is undetectable in synovial fluids in a dose that causes locomotion of lymphocytes in vitro (Combe *et al.*, 1985; Firestein *et al.*, 1987). The chemotactic factors produced in the synovial tissue or the synovial fluid may activate the adhesion molecules that are expressed on the synovial tissue blood vessel endothelial cells which in turn stimulates the adhesion of lymphocytes to the blood vessels (Harris, 1990). Thus lymphocyte chemotactic factors govern the mediation of locomotion of lymphocytes from synovial venules, through synovial tissue into the synovial fluid.

1.2.2. Other variants of rheumatic diseases

1.2.2. A. Osteoarthritis (OA)

Osteoarthritis is the most common joint disorder reported in most populations. It affects hand joints, the distal interphalangeal joints, proximal interphalangeal joints, the carpometacarpal joint of the thumb (frequently involved) and other joints (Cotran *et al.*, 1988). OA is very common in old age, it affects women more often than men, under the age of 45, and occurs with equal frequency above the age of 50. The onset and the aetiology of OA is still unclear. Several investigators have reported many risk factors which are associated with OA including obesity (reported to be associated with bone mineral density) (Anderson and Felson 1988), acute major injuries during sports activities and any heavy repetitive labour (Felson *et al.*, 1988). Another possible cause of OA is crystal depositions in the joints such as calcium pyrophosphate dhydrate which was found in about 60% OA synovial fluid (Schumacher *et al.*, 1981). A further cause of OA is impairment of the integrity of cartilage by collagenase and other protease enzymes which cause synovitis with release of inflammatory mediators, white cell degenerative enzymes and free radicals which in turn lead to cartilage degradation (*et al.*, 1988).

1.2.2 B. Ankylosing spondylitis (AS)

Ankylosing spondylitis (AS) is prototypical of a group of rheumatic disorders collectively called the seronegative spondylarthritides (Moll *et al.*, 1974). These disorders are characterized by chronic inflammation of the spine. The sacroiliac

joints are always affected but the hip, shoulder joints and other joints are rarely involved. The histopathology of the synovial lesions closely resembles that typical of RA (Moll et al., 1974). Classically AS is seronegative for IgM RF (Holborow and Swannell 1983). It affects mostly young men. The aetiology is unknown but over 90% of patients across the world have strong association with the HLA-B27 antigen (Khan et al., 1990). HLA-B27 is an MHC class I antigen (Kellner and D.Yu 1992). Hereditary influence appears to play an important role in AS because about one quarter of close relatives also have asymptomatic or symptomatic spondylitis (Cotran *et al.*, 1988).

1.2.2. C. Psoriatic arthritis (PsA)

This is an erosive chronic, recurrent inflammatory polyarthritis affecting about 5-7% of patients with psoriasis (Stites and Terr 1991). The onset of arthritis may be acute and preceded by skin disease. It differs from rheumatoid arthritis in being consistently seronegative for RFs and anti-nuclear antibodies (ANAs), but in half of the patients there is immune complex circulation and high levels in serum of IgA and IgG. Synovial fluid examination has shown high white cell counts. The interphalangeal joints of the fingers are affected including a considerable periostitis which produces a sausage-like appearance of the fingers (Holborow and Swannell 1983). The disease might progress to ankylosing spondylitis (Khan and Linden 1990). The cause of PsA is unknown. Genetic factors appear to play a role in disease causation. PsA are found in family members of 15% of patients. Patients with PsA have an increased prevalence of HLA-DR4 and HLA-B 27

(Leonard et al., 1978; Stern, 1985). These genetic markers might be associated with an increased susceptibility to unknown infectious or environmental agents or to autoimmune phenomena (Kenneth and Kenneth 1991).

1.2.2. D. Polymyalgia Rheumatica

Polymyalgia rheumatica (PMR) is a subgroup of giant cell arteritis (GCA). The name was introduced in the 1950's to define a syndrome with pain and stiffness of proximal muscle of elderly people (Bengtsoon 1991) with fever, weight loss, and synovitis that may be persist out or recurrent (Chuang et al., 1982). The synovitis is relatively mild confined to several joints, likely to appear at the onset of disease and does not cause deformity or erosive radiographic changes (Healey 1984). The synovial fluid is inflammatory with white cell count ranging from 5,900 to 21,000 cell/mm³. In contrast with rheumatoid arthritis, it has been reported that PMR patients are seronegative RF (Healey 1984). PMR is characterized by general arthritis which involves the large arteries. However the aetiology of this disease is not well defined but several studies reported that the lymphocytes from patients with PMR showed higher transformation responses to arterial antigens than lymphocytes from controls (Ziko *et al.*, 1977;) It has been also observed that patients with PMR have increased prevalence of HLA-DR4 expression in the arterial wall (Richardson *et al.*, 1987; Sakkas *et al.*, 1990). Thus genetic factors may be associated with the autoimmune reaction.

1.2.3. How lymphocytes enter the rheumatoid synovium and sustain their chronic activity.

1.2.3. A. Introduction

The synovium is more vascular than the structures supporting the joints such as capsule, ligaments and tendons. This vasculature in chronically inflamed joints has unique venules with specialised endothelium called high endothelium venules (HEV). There is preferential binding of lymphocytes at these sites which is enhanced by IL-1, TNF and interferon gamma. The high endothelial venules play an important role in blood lymphocyte adhesion and entrance into the synovium (Cavender *et al.*, 1987). This section will examine the current knowledge about the development, **adhesion, locomotion and chemotaxis** of the major lymphoid cells (T cells) which are postulated to play a central role in chronic inflammatory lesions, including joint diseases.

1.2.3.B. Lymphocyte, adhesion, locomotion and chemotaxis

1.2.3. B1. Historical background

Lymphocytes are first formed in the bone marrow (B cells) and thymus (T cells) in adults; in the foetus, the yolk sac, and liver are additional sources. T lymphocytes arise from stem cells (in the bone marrow) migrate to the thymus where they undergo differentiation into mature T cells and then leave the thymus. Although the lymphocytes were first described before the 1900's (McGregor and

MacKanness 1974; Powers, 1989), it was not until the second half of this century that the properties and the function of lymphocytes began to be understood. The classical experiments of Gowans in the rat provided a large step forward in the understanding of lymphocyte circulation in the secondary lymphoid organs through which they recirculated into the efferent lymph and thence back to the blood (Gowans and Knight 1964; Pabst 1988). Lymphocytes are the most predominant cells in the lymphoid organs. Mature T cell recirculate in the blood and migrate to the main secondary lymphoid organs, the periarteriolar sheath of the spleen (PALS), lymph node and gut associated lymphoid tissue (tonsils and peyer's patches), and to other lymphoid accumulations in respiratory and urogenital tracts. The majority of the lymphocytes in blood s (90%) are homogenous lymphocytes of small size (6-10 μm in diameter), with a minority of 10% of large cells (12-16 μm in diameter). The mean size of the recirculating lymphocyte pool in man has been estimated as 23×10^9 with approximately 50 percent of recirculating cells being present in the blood (Scott *et al.*, 1972). The mean blood transit of recirculating lymphocytes is approximately 30 min, (Fig). However despite this information the exact function of lymphocytes was still unclear, until 1961 when Miller reported the crucial role of the thymus in cell mediated immunity. Miller working on a murine leukaemia that originated in the thymus showed that thymectomy of new-born mice resulted in animals that were immunodeficient. They could not reject skin allografts and could not produce effective antibodies to either sheep erythrocytes (SRBC) or to salmonella H

antigens (Miller 1961). Similar studies confirmed these findings and showed that the delayed type hypersensitivity response to tuberculin was also impaired (Cooper *et al.*, 1966). It was also shown that in birds not only did thymectomy had a similar effect, but that extirpation or removal of a secondary lymphoid organ-the bursa of Fabricius also led to immune deficits (Warner 1962) It has been has also noticed that the Bursa of Fabricius contained cells that can produce antibodies (Klaus, 1990) which increased the knowledge about the real function of B lymphocytes. However, in contrast to thymectomy, bursectomy ablated all antibody responses but did not affect graft rejection (Warner and Szenberg 1964). It was then appreciated that there were two major lymphoid organs responsible for the production of lymphocytes and the terms 'T' and 'B' were coined for the cells produced in the thymus and bursa respectively.

Subsequently further investigations have been accumulated which described the role of lymphocytes in delayed-type hypersensitivity, antibody dependent cell mediated cytotoxicity (ADCC), graft versus host disease, antibody production, and cytokine production (Thomson and Prector 1984, Stites and Terr 1991; Abbass *et al.*, 1991; Albert *et al.*, 1989; Reinherz *et al.*, 1979, Hirohata *et al.*,1988). Recent development of monoclonal antibody techniques has led to further designation or phenotyping of lymphocyte surface molecules which defined the immunological activities of lymphocytes in primary and secondary lymphoid organs and allowed examination of their inflammatory role in numerous diseases, including rheumatoid arthritis and other inflammatory arthritides.

1.2.3.B2. Lymphocyte Surface Molecules

Several molecules located on the surface of the T-cells play an important role in the differentiation and function of the cell type. Although these molecules were initially designated by the monoclonal antibodies with which they react, recent internationally accepted terminology has designated them as clusters of differentiation antigens (CD). The CD designation refers to the whole molecule and not simply to an epitope detected by a specific antibody.

With the use of the monoclonal antibodies it has been possible to define several cell markers on immature and mature lymphocytes. In the case of T-lymphocytes many CD molecules such as CD3 and CD2 have been recognized on all peripheral T-cells whereas others define functionally distinct subsets. For example the CD4 marker has been found on approximately 60% to 70% of mature T-cells in the human blood whereas CD8 is expressed on about 30% to 40% of T-lymphocytes. Thus in normal healthy individuals the CD4 to CD8 ratio is approximately 1.5-2%. This may vary according to sex, age and various diseases. Although initially discovered as markers for T cells, subsequent studies documented that these molecules were associated with particular cell functions. For example the CD4+ and CD8+ phenotype of the T-cell is related to its capacity to recognize antigen in the context of either Class II or Class I MHC antigens on the surface of other cells respectively (Biddison *et al.*, 1982, Engelman *et al.*, 1981.). A direct physical contact between antigen and MHC has been demonstrated (Babitt *et al.*, 1986, Townsend *et al.*, 1986), . Class I MHC molecules play an important role as restricting

elements for peptide derived endogenously within antigen presenting cells whereas class II MHC molecules restrict T cells responses to exogenous proteins that are taken up and degraded in the lysosomes of antigen presenting cells to reveal antigenic peptide (Morrison *et al.*, 1986).

The strong association between Class I and Class II MHC restriction and the CD8⁺ and CD4⁺ subsets of T-cells respectively is likely to reflect a fundamental role of the CD4 and CD8 molecules during T cell activation and also during the selection of the T cell repertoire in the thymus. In addition recent studies in the mouse have suggested that CD4 T cell clones can be generated that express specific functional activities (Mosman *et al.*, 1986, Street, *et al.*, 1991). For example Th1 cells are effective mediators of delayed hypersensitivity reactions and the primary producers of IL-2 and interferon-gamma whereas Th2 cells produce IL-4, IL-5, IL-6 and are effective helper cells for B cell differentiation. Further evidence for the existence of Th1 and Th2 subsets came from rheumatoid arthritis studies (Miltenburg *et al.*, 1992) which suggest a relation between Th1-type functions (through the production of large amount of IFN- γ and absence of IL-4 and variable production of IL-2 and IL-6) and chronic inflammation in RA.

In both the human and the mouse CD4⁺ cells are subdivided into memory and naive subsets based on the expression of a variety of markers (Akbar *et al.*, 1991, Sanders *et al.*, 1988, Cerotini *et al.*, 1989). Although these subsets differ in the expression of a number of markers the most useful ones to separate them have

proven to be CD45 RA and CD45RO the isomers of the leukocyte common antigen.

Properties and function of the leukocyte common antigens (CD45)

The transmembrane protein tyrosine phosphatase CD45 comprises a family of 180-220 KD glycoproteins present in high abundance on the surface of all leukocytes (Thomas, 1989). They share a common intracellular domain that exhibits protein tyrosine phosphatase activity (Tonks *et al.*, 1990). Their different extracellular domains are produced through alternate mRNA splicing of three exons (A, B, C) present on the common parent gene (Streuli *et al.*, 1987; Morimoto *et al.*, 1986b). While the exact function and role of the different CD45 proteins chains remains to be determined the CD45RA (RA identified by 2H4; Morimoto *et al.*, 1985; see chapter) and CD45RO (Identified by mAb UCHL1; Smith *et al.*, 1986; see chapter 2) forms have proven to be reliable markers for identifying functionally distinct naive and memory T cells subsets. T cells that express the RA⁺ phenotype are resting lymphocytes. These cells when stimulated with anti-CD3 or phorbol 12, 13 myristate and ionomycin secrete a limited number of cytokines (Akbar *et al.*, 1991; Salmon *et al.*, 1989) including MIP-1 α , IL-8 and IL-2 (Conlon *et al.*, 1995). It has reported that the CD45RA subsets express specific adhesion molecules at low levels (Wallace *et al.*, 1990; Sanders *et al.*, 1988) and respond slowly to primary antigenic stimuli. This phenotype predominates in peripheral blood, especially in the blood of neonates (Demeure *et*

et al., 1994). In contrast, CD45RO⁺ are larger, more activated lymphocytes that express activation markers such as CD25. These cells secrete a large repertoire of cytokines including IL-2, IL-4, IL-5, IL-6, GM-CSF, TNF, INF-gamma, MIP-1 α and IL-8 (Conlon *et al.*, 1995) and express high levels of adhesion molecules including CD2, LFA-1, and CDw29 (Sanders *et al.*, 1988). They also respond briskly to recall antigens (Akbar *et al.*, 1988). T lymphocytes in RA peripheral blood primarily express the CD45RA isoforms (Summers *et al.*, 1994) whereas CD45RO⁺ lymphocytes predominate in the rheumatoid tissue (Hanly *et al.*, 1990; Nakao *et al.*, 1990; Pitzalis *et al.*, 1987; Sew *et al.*, 1992; Lasky *et al.*, 1988) and synovial fluid (Ichikawa *et al.*, 1992). Antigen presentation is believed to be the primary in situ signal that induces CD45RA⁺ lymphocytes to transform to memory cells and concordantly express the RO isoforms. Several in vitro models have been developed to study this transition process including activation of CD45RA⁺ T cells with alloantigen (Akbar *et al.*, 1988) or with mitogens such as Con A, PHA, or anti-CD3 mAb 2 (de Jong *et al.*, 1991; Akbar; *et al.*, 1988).

1.2.3. B3. Lymphocyte adhesion

The extravasation of lymphocytes from blood into tissue is mediated by specific interactions between lymphocytes and the endothelial cells lining post-capillary venules. These interactions are a crucial step for migration of lymphocytes into chronic inflammatory sites including synovial compartments in rheumatoid arthritis (RA) where HEV-like morphology resembles that of lymphoid tissues

(Freemont 1983; Janossy *et al.*, 1981) and where HEV can bind to normal peripheral blood lymphocytes (Fan and Dale 1994).

The molecular mechanisms which direct lymphocyte migration through endothelium into inflamed tissue have been the subject of intensive investigation. Over the past decades it has been found that several molecules (called adhesion molecules or homing receptors) influence the first step of lymphocyte-endothelium interactions and entry into tissue. Many of these molecules have been identified and characterised using monoclonal antibodies (Osborn *et al.*, 1989; Staunton *et al.*, 1989; Picker *et al.*, 1992) and also several adhesion regulatory factors have been identified such TNF- α , IL-1, INF- γ , IL-4, and bacterial lipopolysaccharide (LPS) (Wilkinson 1994; Hamblin 1994; Panayi 1994). When endothelial cells are exposed to any of these factors *in vitro* marked lymphocytes emigration across the endothelium is seen (Pettiffer *et al.*, 1986; Munro *et al.*, 1989). Combinations of these factors enhance adhesiveness for T cells further. The combination of TNF and IL-4 was found to be more potent than the combination of TNF and IFN- γ (Thornhill *et al.*, 1991).

The first adhesion molecule characterized as a homing receptor was the L-selectin which was previously named lymphocyte-endothelial cell adhesion molecule-1 (LECAM) or murine MEL-14, LAM-1 or Leu 8 (Gallatin *et al.*, 1983; Picker *et al.*, 1991). It belongs to a family of cell surface receptors characterized by a lectin binding site recognizing carbohydrate ligands (Lasky *et al.*, 1989; Siegalman *et al.*, 1989; Tedder *et al.*, 1989; Spertini *et al.*, 1991; Camerini *et al.*, 1989) on the

following HEV glycoproteins: glycosylation-dependent cell adhesion molecule (GlyCAM-1,) CD34 and mucosal addressin cell adhesion molecule (MAdCAM-1) (Girard, J.P and Springer 1995). It is not clear whether these molecules are involved in lymphocyte traffic into inflamed tissue.

Another adhesion molecule expressed on the surface of human lymphocytes which plays a role in lymphocyte migration is CD44 (Pgp-1, Hermes antigen). Antibodies against CD44 have been found to inhibit lymphocyte adhesion to peripheral blood lymph node, and synovium high-endothelial venules (Jalkanen *et al.*, 1987). In addition numerous studies have reported another family of adhesion molecules which mediate lymphocyte adhesion to endothelium *in vivo*. This family consists of three surface membrane heterodimeric glycoproteins named the β_2 integrins (CD11a, 11b and 11c /CD18). CD11a/CD18 is commonly called leukocyte function antigen-1 (LFA-1, on lymphocytes) (Dana and Arnaout 1988; Dahams and Hart 1986; Dahmas and Hart 1985; Haskard *et al.*, 1986; Hamann *et al.*, 1988;) and α_4/β_1 or β_7 integrins also known as very late activation antigen-4 (VLA-4)(Holzmamm *et al.*, 1989; Elices *et al.*, 1990) that appear 2 to 4 weeks after T cell proliferation *in vitro* at a time that is late relative to the expression of other activation antigens. There are about six VLA antigens which are variably expressed depending upon cell lineage and activation (Hemler 1990). It has been reported that lymphocyte adhesion via integrin (LFA-1 (CD11a), VLA-4 (CD49d) and some non integrin (e.g., CD2 CD43) receptors provides costimulatory signals to T lymphocytes engaging antigen via the T cell receptor/CD3 complex as well as

to B lymphocytes that recognize antigen on follicular dendritic cells. When these accessory and costimulatory adhesion are blocked with antibodies in vitro, greater than 100-fold higher concentrations of antigen are required to initiate lymphocyte responses (Wegner *et al.*, 1993) whereas α_4 integrins seem to be involved in homing into mucosal tissues and into inflamed sites. It has been shown that VLA-4 (on lymphocytes) mediates the adhesion of lymphocytes to fibronectin (that have separate tissue cell-binding sites) which is abundant in synovium and throughout the lining, blood vessels and interstitium (Isssekutz and Isssekutz 1991; Van Dinther-Janssen *et al.*, 1991). VLA-4 binds to the cell surface molecule VCAM-1 (Vascular-cell adhesion molecule-1). Endothelial ligands for these β_1 integrins such as intracellular adhesion molecule-1 (ICAM-1 or CD54) (Diamond *et al.*, 1991) and Vascular cell adhesion molecule-1 (VCAM-1) (Osborn *et al.*, 1989; Thornhill and Haskard 1990) have reported to be stimulated by cytokines produced in the inflamed synovial tissue suggesting a role in lymphocyte infiltration into inflamed synovial tissues (Pober and Cotran 1990). ICAM-1 is widely distributed among synovial tissue cells including fibroblasts, macrophage-like synovial lining cells and the endothelium of blood vessels in the lining as well as deep in the subsynovium. It has been found in vitro that treatment of synoviocytes (B cell type) with TNF- α , IL-1 or INF- γ upregulated ICAM-1 expression (Chin *et al.*, 1990). Also it has been found that $\alpha_L\beta_2$ (LFA-1) is present on synovial T cells and could play a role in the ingress and retention of T lymphocytes. Also it has been found that ICAM-1 expression is upregulated by

TNF- α and IL-1 with an expression peak of 24-48 hours after cytokine treatment and remains elevated as long as cytokine is present (Pober et al., 1986; Pober and Cotran 1990; Osborne et al., 1989; Marlin and Springer 1987). On the other hand it has been observed that TNF- α and IL-1 has the same effect on VCAM-1 with similar time courses of expression as ICAM-1. It has been reported that the rheumatoid synovial T cells exhibit a higher avidity than peripheral blood T lymphocytes for VCAM-1, the endothelial cell ligand for $\alpha 4\beta$ and $\alpha 4\beta 7$ integrins as well as for several extracellular matrix proteins (Arroyo *et al.*, 1995).

The lymphocytes-endothelial interaction involves the following multistep cascade (Fig) (Ager 1994; Girard and Springer 1995). i) attachment and rolling mediated by L-selectin, which recognizes CD34 and GlyCAM-1. ii) Triggering and stable adhesion mediated by LFA-1 ($\alpha_L\beta 2$; CD11a/CD18) integrins on leukocytes (following their activation by chemoattractants) and endothelial counter receptors ICAM-1 and ICAM-2. iii) Transendothelial migration mediated by unknown factors.

However adhesion of lymphocytes to the luminal surface of endothelium is not sufficient to promote migration and accumulation of cells into tissues. As will be discussed below **locomotion and chemotaxis** are excellent mechanisms, for cell accumulation, in acute or chronic inflammatory lesions.

1.2.3.B4. Lymphocyte Locomotion and Chemotaxis

1.2.3.B4.1 Historical background

Although several studies by many researchers in the middle of this century gave surprising amount of insight into the ways in which cells moved, chemotaxis studies were hampered by the lack of suitable techniques for testing soluble substances for chemotactic activity and for collecting quantitative data on the movement of the cells. In 1962, Boyden developed a method (Filter assay) which provided a simple way of quantifying the locomotion stimulus given to a cell by a chemical. Subsequently a number of articles were published using this assay to determine the chemotactic potential of complement component C5a (Shin *et al.*, 1968; Synderman *et al.*, 1970).

However despite the progress of chemotaxis methods the definitions of different forms of locomotor behaviour were unclear until 1977 when Keller and Wilkinson revised the terms describing leukocyte locomotion and presented a proposal for a standardisation of locomotor terminology. There was serious confusion in misuse of terms (relating to the locomotion of leukocytes). First the term, chemotaxis was used in many different ways such as interchangeably using the term chemotaxis (directional locomotion) to describe any form of leukocyte movement. Second confusion is use of the terms random migration or random locomotion to describe the intrinsic locomotor capacity and change in the speed of locomotion.

Current definitions of leukocyte locomotor behaviour are as follow: a) **Chemotaxis** (Pfeffer, 1884) is a reaction by which chemical substances determine the directional of locomotion of cells. It is usually accompanied by morphological orientation of the cells towards the source of concentration gradients of a chemical substance (positive chemotaxis) or away from the source (negative chemotaxis) (Wilkinson 1994); b) **Chemokinesis** (Rothert 1901; Wilkinson, 1985) is used in many different contexts but in the case of leukocytes it refers to the reaction by which chemical substances determine the speed or frequency of locomotion of cells in their environment (orthokinesis); c) **Haptotaxis** (Carter 1967 R27) is defined as chemotaxis along a gradient in which the attractant molecules are surface bound rather than in the fluid (Wilkinson 1995 in press); d) **Random locomotion** is a term which describes the random direction of cell movement along a path which is not oriented in relation to the stimulus. (Wilkinson 1995; Abercrombie 1965; Gail and Boone 1970) e) **Directional locomotion** Locomotion with a preferences for (or avoidance of) a particular direction. The axis of the migrating cell is oriented in relation to the stimulus (Wilkinson 1977; and Wilkinson 1995).

1.2.3.B4.2 Methodology

As mentioned above chemotaxis studies have been faced with a lot of difficulties in finding reliable methods to quantify cell locomotion. For this reason numerous

reports have been published in scientific journals using assays to determine the chemotactic migration of cells *in vitro*. These are as follows:

i. Microfilter assays (Boyden chamber)

This assay was first introduced by Boyden in 1962 and proved to be a reliable method for quantifying the chemotactic migration of neutrophils, and was also used to measure the chemotactic activity of various factors which might cause the locomotion of lymphocytes. The principle of this assay is as follows: A porous filter paper (of size large enough to allow cells to move into the filter by active migration) is separated into two compartments, one containing the chemoattractants (lower chamber) and one containing the cells (upper chamber). The attractant diffuses up through the filter to form a gradient to which the cells respond by migrating through the pores of filter towards its lower surface and then after incubation the filters are fixed. The evaluation of locomotion by counting cells on the bottom of the filter is inaccurate (Keller *et al.*, 1972). However a leading front count method developed by Zigmond and Hirsch (1973) is a more accurate method for evaluation of locomotion using the filter assay.

ii. Polarization assay

As mentioned above the accumulation of lymphocytes at the site of inflammatory tissues requires adhesion and locomotion. Differentiation between these processes, needs assays independent of adhesion to evaluate the locomotor activity of

leukocytes in response to chemoattractants (Wilkinson 1995 in press). An assay which achieves this is the polarization assay (Haston and Shields 1985). It is carried out on cells in suspension (for more details see chapter 2) and it enables leukocytes polarized in response to a chemotactic factor to be scored visually. Compared with the filter assay the advantages of this assay are, short time incubation, low cost, accuracy, and excellent dose response data (Wilkinson 1995 in press; for details, see chapter 3). This assay has been used to study the activity of various chemotactic factors which cause locomotion of either neutrophils (such as FMLP, Casein, LPS-activated serum; Haston and Shields 1985) or lymphocytes such as IL-2 and MIP-1 alpha (Wilkinson and Newman 1994) IL-8 (Wilkinson and Newman 1992), IL-15 (Wilkinson and Liew 1995), synovial fluid (Al-Mughales *et al.*, 1995 submitted for publication), IL-4 and IFN- γ (Wilkinson and Islam 1989) Phorbol ester (Wilkinson *et al.*, 1988) MCP-1 (Wilkinson unpublished observation)

However this assay is not a direct measure of locomotion, but simply of the morphological change that accompanies locomotion but data collected from this assay do correlate very well with locomotion assays, such as collagen gel invasion (Wilkinson 1986 ; Al-Mughales *et al.*, 1995 submitted for publication)

iii. Collagen gel invasion assay

As mentioned above polarization of lymphocytes in response to various stimuli does not allow full evaluation of locomotion. Polarization studies (using

polarization assay) needs to be supplemented with migration studies. In 1982 and 1985 Haston *et al* and Wilkinson respectively, reported the locomotion or migration of normal lymphocytes into a 3- dimensional collagen gel, using the collagen gel invasion assay. This assay analyses very well the invasion of cells into three dimensional collagen gels (Haston, Shields and Wilkinson 1982; Schor *et al.*, 1983) or fibrin gels (Wilkinson and Lackie 1983; Lanier *et al.*, 1988). The collagen gel invasion assay has been used to evaluate the locomotion activity of lymphocytes in response to many chemoattractants.

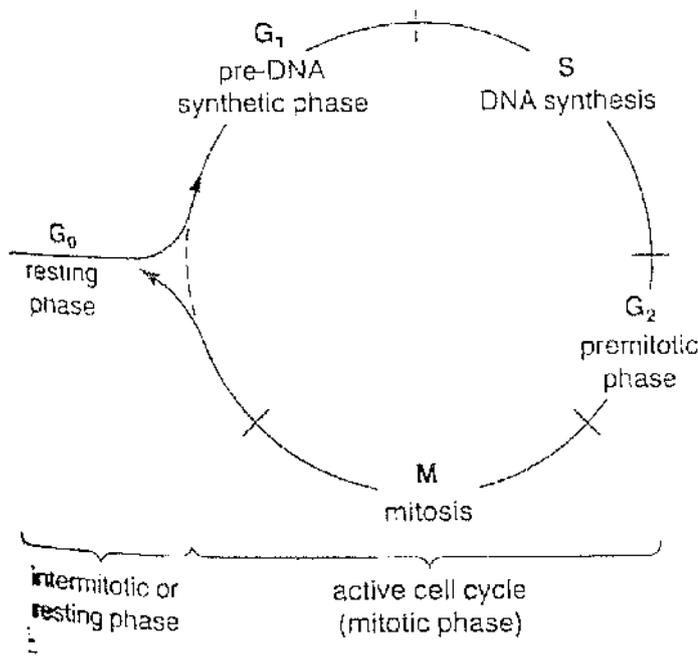
1.2.3.B4.3. Activation of lymphocyte locomotion in vitro

Studies of the behaviour of lymphocyte locomotion showed that most lymphocytes separated from peripheral blood are immotile. These cells are not in cell cycle (G_0) (see Fig 1.1). Several studies have shown that the locomotor capacity of resting T cells is enhanced if they are cultured (24-48h) in the presence of activators of growth, such as phytohemagglutinin (PHA), Con A, mixed lymphocyte culture, OKT3 (anti-CD3), PPD and FCS (Wilkinson 1986; Wilkinson and Higgins 1987; Wilkinson 1987; Wilkinson and Islam 1989; Wilkinson 1992). The idea that lymphocyte locomotor capacity is increased in culture is supported by the finding that these locomotor cells increase in size (Wilkinson 1986) and in uptake of tritiated uridine or leucine. Polarization occurs when lymphocytes move from G_0 to the G_1 phase of growth and polarized G_1 cells show increased synthesis of RNA

and proteins. In addition it has been found that the acquisition of locomotor capacity (G_0 - G_1 phase) can be inhibited by cyclosporin A (Wilkinson and Higgins 1987a) and FK506 (Wilkinson and Watson 1990). Recently it has been found that corticosteroids such as dexamethasone and prednisolone inhibit the acquisition of lymphocyte locomotion. The activation of lymphocyte locomotion occurs during the first 24h of growth and is accompanied by protein and RNA synthesis but precedes DNA synthesis (48 hours). As will be discussed below, once the lymphocytes acquire locomotor capacity they can respond to a variety of chemoattractants which mediate the lymphocyte locomotion either *in vivo* or *in vitro*. This locomotion activity can be blocked either by Cytokine-specific antibodies (Al-Mughales *et al.*, 1996 submitted for publication) or by a variety of anti-rheumatic drugs.

Fig 1.1. Lymphocyte Growth Cycle.

This figure shows 4 phases in mitosis : G_1 (the pre- DNA synthetic phase which is the stage at locomotor capacity develops), S (the DNA synthetic phase), G_2 (the premitotic phase) and M (the actual mitosis). (Adapted from Basic and clinical Immunology. Stites & Terr, 7th ed 1991).



1.2.3.B4.4. Factors Mediating Lymphocyte Locomotion

As mentioned above adhesion is not sufficient to organize and ensure the accumulation of leukocytes at the site of inflammation, because, during the process of adhesion (to endothelial cells at site of inflammation) lymphocytes may be exposed to chemoattractants derived from extravascular sources. These stimuli are thought to regulate extravasation of leukocytes by stimulating movement and directional locomotion (chemotaxis) (Parrott and Wilkinson 1981). The description of these chemoattractants will be discussed below in more detail with special reference to lymphocytes attractants, such as IL-2 (Wilkinson and Newman 1994), IL-8 (Wilkinson and Newman 1992), IL-15 (Wilkinson and Liew 1995), MLP-1 α (Taub *et al.*, 1993; Schall *et al.*, 1993; Wilkinson and Newman 1993), MCP-1 (Carr *et al.*, 1994; Taub *et al.*, 1995).

i. Interleukin-2 (IL-2)

IL-2, was first discovered during stimulation of normal human blood lymphocytes with PHA (Morgan *et al.*, 1976). Subsequently it was reported to have several activities (Ruscetti *et al.*, 1971; Robb, 1984) as a T cell growth factor (TCGF), thymocyte stimulation factor (TSF), T cell replacing factor (TRF), thymocyte mitogenesis factor (TMF), and Killer helper factor and (KHF). Its molecular weight is of 19 Kd by gel filtration chromatography and 14-16 by SD-PAGE (Robb *et al.*, 1981; Kuziel and Greene, 1991; Waldmann 1993; Minami *et al.*, 1993).

IL-2 plays a central role in the regulation of cellular immune responses by expansion of the function of T-lymphocytes (Farrar *et al.*, 1982) proliferation of murine NK cells (Biron *et al.*, 1990) and production of IFN- γ by T cells (Kashara *et al.*, 1983; Yamamoto *et al.*, 1982; Kelin *et al.*, 1983). Several researchers also confirmed the important role of IL-2 in many aspects of the immune response. Blocking IL-2 production in the thymus interrupts T-cell development (Tentori *et al.*, 1988), blocking IL-2 with anti-IL-2R in vivo inhibits the development of virus specific cytotoxic T cells (Leist *et al.*, 1989), blocking the activity of IL-2 may have potential in prevention of allograft rejection (Kirkman *et al.*, 1985; Kelley *et al.*, 1988).

The biological activities of IL-2 are mediated by the binding to a receptor comprising α , β , and γ chains (Tsuda *et al.*, 1986; Sharon *et al.*, 1986; Smith *et al.*, 1989; Takeshita *et al.*, 1990). The α -glycoprotein chain has a molecular weight of 55 KD (IL-2R α ; also known as Tac antigen and CD25) (Nikaido *et al.*, 1984; Cosman *et al.*, 1984). The β -chain has a molecular weight of 75 KD (IL-2R β Hatakeyma; *et al.*, 1989; Minami *et al.*, 1993). The γ chain is 65 KD a transmembrane protein, necessary for high affinity binding and signalling (Takeshita *et al.*, 1992; Cosman *et al.*, 1993).

Although it has been reported that IL-2 is a T cell stimulating factor in rheumatoid synovial tissue, the biological activity of IL-2 in the rheumatoid arthritis is reported to be deficient. Several studies suggested that IL-2 production in vitro by lymphocytes from RA synovial fluid or RA peripheral blood lymphocytes is

very low (Miasaka *et al.*, 1984; Combe *et al.*, 1989; Kitas *et al.*, 1988) and also the IL-2 level in the rheumatoid synovial fluid was found to be low. The lack of this factor may be due to the activity of immune response during the disease activity or due to treatment effects such as steroids which inhibit the IL-2 production (Arya *et al.*, 1984).

It has been reported that IL-2 has chemoattractant activity for human T lymphocytes (Kornfeld *et al.*, 1985). Wilkinson and Newman (1994) reported that IL-2 is a stronger chemoattractant for T lymphocytes, than other chemokines. (see below). Although the IL-2 receptor has several chains (Uchiyama *et al.*, 1981; Miyjima *et al.*, 1992; Sharon *et al.*, 1986) it has been reported that the β chain of the IL-2 receptor is sufficient for activation of locomotion of IL-2 as judged by complete blocking of the locomotion activity of lymphocytes in response to IL-2 by α IL-2R β -chain, and not by α IL-2R α (Wilkinson and Newman 1994).

ii. Interleukin-8 (IL-8)

IL-8 was originally described as a neutrophil activating peptide that was purified in several laboratories (Schroder *et al.*, 1987; Yoshimura *et al.*, 1987; Walz *et al.*, 1987;). These various studies led to the identification of several peptides with 79, 77, 72 or 69 amino acids (Schroder *et al.*, 1990; Gimbrone *et al.*, 1989). The 72- (secreted predominantly by T cells and monocytes) and 77- amino acid peptide forms (secreted predominately by endothelial cells) are reported to be the most

amino acid peptide was suggested to be the most potent form of NAP-1/IL-8 (Nourshargh *et al.*, 1992; Clark-Lewis *et al.*, 1991).

NAP-1/IL-8 has been known under variety of names. It was initially called monocyte-derived neutrophil chemotactic factor (MDNCF; Yosimura *et al.*, 1987); lymphocyte-derived neutrophil-activating peptide (LYNAP; Gregory, II *et al.*, 1988), neutrophil activating factor (NAF, Walz *et al.*, 1987); and the term neutrophil-activating peptide (NAP)-1/IL-8 became widely accepted (Baggiolini *et al.*, 1989). Presently due to studies showing that that NAP-1 IL-8 has chemoattractive/activating properties for different inflammatory cells, the **term IL-8** is most widely used.

Several articles reported that IL-8 is a member of the α chemokine superfamily of 8-10 KD molecular weight (Schall *et al.*, 1994; Murphy *et al.*, 1994; Oppenheim *et al.*, 1991; Baggiolini, *et al.*, 1994; Taub, *et al.*, 1993) characterized by four cysteine residues at identical relative positions. This family is called the α subfamily or -Cys-X-Cys-(C-X-C) (i.e the first two cysteine amino acids are separated by variable amino acids). Other members of this family are , melanoma growth-stimulating activity (MGSA/gro; Schroder *et al.*, 1990; Moser *et al.*, 1990) and neutrophil activating protein (NAP-2; Walz *et al.*, 1989). The gene for these chemotactic factor is found on **chromosome 4q**.

The receptors for IL-8 have isolated and characterized (Samanta, *et al.*, 1990). It has been shown that human IL-8 has two receptors termed IL-8 RA and IL-8 RB (Holmes *et al.*, 1991). The IL-8 RA is a 350 amino acid length with five potential

N-linked glycosylation sites and seven hydrophobic membrane spanning stretches characteristics of the G-protein linked family of molecules (Strosberg 1991). The IL-8 RB is 355 amino acids in length with one potential N-linked glycosylation site . It was also observed that in the rabbit, F3R, which was reported to be an N-formyl-Met-Leu-Phe (fMLP) receptor has turned out to be the rabbit IL-8 receptor (Lee, J. *et al.*, 1992).

Different cellular sources have been reported to produce IL-8. Although human fibroblasts are important for IL-8 production in response to IL-1 or TNF (Strieter *et al.*, 1989; Schroder J *et al.*, 1990; Van Damme *et al.*, 1989; Larsen, C *et al.*, 1989), human macrophages appear to be the principal source of IL-8 (Koch *et al.*, 1991). Human synovial cells also release neutrophil stimulating activity when challenged with IL-1 (Watson *et al.*, 1988), and also other cells produce IL-8 such as synovial chondrocytes (Brennan *et al.*, 1990), and articular chondrocytes (Lotz *et al.*, 1992). IL-8 is reported to play a major role in neutrophil diapedesis through vascular endothelium and focal recruitment at inflamed sites. This study is supported by the observations that upon exposure to IL-1 or TNF, endothelial cells not only express adhesion proteins (Bevilacqua *et al.*, 1985; Pohlman *et al.*, 1986) that promote their interaction with circulating neutrophils but also produce IL-8 (Srieter *et al.*, 1988; Schroeder *et al.*, 1989).

IL-8 is also effective in vivo. Intradermal injection in rabbits results in plasma exudation and massive neutrophil infiltration (Colditz *et al.*, 1989; Endo, H. *et al.*, 1991).

Several studies have also reported that IL-8 has chemoattractive/ activating properties for neutrophil in the joint cavities of RA and other arthritides. Although a large proportion of the chemotactic activity for PMN in the synovial fluid was due to IL-8 (Elford and Cooper 1991; Koch A. *et al* ., 1991) no correlation existed between the concentration of IL-8 (Koch et al., 1992) and accumulation of neutrophils or lymphocyte count in the RA and non RA.

The effect of IL-8 on the *in vitro* locomotion of human activated lymphocytes was studied. It was observed that IL-8 induced chemokinesis and chemotaxis in these cells as determined by several locomotion assays (Wilkinson and Newman 1992).

IL-8 was released into the medium when mononuclear cells were cultured with a CD3 or PPD and induced locomotion and chemotaxis of the activated population of cultured lymphocytes. This might suggest that *in vivo* such activated lymphocytes would respond to specific signals from IL-8 in inflammatory sites by leaving the circulation and migrating into the lesion.

iii. Interleukin-15 (IL-15)

Recombinant IL-15 (14-15 Kd on SDS-PAGE) was originally purified from a simian kidney epithelial cell line and shown to have lymphocyte activating properties like IL-2 (Grabstein, K *et al.*, 1994). Activation of T cells by IL-15 is inhibited by preincubating the cells with antibody to the β chain of the IL-2 receptor. Modelling studies have suggested that IL-15 shares a similar three dimensional structure with IL-2 but has a different sequence from IL-2. IL-15

exerts its biological activity through the β and γ chain of the IL-2 receptor (Grabstein, *et al.*, 1994; Giri, *et al.*, 1994). Subsequent investigations reported that IL-15 mediates T cell (Burton *et al.*, 1994;), B cell (Armitage *et al.*, 1995) and NK cell proliferation (Bomford *et al.*, 1994; Carson *et al.*, 1994).

The chemoattractant activity of IL-15 for human lymphocytes was studied. Wilkinson and Liew reported that IL-15 stimulates locomotion of T lymphocytes as judged by several locomotion assays. IL-15 is widespread in the RA synovium and IL-15 levels in the synovial fluid are high (MacInnes *et al.*, 1996). Moreover it has been found that the immunological activities of T helper cells, B cells and NK cells in the *in vivo* immune response of IL-2 knockout mice were normal (Kundig *et al.*, 1993) which might suggest an important alternative role for IL-15.

iv. Macrophage Inflammatory-Protein-1 (MIP-1 α)

MIP-1 α (7.8-KD on SD-PAGE) is a heparin binding protein, which was originally identified during stimulation of murine macrophages with Lipopolysaccharide (LPS) (Oppenheim, J. *et al.*, 1991; Davatelis, G. *et al.*, 1988; Wolpe *et al.*, 1988; Wolpe and Cerami 1989). MIP-1 α , belongs to the family of chemokines that have been called the intercrine β subfamily, defined by the Cys-Cys- (C-C) motif. This family includes MIP-1 β , monocyte chemotactic and activating factor (MCAF, MCP-1), hRANTES, Hact-2h, and MJF,. The cellular sources of this molecule are various such as T cells, B cells, monocytic cells, mast

were shown to express significant levels of MIP-1 α via immunochemistry, both spontaneously and in response to lipopolysaccharide (LPS) (Denis and Ghadirian 1994).

Several investigators have shown that MIP-1 α exerts a variety of biological activities such as chemoattraction of monocytes and eosinophils (Taub *et al.*, 1993) as well as induction of chemotaxis for neutrophils (Wolpe *et al.*, 1988; Alam *et al.*, 1992), basophils and mouse mast cells (Alam *et al.*, 1992). In addition it has been reported that MIP-1 α increases the adherence of T cells into inflammatory sites (Taub *et al.*, 1993). The chemoattractant activity of MIP-1 α for human lymphocytes was first reported by Taub *et al.*, 1993 and Schall *et al.*, 1993. Subsequently Wilkinson and Newman reported that MIP-1 α is chemoattractant for normal human lymphocytes as judged by several locomotion assays (Wilkinson and Newman 1994). In addition it has been shown that MIP-1 α in supernatants of alveolar macrophages has strong chemotactic activity for highly purified CD8⁺ T cells as judged by neutralizing the activity of MIP-1 α (Denis and Ghadirian 1994).

v. Macrophage chemotactic protein-1 (MCP-1)

MCP-1 (alternative designations: JE, monocyte chemotactic and activating factor, tumour-derived chemotactic factor) is a 76 amino acid protein that structurally belongs to the C-C supergene family of chemokines (see above) and has specific

chemoattractant and activating properties for monocytes (Leonard, *et al.*, 1990; Schall *et al.*, 1991; Van. Damm *et al.*, 1989 MCP-1 9). The human MCP-1 gene maps to chromosome 17 (q 11.2 -12; Miller and Krangel 1992). However MCP-1 is produced by various cell types, including PHA-stimulated blood mononuclear cells (Yoshimura, T. *et al.*, 1989), fibroblasts (Lukacs *et al.*, 1994), tumour cells, LPS treated macrophages, human liver fat storing cells, endothelial cells and smooth muscle (Christensen *et al.*, 1993; Marra *et al.*, 1993;).

The biological function of MCP-1 has been reported by many investigators., MCP-1 is a potent stimulus of histamine release from human blood basophils (Bischoff, *et al.*, 1992; Kun *et al.*, 1992), stimulates macrophage infiltration of tumours, which suggests its ability to stimulate the killing of tumour cells in animal models (Charo *et al.*, 1994; Matsushima, K *et al.*, 1989). It is also suggested that monocyte recruitment into the artery wall is a critical step in the initiation of atherosclerosis, MCP-1 was reported to be abundant in the active macrophage-rich areas of human atherosclerosis plaques (Charo, *et al.*, 1994). In addition MCP-1 is reported to mediate monocyte recruitment of tissues in various inflammatory diseases such as alveolitis (Jones *et al.*, 1992) and rheumatoid arthritis (Koch, *et al.*, 1992).

Recently it has been found that MCP-1 has chemoattractant activity for human lymphocytes in vitro. Carr *et al.* (1994) reported that MCP-1 is the major lymphocyte chemoattractant secreted by mitogen-stimulated peripheral blood mononuclear cells and is capable of acting as a potent T- lymphocyte as well as

monocyte, chemoattractant. This may explain why monocyte and T lymphocytes of the memory subset are always found at sites of antigen-induced inflammation (Carr et al 1994). MCP-1 has a chemoattractant activity for human blood lymphocytes in vitro at optimal concentrations of about 1ng/ml (Wilkinson 1995; unpublished observation).

vi. Foetal Calf Serum

The chemoattractant activity of FCS serum was investigated by several researchers. It has been reported that culture of lymphocytes in FCS cause a large proportion of lymphocytes to change from a round to a polarized shape (O' Neill and Parrott, 1977). A Similar study reported that neat or 20%, FCS can stimulate the locomotion activity of lymphocytes (45% or 20% respectively) as judged by short term polarization assays (Wilkinson 1986).

1.2.4. Laboratory evaluation of systemic and chronic rheumatic inflammatory diseases.

1.2.4.A. Introduction

The inflammatory immune response in rheumatic diseases mentioned above play an important role in the immunopathogenesis of these diseases.

Several laboratory methods have been used to evaluate the metabolic changes in the joint and peripheral blood including histopathology, immunochemistry, blood analysis. The major important changes noted are in the increase of concentration of acute phase proteins such as complement components, α_1 antitrypsin, fibrinogen, haptoglobin and major important changes have been observed in the concentration of C-reactive protein and erythrocyte sedimentation rate (Several hundred fold the normal value) which have become widely employed indicators of the acute phase response in the rheumatic diseases and other systemic diseases.

1.2.4B. Erythrocyte sedimentation rate

The ESR is a single most important laboratory test of inflammatory activity. The increased rate of settling of erythrocytes in the blood of patients with inflammatory diseases has been known for many years. Measurement of the ESR was first applied to the study of the acute and chronic rheumatism by Herman in 1924. The principle of this test relies on the concentration of fibrinogen and other acute phase proteins which cause increase aggregation of erythrocytes suspended

in plasma (rouleaux formation) causing them to fall more rapidly (Ballou and Kushner 1993). The international committee for standardisation in haematology has recommended a method for ESR determination called Westergren's method (ICSH. 1977).

1.2.4C. C-reactive protein.

CRP is present in all RA patients with clinical evidence of disease activity. C-reactive proteins were originally detected in human serum because of their ability to interact with somatic C-polysaccharide of the pneumococcus (Ballou and Kushner 1993). Its serum level rises and falls faster than ESR which gives an indication of the current inflammatory activity of patients more accurately (Morley et al., 1982). Following acute inflammation the serum concentration increases within a few hours and there is an increase in numbers of hepatocytes producing C-RP (Powell. 1983). The most widely used method for C-RP determination is immunoturbidometry assay.

1.2.5 Control of systemic and chronic inflammation in rheumatic diseases using anti-inflammatory drugs.

1.2.5A. Non Steroidal Anti-inflammatory Drugs (NSAIDs)

In 1977 Brook and Corbett reported that the serious complications in RA occur within 2 years of disease onset. Hence rheumatologists have suggested that treatment with arthritis should be started with simple antirheumatic drugs (first line drugs) such as analgesic and non-steroidal antiinflammatory drugs (NSAIDS) (Brooks 1985). These are relatively lipid soluble, weak acids (they have a carboxylic acid, keto-enolic moiety giving them a pKa of 3-6 which enables them to accumulate in inflammatory sites where the pH is about 6-9.5) (Rainsford 1994), and almost all are absorbed from the gastrointestinal tract (Langman 1989; Fries et al., 1989). The presence of these drugs at the inflammatory site including synovial tissue is critical for their manipulation of inflammation and other undesirable inflammatory processes such pain (pain controlled by NSAIDs may be at the level of the central nervous system by regulation of neurokinines). The mechanism of action of NSAIDs is as follows: They inhibit the biosynthesis of prostaglandin, thromboxane and prostacyclin by irreversibly blocking the enzyme cyclo-oxygenase (prostaglandin synthetase) which catalyzes the conversion of arachidonic acid to endoperoxide compounds (Rinsfoord 1994; Forest and Brook 1988; Brook and Day 1991; Allen K 1995). They can also exert other functions including (c.g Aspirin indomethacin, Ibuprofen) suppression of lymphocyte

transformation (Opeiz *et al.*, 1973;), inhibition of cartilage degeneration (Palmoski and Brandt 1983). In addition it has been reported that NSAIDs inhibit the migration, adhesion, and locomotion of leukocytes as well as the release of lysosomal enzymes (Brown and Collins 1977; Spisani *et al.*, 1979; Higg *et al.*, 1980). A Further action of NSAIDs is the interfering with the kallikrein system (Kallikreins are proteinases which generate small peptide molecules, kinins, from a protein substrate, kininogen) (Cuthbert 1994) and cell membrane activities (such as enzyme, NADPH oxidase and phospholipase). New generation of NSAIDs can inhibit the production or activity of cytokines such IL-1 (Rainsford 1994). For more detail about the sites of action of NSAIDs see Fig 1.2.

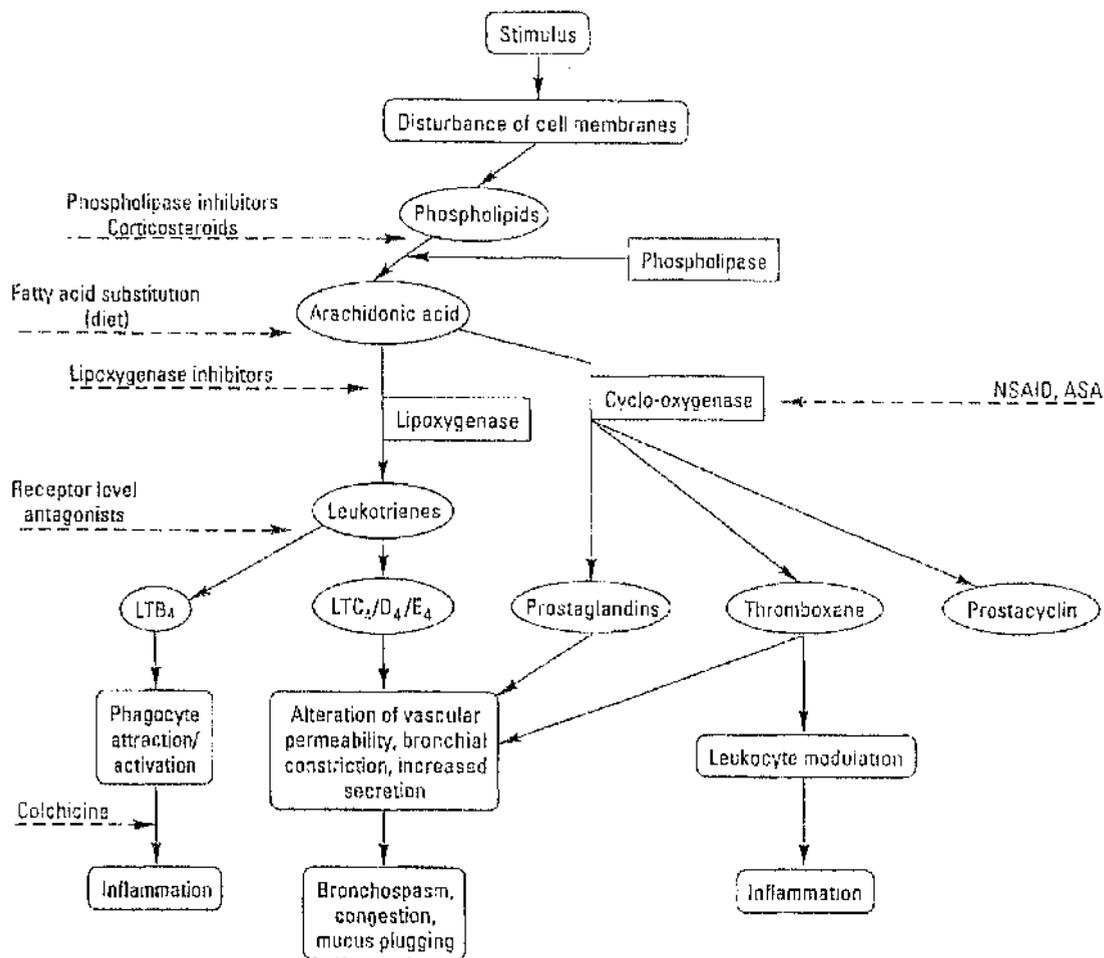
1.2.5B. Disease-modifying antirheumatic drugs

The second goal of rheumatic disease treatment is to alter the disease progression and outcome using second line or disease-modifying antirheumatic drugs (DMARDS). Examples are Gold, D-penicillamine and anti-malarial drugs.

There have been several trials using combined DMARDS to rheumatoid arthritis, such as hydroxychloroquine, penicillamine and hydroxychloroquine, penicillamine and chloroquine, penicillamine and salphazalazine (Dawes *et al.*, 1987; Bitter 1984; Gibson *et al.*, 1987). The mechanism of action of these drugs as follows:

(i) Gold compounds were introduced for treatment of RA in 1929 (Forestier, J., 1935; Kean *et al.*, 1985) and most commonly administrated gold preparations are aurothiomalate and aurothioglucose (Harfall *et al.*, 1937). Gold compounds

Figure 1.2. Scheme for mediators derived from arachidonic acid and site of action of NSAIDs (adapted from Basic and clinical pharmacology, Katzung, D.G, 4th ed 1989).



available for therapeutic use contains elemental aurous gold (Au⁺) combined with a sulfur-containing ligand. The mode of action of gold in RA still unclear. Several investigators reported that gold suppresses activity of lymphocytes, prostaglandin and leukotrienes (Lies, *et al.*, 1977; Lipsky and Ziff 197).

(ii) D-penicillamine (D-(-)-2-amino-3mercapto-3methylbutyric acid) is a metabolite of penicillin and can be prepared from it by acid hydrolysis (Lock *et al.*, 1986; Joyce and Day 1990). The mechanism of action in RA still unclear, but treatment with this drug interferes with the lymphocyte membrane receptors and inhibits the production of RF by binding to the disulphide bonds (S-S). It has been reported that D-penicillamine in the presence of copper ion markedly inhibited mitogen--induced human T lymphocyte DNA synthesis. The mechanism by which D-penicillamine in the presence of copper inhibits T lymphocytes responsiveness was reported to be due to the production of the generation of hydrogen peroxide (Lipsky, 1984).

(iii). Antimalarial drugs

Chloroquine or hydroxychloroquine have been used extensively in the treatment of RA. Anti-malarial drugs include 4-aminoquinoline-chloroquine, 8-aminoquinoline-primaquine, and others (Golsmith 1989). Treatment with these drugs interferes with plasma proteins, RNA/DNA polymerase (Salako, 1985; McChesney *et al.*, 1966). Their interaction with phospholipids decreases the lymphocytes fluidity (Beccrericica, 1989). In addition it has been shown that the

anti-malarial drugs (e.g Chloroquine) interfere with antigen processing and antigen presentation (Davis P, 1994).

1.2.5C. Cytotoxic drugs

Cytotoxic drugs are used for the treatment of rheumatoid arthritis patients if their illness has failed to respond to less aggressive drugs or has been complicated by systemic vasculitis (Hofman, 1993). Concern about toxicity, including the induction of malignancies has until recently limited more routine use and reassessment of the morbidity in rheumatoid arthritis has lead many rheumatologist to re-evaluate their strategies of when such drugs should be used in the manipulating of the progressive disease (Hofman 1993). As discussed below cytotoxic drugs such as cyclosporin A , rapamycin cyclophosphamide and Glucocorticoids (including dexamethasone, prednisone and prdednisolone) interfere with specific phases of the mitotic cell cycle (Fig). There are 4 phases in mitosis : G1 (pre-DNA synthetic phase), S (the DNA synthetic phase), G2 (the premitotic phase) and M (the actual mitosis). Cells in a prolonged intermitotic period are consider to be in a G0 phase (Albert *et al.*, 1989). However any suppression effect on the cell RNA/DNA cell replication and proliferation leads to suppression of production and function of immune competent cells and other cells, in manner that dose not discriminate against those cells that may be of most pathologic significance.

i. Cyclosporin A

Cyclosporin A (CsA) is a biologically active metabolite extracted from two species of fungi called a *Cylindrocarpon Lucidum Booth* and *Trichoderma Polysporum Rifai* (Borel *et al.*, 1976). It is used widely as an powerful immunosuppressive drug (Borel, *et al.*, 1977) in the treatment of several diseases including rheumatoid arthritis (Douglas *et al.*, 1988). Structurally CsA is a cyclic polypeptide consisting of 11 amino acids (one of them unsaturated C-9 that is unique to this molecule) (Hess & Tuschka 19980), 1976) and is insoluble in water but it can dissolve in ethanol. Several experimental animal studies have been reported that CsA inhibits antibody formation to T cell -dependent antigens, the prolongation of skin graft survival and suppression of delayed-type hypersensitivity reactions (Borel *et al.*, 1988). CSA also exerts its immunosuppressive effects by selectively inhibiting the transcription of messenger RNA of IL-2 and IL-2 receptors by CD4^+ T cells by at an early stage of growth (G1) and other cytokines including IL-3, IL-4, IL-5, IL-6 (Grilly *et al.*, 1995; Hedges 1993). CsA was also found to inhibit the mitogen activated but not phorbol ester-activated locomotion of human lymphocytes *vitro* (Wilkinson and Higgins 1987). In addition a recent study reported that CsA combined with methotrexate had a significant effect on the improvement of patients with sever RA (Tugwell *et al.*, 1995). It has been observed that CsA mediates its immunosuppressive effect through an abundant cytoplasmic protein termed cyclophilin of 17 kda. (Sigal & Dumont 1992).

ii. Rapamycin

Rapamycin was originally extracted from soil micro-organisms and became widely used as an antibiotic and as an immunosuppressive drug (Vezina *et al.*, 1975; Martel *et al.*, 1977). Like CsA it can affect a wide variety of cellular activities but its mechanism of action involves binding to DNA through intercalation with a consequent blockade of the synthesis of DNA and RNA (Sigal and Dumont 1992). Rapamycin has similar, but not identical actions to cyclosporin A, and may also prove a less toxic immunosuppressant than the former (Fan and Rainsford 1994). Several studies have reported that rapamycin inhibits murine and human T cell proliferation. It interferes at a later time in the activation processes than does CsA which act at an early stage of T cell signal transduction (Sigal and Dumont 1992). Further studies reported that rapamycin delays the growth of tumour cell lines transplanted in mice, suggesting that this compound is a general anti-proliferative agent (Sigal and Dumont 1992).

iii. Cyclophosphamide

Cyclophosphamide is one of the alkylating agents that have been widely used to treat rheumatic disease (Clement *et al.*, 1974). Its mode of action is inhibition of a wide variety of cellular processes including T and B lymphocytes (Stockman *et al.*, 1973). The alkylation of guanine in DNA can lead to miscoding, destruction of the purine ring and inhibition of DNA replication through cross-linking. Although the alkylating agents are not cell cycle specific, cells are most susceptible to

alkylation in late G1 and S phase of the cell cycle and express blockage in G2 (Kovarsky 1983).

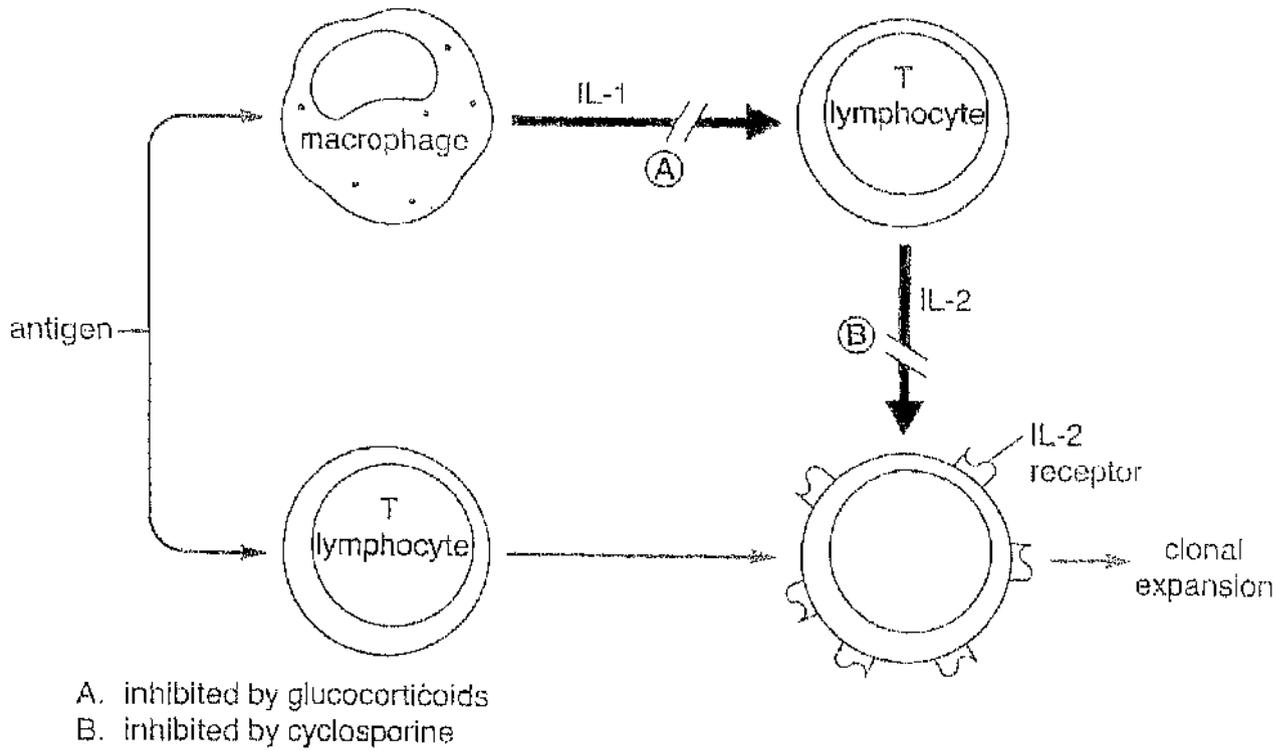
1.2.5D. Glucocorticoids

Glucocorticoid hormones (GcH) are used widely in the therapy of inflammatory diseases including rheumatoid arthritis (Morand *et al.*, 1994). They mediate their action through cytosolic receptors. The glucocorticoid-receptor complex then moves quickly into the nucleus. They bind to the to glucocorticoid-responsive elements of genes, thereby influencing gene transcription. In the immune system GcH acts primarily by inhibition of cytokine production and lymphocyte activation.

Several studies have reported the inhibitory effect of GcH upon lymphocytes and their response to mitogens (Dugherty, 1952). In addition it has been reported that GcH have an inhibitory effect on RNA, DNA and protein synthesis in lymphocytes (Makman *et al.*, 1968). The number of circulating lymphocytes is reduced within 24h of GcH injection and more CD4+ T cells are affected than B cells. Also cells of the monocyte-macrophage lineage, NK cell (Parillo and Fanci 1979) and neutrophil functions are affected. It has been observed that lung inflammation is down regulated by dexamethasone therapy which affects the release of inflammatory mediators and the influx of neutrophils into the airways (Groneck *et al.*, 1993). The activity of GcH on cytokine production has been extensively studied. For example it has been found that GcH acts primarily by

inhibition of cytokine production and T cell activation. GcH inhibits the production of IL-1 (see fig 1.3), IL-6, IL-2, IL-4, TNF- α , and INF- γ (Kavelaars *et al.*, 1995; Rakasz *et al.*, 1993; Schlaghecke *et al.*, 1994). Taken together inhibition of lymphocyte activation by GcH may operate by the following mechanisms (Mendelsohn *et al.*, 1977): (1) they suppress recruitment of cells from G0 to G1 phase of the cell cycle. (2) they inhibit progression from G1 phase into S phase (3) they reduce the rate of DNA replication.

Figure 1.3. Sites of action of glucocorticosteroids and cyclosporin A.(adapted from Basic and Clinical Immunology, Stites and Terr, 7th ed 1991).



Section 2: OBJECTIVE OF PRESENT RESEARCH.

i. Introduction

Tissue lymphocyte infiltration occurs in response to different pathological stimuli. Lymphocyte locomotion across vascular endothelium venules (HEV) into inflamed sites plays a crucial part in initiating and modifying the disease process. Despite their importance, our knowledge of factors stimulating and controlling lymphocyte locomotion and chemotaxis into rheumatoid lesions is still unclear. Although it would be advisable to examine synovial tissue from each patient with RA to compare histological changes with those from previous specimens and assay for lymphocyte subset and chemotactic factors production by these cells or synovial cells, this is very difficult and impractical. Synovial fluid is a good source; by examination of its characteristics (Chemoattractant activity, cell count, chemotactic factors levels), one can gain a good appreciation and learn more about the extent of inflammation and events occurring in the synovium itself.

ii. AIM of research.

To study the role of chemotaxis in the accumulation of T lymphocytes in rheumatoid and other inflammatory joint diseases.

I. Examine the chemotactic activity of synovial fluids from RA in comparison to OA and other inflammatory arthritides where the lymphocytes infiltrate is much less. The Study involves the response of normal blood T cells to joint fluids.

II. To identify and quantify T lymphocyte chemotactic factors in joint fluid and to use specific monoclonal antibodies to inhibit their activity.

III. To study the response of synovial tissue T cells to joint fluids and to cytokines in comparison with cells from blood.

IV. To study the role of activation of T cells in the locomotor response to joint fluid. Phenotyping of responsive cells for activation markers, e.g CD45RO.

V. Study of the inhibitory effects of anti-rheumatic drugs on the locomotion of T cells.

CHAPTER 2.

PATIENTS, MATERIALS AND METHODS

2.1 Patients

The clinical categories of the patients used in this study are summarised in table 1 and 2. Synovial fluid was obtained from joints of patients attending the Rheumatology day ward at Gartnavel General Hospital and Glasgow Royal Infirmary for aspiration and corticosteroid injection into their painful joints. The fluid was taken by rheumatologists from knee joints since synovial fluid is most easily obtained. Synovial fluid was transferred to transport medium and was in the laboratory within about 1-2 hours. The fluid was aspirated using full aseptic precautions and before any steroid was injected. Fifty one patients with a confirmed clinical diagnosis of rheumatoid arthritis or other arthritides were studied. The patients were classified into two groups, RA and non RA. The RA patients (n= 35 median age 56; range 33-91 years) diagnosed according to the American College of Rheumatology criteria (Arnett, *et al* 1988) were classified according to disease duration (see Table 1a, 1b, 1c). The non RA group were Osteoarthritis (n=7 median age:76; range 59-78), ankylosing spondylitis (n=5 median age: range 30-70) psoriatic arthritis (n=3 age 48 and 70 years) intermittent hydroarthrosis (n=1 age 32), polymyalgia rheumatica (n=1 age 48) and pyrophosphate arthropathy (n=1, age 77). The drug regimen in the RA group

consisted of non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen, Voltarol, Indocid, and disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate, azathioprine, sulphasalazine, or gold. Corticosteroids such as prednisolone were also used. Cytotoxic drugs were occasionally used. The non RA group were either treated with NSAIDs or not treated. Only a few patients were treated with DMARDs. The Westergren erythrocyte sedimentation rate (ESR) , and C-reactive protein (Beckman), antinuclear antibodies (Immunofluorescence) and rheumatoid (Rose-Waaler or ELISA) factor were routinely measured in all patients.

Table 2.1A . Descriptive characteristics of the patients with RA

disease duration (1-10 years).

Code	Disease duration (years)	Age (years)+Sex	Overall severity+Erosion	Drugs
W.C	4	67 (F)	SEV+Eros (++)	Feldene+SASP
M.G	8	57 (F)	MOD+Eros (++)	Ibuprofen+SASP
G.H	6	76 (M)	SEV+Eros(++)	Oruvail
C.W	9	63 (F)	MOD+none	Relifex+Meptide
R.M	4	82 (F)	SEV+Eros(+++)	Voltarol+Gold
M.MA	9	66 (F)	SEV+Eros(++)	SASP+Feldene
M.MO	8	52 (F)	MOD+Eros(++)+nodules	Ibuprofen+Penicillamine
D.G	7	35 (M)	MOD+none	Voltarol+Gold
E.M	9	59 (M)	SEV+Eros(++)+anemia	Ibuprofen+Gold
R.E	9	50 (M)	SEV+Eros(++)	Indocid+penicillamine
H.M	7	44 (M)	SEV+Eros(+++)	Methotrexate+Prednisolone
M.EZ	4	70 (F)	SEV+Eros(++)	NA
H.Y	6	88 (F)	V.seve	Sulindac+acupan
JK	9	60 (F)	NA	Penicillamine+Naproxen+ Co-cadamol
IS	9	39 (F)	NA	Indomethacin+Imuran+ Co-cadamol
LM	6	37 (F)	NA	Gold

2.1B: RA, Disease duration (11-20 years)

Code	Disease duration (years)	Age (years)+Sex	Overall severity+Erosion	Drugs
IF	20	81 (F)	MOD+Eros (++)	Voltarol+SASP
M.B	19	80 (F)	SEV+Eros(++)	NA
S.S	17	53 (F)	SEV+Eros(+++)+Raynaud's	Sulindac+SASP
J.C	20	56 (M)	SEV+Eros(++)	Relifex+Penicillamine
R.L	16	51 (M)	V.SEV+Eros(++)+NFA	Feldene+cyclophosphamide
S.K	11	33 (M)	MOD+none	SASP+Naproxen
D.R	14	67 (F)	NA	Feldene
B.L	19	77 (F)	NA	NA
C.M	13	55 (F)	MOD+Eros(+++)	Voltarol
E.P	16	56 (F)	SEV+Eros(++)	Aspirin+Gold
G.J	18	41 (F)	NA	NA
M.N	13	60 (F)	NA	Copraxamol+Methotrexate+ Analgesics

2.1C : RA, Disease duration(>20 years).

Code	Disease duration (years)	Age (years)+Sex	Overall severity+Erosion	Drugs
A.M	25	60 (F)	MOD+Eros (++)	Indocid+SASP
W.M	23	80 (F)	SEV+Eros (+++)	SASP
M.J	51	50 (F)	SEV+Eros (++)	Naproxen
G.W	40	47 (M)	SEV+Ero (++)	Indocid+Penicillamine
D.M	23	59 (F)	SEV+Eros (++) +Nodules	Voltarol
J.A	31	53 (M)	MILD+Eros (++)	Indocid+SASP
M.S	40	74 (F)	Mild+Eros (++)	Indocid

Synovial fluid was collected from different patients with rheumatoid arthritis (n=35) 16 patients with disease duration of 1-10 years (Table 1a), 12 patients of 10-20 years (Table 1b) and 7 patients with >20 years disease duration (Table 1c) were in this study. RA patients were treated with NSAIDs: Ibuprofen, indocid, Voltarol, and Disease modifying antirheumatic drugs (DMARDs): gold, D-penicillamine, salphasalazine (SASP), methotrexate, naproxen and aziothioprine; or corticosteroids such as prednisolone or cytotoxic drugs such as cyclophosphamide A, Eros=erosion, NA=information not available, RA=rheumatoid arthritis, Sev=severe, Mod=moderate, M=Male, F=Female.

Table 2.2. Clinical description of the patients with non RA.

Code	Provisional diagnosis	Disease duration	Age (years)+Sex	Overall severity	Drugs
H.Me	OA	5	78 (F)	Mild+Eros	NA
D.SC	OA	5	76 (M)	Mod	Voltarol
A.SO	OA	10	59 (F)	Mod	Voltarol
E.Mc	OA	15	72 (F)	Mild	Paracetamol
D.Ba	OA	12	78 (M)	Mild	Paracetamol
A.La	OA	10	68 (M)	Mild	Co-Cadamol
E.Ch	OA	10	66 (F)	Mild	NSAIDs+ Paracetamol
D.SM	AS	8	44 (M)	V.SEV+Eros	Gold+Ibuprofen
D.SH	AS	5	30 (M)	Mod	Voltarol
W.G	AS	30	70 (M)	Mild	Ibuprofen+ Coproxamol
R.Mc	AS	15	NA	NA	NA
C.Ca	AS	2	54 (F)	Seve+Eros	Salazopyrn+ Voltarol+ Paracetamol
M.T	PSA	7	48 (F)	NA	Paracetamol
W.S	PSA	8	70 (M)	Mild	Ibuprofen
B.T	PSA	4	58 (M)	Seve+Eros	Lederfen+ Copraxamol
S.T	I.M.H.A	10	32 (F)	Mild	NA
B.F	PMR	2	58 (M)	Mild	Prednisolone
G.M	PARP	1	77 (F)	NA	Paracetamol

SF sample from patients with non RA (n=18). Osteoarthritis (OA, n=7), ankylosing spondylitis (AS n=5), Psoriatic arthritis (PSA n=3), intermittent hydroarthritis (IMHA, n=1), Polymyalgia Rheumatica (PMR n=1) and pyrophosphate arthropathy (PARP, n=1). Patients in this group were untreated or treated either with analgesics and NASIDs or DMARDS.

NA=information not available, M=Male, F=Female, SEV=Severe.

2.2 Materials

2.2.1 Preparation of Media

Hanks' balanced salt solution (**HBSS**), and calcium and magnesium free salt solution (ICN Flow Ltd., High Wycombe, U.K) were obtained as 10x strength solutions. These were diluted to 1x strength with distilled water and were buffered to pH 7.4 with (3-[N-morpholino] propane sulphonic acid (MOPS: Sigma Chemical Co, Ltd., Poole, UK) or **HEPES** (Gibco BRL, Life Technologies Ltd., Paisley, U.K) . **Hepes** (N-2-[hydroxyethyl] piperazine-N-[2-ethane sulphonic acid) was used at 20mM. The pH of HBSS was adjusted to 7.4 using 2M NaOH or HCL (BDH), if necessary.

RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley) was obtained as a 1x strength solution with sodium bicarbonate. This was supplemented with 10mM HEPES buffer, 2mM L-glutamine, 100u/ml penicillin, and 100µg/ml streptomycin

2.2.2. Foetal calf serum (FCS)

FCS (Flow , Rickmansworth, Herts, England) was heat inactivated at 56 °C for 30 minutes and then diluted in HBSS. For routine use FCS was diluted either in HBSS as 25% (v/v) or 5-10% in RPMI. A 25% FCS was used to culture the cells for long periods (24, 48, hrs).

2.2.3. Human serum albumin (HSA)

HSA (Behringwerke, Marburg,FRG) was used at 10 mg/ml in HBSS (HBSS/HSA).

2.2.4. N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma)

was prepared as a stock solution of 10^{-2} M in dimethyl sulphoxide (DMSO , Grade I, Sigma) and stored at -20°C .

2.2.5. Preparation of buffers

2.2.5. A1 MOPS

One molar **MOPS** [3-(N-morpholino) propanesulphonic acid] pH 7.4, was used as standard buffer in most media preparations. MOPS (Sigma) was prepared by dissolving 20.93g in 90ml of distilled water . Final volume was adjusted to 100 ml by adding sterile distilled water. Then the pH was adjusted to 7.4 by adding an appropriate amount of sodium chloride (BDH) and stored at 4°C . This buffer was routinely used as 10mM.

2.2.5.A2. Phosphate Buffered Saline

PBS (pH7.4) was prepared at 25x strength in one litre of distilled water by dissolving 200 g of NaCl (BDH); 5g of KCL BDH) ; 5g of KH_2PO_4 (BDH) and 28.75g of NA_2HPO_4 (BDH) . For routine use it was diluted to 1x strength in

distilled water.

2.2.5.A3. Tris-buffered saline (TBS, pH 7.6)

It was prepared as stock 0.5 M Tris-HCL in water and was diluted 1/10 in saline.

This buffer was used throughout as diluent for APAAP substrate, diluting of antibodies and for washing slides between incubation steps.

2.2.5A4. IL-15 ELISA coating buffer

1.59g of Na_2CO_3 (BDH), 2.9g of NaHCO_3 (BDH), were dissolved in , one litre of distilled water and pH was adjusted to pH 9.6, or 2.9 g of 0.1M Sodium bicarbonate was dissolved in 250 ml of distilled water and the pH was adjusted to pH 8.2. The coating buffer can be stored for 2 weeks at 4 °C.

2.2.5A5. Substrate buffer for IL-15 ELISA

$\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ (0.10g, BDH), and 0.20g of sodium azide (NaN_3 , BDH) were dissolved in 800ml of water and then 97ml of diethanolamine was added and the pH was adjusted to pH 9.8.

2.2.5.A6. Washing buffer for IL-15 ELISA (PBS/Tween)

Tween 20 (0.5ml; Sigma) was mixed with 1 litre of PBS and stored at 4°C for 2 weeks.

Saline

This was prepared by dissolving NaCl (8.5g/l; BDH) in distilled water and the solution was sterilised at 121⁰C for 15 min or it can be prepared as 25X without sterilisation.

2.2.6. Preparation of fixative.

To ensure the best preservation of structural detail of lymphocytes, different fixatives were used in this study as follows:

2.2.6A1. Glutaraldehyde

Grade II glutaraldehyde (25% aqueous solution, Sigma, Poole, Dorset) was used as a 2.5% solution. A 2.5% stock was prepared from 10 ml of 25% aqueous solution in 90ml saline and stored at 4 °C. It was used as the routine fixative in polarisation and collagen gel assays. Glutaraldehyde was not suitable for fluorescence studies because it gives strong background fluorescence (Nicholas, *et al.*, 1992). Therefore, for these studies, paraformaldehyde was used.

2.2.6A2. Paraformaldehyde

Paraformaldehyde (BDH) was prepared at 1% (w/v) by dissolving paraformaldehyde powder in PBS at 60-70 °C for 45-60 min. A stock solution was stored at 4 °C. This fixative allows both a reasonable preservation of cell

morphology and antigen detection (Nicholas, K., 1992).

2.2.6.A3. Methanol /acetone (BDH).

This was prepared by adding one volume of methanol to one volume of acetone and stored at room temperature.

Preparation of sodium azide (NaN₃)

Stock sodium azide (BDH) was prepared at 10% (w/v) in distilled water and was used at 0.1%.

2.2.7 Preparation of substrates

2.2.7.A1 APAAP substrate

Levamisole (1mg/ml , Sigma) , 5.5 ml of water and 4 mg/ml fast red (sigma) were mixed with 0.5ml of naphthol AS-MX phosphate (0.25% alkaline solution; sigma). The whole solution was filtered using Whatman filter paper (Kent; UK).

2.2.7 A2. IL-15 ELISA substrate

This was prepared by dissolving one tablet of para-nitrophenyl in 5ml of substrate buffer.

Preparation of Mounting Media

This was prepared by diluting 4 volumes of glycerol (Hycor. Biomedical. INC. CA) in one volumes of PBS 7.4.

2.2.8. Preparation of primary phenotypic antibodies

All primary antibodies used in APAAP and FACS (see below) were monoclonal and of murine origin and unconjugated. Anti CD3 (Reinherz and Schlossman, 1979) was from Orthoklein (Rahway, NJ, USA) or the Scottish antibody production unit, (Carlake, U.K.) Anti-CD4 (Reinherz *et al.*, 1979), anti-CD8 (Reinherz *et al.*, 1980) and Anti-CD22, (Dorken *et al.*, 1986) and antiCD56 (Griffin *et al* 1983; Hercend *et al* 1985) were from SAPU (Carlake, U.K). Anti CD 14 (Dimitriu-Bona *et al.*, 1983) was from Dako Ltd (High Wycombe, U.K). Anti-CD45RA (2H4; Morimoto *et al* 1985a) was from Coulter Electronic (Luton, U.K.) and was used at a final dilution of 1/10. Anti-CD45RO (UCHL1; Smith *et al.*, 1986) was a gift from Prof. P.C.L. Beverley, (ICRF, London, U.K) and was used as final dilution of 1/2.

Preparation of negative control antibodies.

Unconjugated mouse IgG₁ (Dako) or FITC-conjugated mouse IgG₁ anti-human tissue and serum protein (Coulter) were used.

2.2.9. Preparation of cytokines and cytokine specific antibodies.

i) Cytokines

Recombinant human (rh) IL-2 was provided as a gift to Dr D.I. Stott of this department by Glaxo, Geneva, Switzerland. This material gave a single band at MW 1500 on reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SD-PAGE) indicating >99% purity (Wilkinson and Newman 1994). It was used at 100ng/ml. Recombinant human (rhu) IL-8 was from British Biotechnology (Oxford, U.K) and was used at 100ng/ml (Wilkinson and Newman 1988; Wilkinson and Watson 1990). Recombinant human MIP-1 α (LD78) was a gift from Dr. G.J. Graham (CRC Beatson Institute, Glasgow, U.K.) and originated from R&D (Minneapolis, MN). Recombinant human MCP-1 was from PeproTech EC LTD (London, U.K) and was used at 1ng/ml (Wilkinson P.C. unpublished observations). Purified recombinant human (rh) IL-15 derived from a simian kidney epithelial cell line was provided by Immunex Corp., Seattle, WA and was used at 100ng/ml (Wilkinson and Liew 1995). This material gave a single band at 14-15 kd on SD-PAGE and induced proliferation of PHA-activated human T-cells at concentrations between 0.1 and 10ng/ml (Grabstein, *et al* 1994).

i) Cytokines specific antibodies

Anti-IL-2 (Tsuda *et al* 1989) was from R&D and was used at 1/500. Rabbit anti-human anti-IL-8 (aIL-8), originally documented by Strieter *et al* 1989 was provided by Dr. S. Kellie (Yamanouchi Research Oxford, U.K) and was used at 1/500. This antibody was shown earlier not to react with cytokines of the β -thromboglobulin gene family (Huber *et al.*, 1991). Anti-human MIP-1 or anti-LD78 was a gift from Dr. G.J. Graham (CRC Beatson Institute, Glasgow, U.K.) and was used at 1/500. Monoclonal mouse anti-human IL-15 (Mouse IgG1) was provided by Immunex Corp (Seattle, WA) and also was used as 1/500. Goat anti-human MCP-1 was provided by R&D and was used at 1/50.

2.2.10. Preparation of drugs

Aspirin; Indomethacin; Ibuprofen; Cyclophosphamide (Kovarsky 1983), D-penicillamine, Gold (Karlsson-Parr, *et al.*, 1986., Vini, *et al.*, 1994), 8-aminoquinoline (Mihaly *et al.*, 1985) dexamethasone (Groneck *et al* 1993), prednisone and prednisolone were from Sigma. Cyclosporin A (Borel., 1972., Yocum *et al* 1990; Wilkinson and Higgins 1987) was a gift from Dr A.W. Thomson University of Aberdeen and originally from Sandoz (Basel Switzerland). Rapamycin was also a gift from Dr A. W. Thomson. The drugs were dissolved in ethanol as stock solutions at 50-100mg per ml . Cyclosporin A and rapamycin were dissolved in ethanol and DMSO respectively as stock solutions at 10 and 3 mg per ml.

2.2.11. Preparation of collagen.

The preparation of collagen gels was originally described by Elsdale and Bard (1972) and modified by Hatson and Wilkinson (1988). Tails from more than five adult rats were collected and fixed in 70% ethanol for 5min and stored at -20 or -70 °C . Then the tails were thawed and the skin was removed. The longitudinal bundles of tendons were stripped and collected in 200 ml acetic acid (BDH) diluted to 3% (v/v) in water , and incubated for 24-48 hours to allow collagen to dissolve. After the incubation the tropocollagen was allowed to settle down in solution (Gross et al ., 1954) . Then the whole solution was filtered through 100µm Nitex guaze (R. Cadish and Sons London UK) and centrifuged at 800 xg for 30 min to deposit any debris. The collagen was mixed with 10-20% w/v sodium chloride and left to precipitate overnight. The precipitate was removed into a sterile flask and centrifuged for 30 min at 600g and then the precipitated collagen was collected and redissolved in 3% acetic acid overnight and centrifuged 2x for 15 min at 800g . The collagen solution was dialysed against distilled water, and adjusted to pH 4.0 with HCl. The optical density of the solution was estimated according to Schor, 1980; and Haston and Wilkinson 1988a by comparing the optical density value at 230nm (using matched cuvettes in a Pye Unicam spectrophotometer , SP 1800) to a freeze-dried sample of known concentration.

2.3 Cells: preparation and culture.

2.3.1 Peripheral blood mononuclear cells (PMNC).

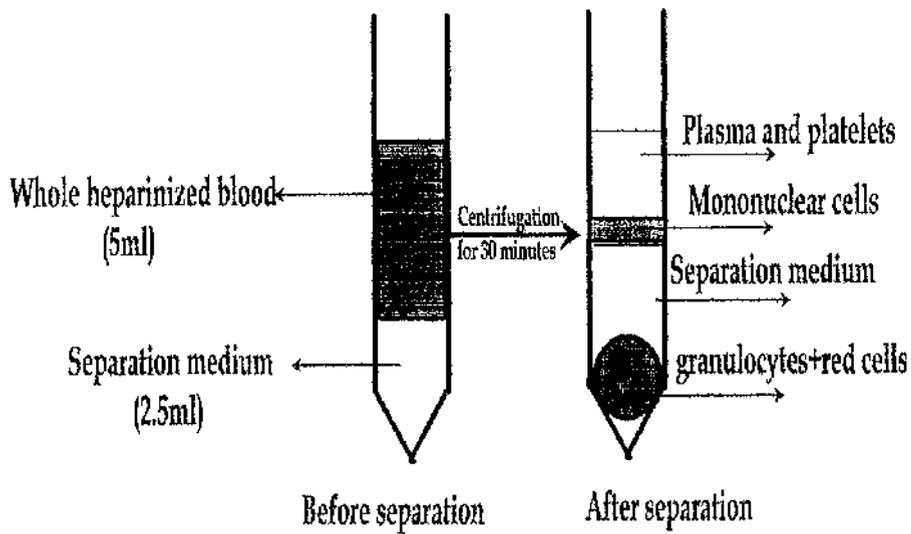
Separation principle

Peripheral blood mononuclear cells were separated from red cells and granulocytes on the basis of their differing densities on a dense osmotically balanced solution, Ficoll-Hypaque (Boyum 1968). Following centrifugation, cells separate as in Fig 2.1.

Separation method

Human blood was collected from normal individuals by Venepuncture, anticoagulated with heparin (CP pharmaceuticals Ltd, Wrexham U.K) at 2 units per ml blood in sterile plastic universal tubes . Five ml of whole blood were carefully layered onto 2.5ml lymphocyte separation medium (Lymphoprep™ Nycomed pharma Oslo, Norway) Tubes were spun at 400g, for 30 minutes at room temperature. Using a fine plastic sterile pipette (Sterilin Ltd Staffordshire UK) the mononuclear cells were carefully separated from the interface between the platelets, plasma and the separation medium (Fig 2.1) into 10-12 ml tubes. The cells were then washed 2x in HBSS (i.e centrifuged for 10 minutes each wash at 1100rpm) and resuspended in 10 ml of HBSS-HSA. For purification of

FIGURE: 2.1 Principle of Mononuclear cells separation.



As shown in the diagram after centrifugation, mononuclear cells can be carefully separated from the layer between the separation medium and plasma. This method gave about $10-20 \times 10^6$ cells/20 ml of whole blood.

lymphocytes, cells were mixed with 25% FCS+sodium bicarbonate and seeded into 24-well plates (ICN Flow) at $0.5-1 \times 10^6$ per ml. Then cells were cultured at $37^\circ\text{C} + 5\% \text{CO}_2$ for 24 hours to allow adherence to the plastic plate. Non-adherent cells were gently removed to 10-12ml tubes and washed 2x in HBSS-HSA. This method gave about $15-20 \times 10^6$ cells/20ml of whole blood. 95% of the cells were viable by trypan blue stain or morphology using phase contrast microscopy. The non-adherent cells (>90% T lymphocytes) were routinely used for locomotion studies. Monocytes were not removed from the cell preparation, because G0 lymphocytes (resting cells) are poorly motile and the locomotor capacity is acquired as the cells enter G1. T cells requires contact with appropriate accessory cells during G1 to increase their locomotion activity in the culture (Wilkinson and Higgins 1987 and, Wilkinson and Newman 1992).

2.3.1B. Peripheral blood polymorphonuclear cells.

AS mentioned above 5 volumes of human heparanized blood was layered directly over 2.5 volumes of lymphocyte separation medium. After 30 min centrifugation at 1100/ rpm, plasma, platelets, mononuclear cells and the rest of separation medium were removed. Two ml of dextran 110 (Mwt 110,000 Fison Ltd., Loughborough) was added to the pellet of heparinized blood (contains red cells and polymorph) then mixed with 6ml HBSS and incubated for 45-60 minutes at room temperature. The neutrophil-rich supernatant was collected and

washed 3x in HBSS to remove red cells and debris . One ml of distilled water was added for 30 seconds to lyse the remaining red cells. Then the pellet was resuspended in HBSS and washed 1x . The polymorphonuclear cells were counted. This method gave about $2-3 \times 10^6$ cells/ml of blood (>95% were round neutrophils as stained by Leishman and examined under light microscopy at x40). The cells were used at $0.25-0.5 \times 10^6$ /ml for locomotion studies within a short time after separation.

2.3.1C. Synovial tissue cells

Rheumatoid synovial membranes were collected in RPMI 1640 medium from patients with rheumatoid arthritis receiving surgical treatment. Using forceps, scissors, scalpel and Disposable micro-touch latex medical gloves, fat, fibrous material and blood clots were carefully removed. Then the synovial membranes were dissected and finely minced with scissors in RPMI 1640 medium with heparin, 10% FCS, and $10 \mu\text{g/ml}$ Penicillin-Streptomycin 10 ml/ml (Abrahamsen *et al* 1975) . The minced tissue was mixed with 20 volumes of RPMI 1640 containing 5mg/ml collagenase (Worthington Biochemical Corporation, New Jersey, U.S.A). After constant stirring in a small flask for 90 minutes at 37°C , the cell suspensions were filtered through sterile nylon sieves and washed 3 times. The mononuclear cells were purified by separation medium for 30 min and were seeded on petri dishes at an approximate cell concentration of $0.5-1 \times 10^6$ cells/ml.

After incubation for 1-3hr at 37°C, in 5% CO₂, non adherent cells were removed by gently washing the plate three times with RPMI 1640 and were collected for use in locomotion studies.

2.3.1D Synovial fluid cells

As mentioned above synovial fluids were collected from patients with rheumatoid and non rheumatoid arthritis. Fluids were initially spun for 10 minutes at 400g to deposit cells and debris. The supernatant fluid was removed aliquoted and stored at -70 °C for locomotion studies. The cells in the pellet were washed 2x and resuspended in 10ml HSA/HBSS. Total WBCs were counted using an improved Neubauer haemocytometer (depth 0.1mm Weber England) and cytocentrifuge slides were prepared using cleaned plastic cytofunnels in contact with methanol-cleaned slides and filter cards (Southern Instruments, Swickely, P.A., U.S.A). Trypan blue stain was used to check the cells' viability. May - Grünwald-Giemsa- or Leshman stain were used for differential counts. Separation of neutrophil and mononuclear cells was performed as for blood cells.

2.3.2 Cells: Checking the viability

As it is important to know how many viable cells to work with ,various dyes are used to stain live or dead cells. Trypan blue (see stain method) for example is excluded by viable cells . Cells which stain blue have become permeable and are considered dead.

i. Trypan blue (Stain method)

A cell suspension (50 μ l) was mixed with 450 μ l of trypan blue solution (BDH) 1% v/v in PBS). Without delay the suspension was run into a haemocytometer. One minute was allowed for cells to settle. Within 3 minutes the number of stained versus unstained white cells were counted.

2.3.3 Cells: Staining, differentiation and counting

As it is difficult to differentiate between unstained neutrophils and lymphocytes under phase contrast microscopy or after separation of these cells from inflamed materials such as synovial fluid or synovial tissue, May -Grünwald's-Giemsa and Leishman stains were used in this study.

i. May and Grünwald-Giemsa

After the cytopspin preparation the slides were fixed in methylated spirit for 5 min and stained in May-Grünwald's stain (BDH) for 4 min. The slides were washed 2x in and covered with distilled water for 4min. 2 volume of Giemsa stain (BDH) were mixed with 3 volume of water and filtered using Whatman filter paper. Then the slides were covered with Giemsa for 10 mins, washed 2x mounted and were read under light microscopy. Using the x100 objective 100 cells were counted and then the differential count was calculated.

ii. Leishman stain

Fixed cytopspin slides were covered with neat leishman stain for 2 min and then covered with distilled water for 5min, and mounted under a cover slip for counting.

2.4 METHODS

2.4.1 Polarisation assays

.i. Background

The measurement of the change from spherical to polarised shape as the earliest event of locomotion of leukocytes including neutrophils and monocytes was reported by several groups (Smith *et al.* 1979; Keller *et al.* 1983; Shields and Haston 1985; Cianociolo and Synderman 1981; Islam and Wilkinson 1988). In 1985 Haston and Shields reported a method to measure the polarising response of neutrophils to various factors using an assay that measures the proportion of polarised cells in a population in suspension and suggested that this method could be used both clinically and experimentally to give rapid and less equivocal results than those obtained by the Boyden filter technique (1962). The shape change assay was also used to measure the response of lymphocytes to a range of stimulants in short-term assays and also to measure the change in numbers of locomotor cells in long term cultures with and without activating agents (Wilkinson 1986; Wilkinson and Higgins 1987a,b). In this study I used this assay to investigate the polarising response of lymphocytes and neutrophils (from normal human blood and from synovial fluid and synovial tissue) to synovial fluid and to other chemotactic factors.

ii. Assay principle

Unstimulated lymphocytes or neutrophils remain rounded, but within a few minutes of adding chemotactic agents (such as synovial fluid or other chemotactic factors), cells changed to locomotor morphology (an elongation or ruffling at one edge or polarized morphology with constriction ring and/or uropod), which can easily be scored and quantified to obtain an accurate dose response curve (Haston and Shields, 1985). These motile cells are scored using a 40x objective and phase contrast.

iii. Method of assay (short-term effects)

In all polarisation experiments using either lymphocytes or neutrophils the polarisation assay was performed as follows. $0.25-0.5 \times 10^6$ cells/ml were added to different dilutions of synovial fluid or different concentrations of cytokines and incubated for 30 minutes at 37°C . Cells were then fixed in an equal volume of 2.5% glutaraldehyde for 10 min at room temperature and washed 2x in saline at 2000rpm, 10 minute each wash. The supernatant was removed and the pellet was resuspended in the remaining droplet of fluid. Then 10 μl of fluid was added to the slide and 200-300 cells were counted using phase contrast microscopy (Olympus BH-2 microscope) at x40. As mentioned previously cells that were spherical were scored as immotile and cells that showed ruffling at one edge or polarized morphology were scored as motile (Fig 2.2 a). HBSS-HSA alone was

used as the negative control (i.e the percentage of cells polarized in response to HBSS-HSA is subtracted from the values obtained by SF or other chemoattractants; Fig 2.2 b). As FCS consistently stimulated locomotion in short term assay, 25% FCS was used routinely as a positive control.

iv. Method of assay (long-term effects)

To measure locomotor morphology in cultured lymphocytes, the cells were cultured with an appropriate amount of either synovial fluid or anti-CD3 for 24, 48 and 72 hours at 37 °C/5% CO₂. Cell were then fixed with an equal amount of 2.5% glutaraldehyde. After washing a small number of fixed cells were examined under phase-contrast microscopy (Nikon, Japan) x 40 and cells were scored as mentioned above.

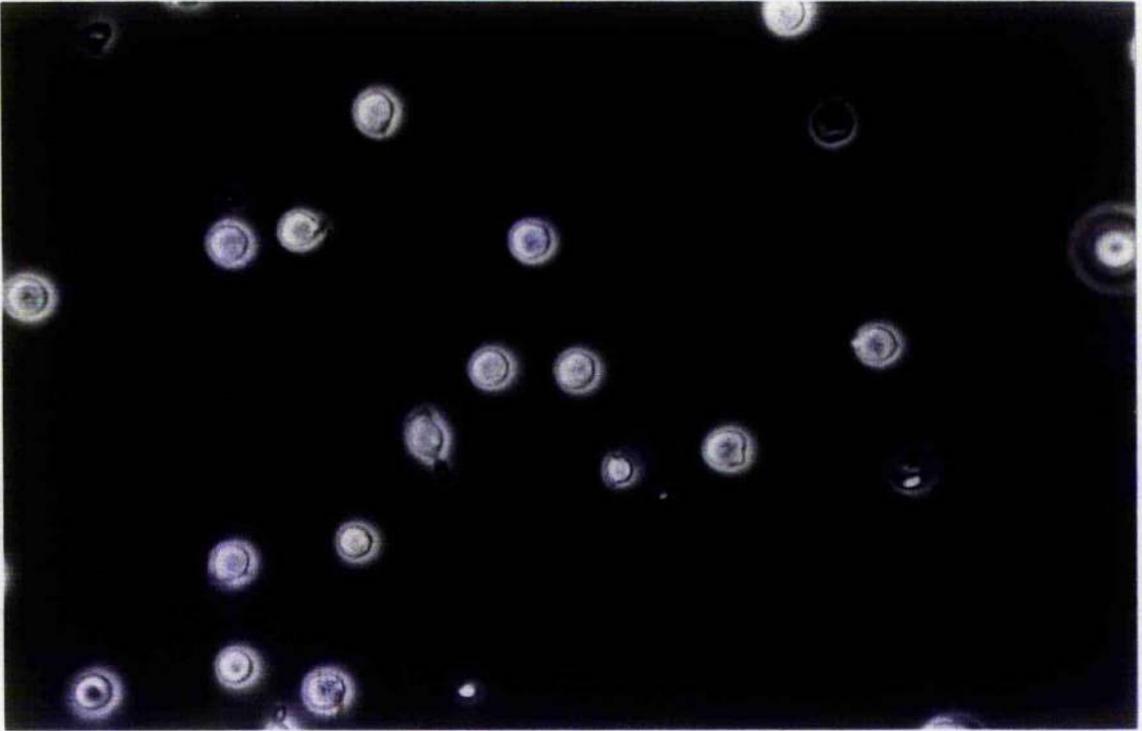


FIG 2.2.(a) Phase-contrast photographs of lymphocytes direct from blood in HBSS-HSA showing most cells are immotile.

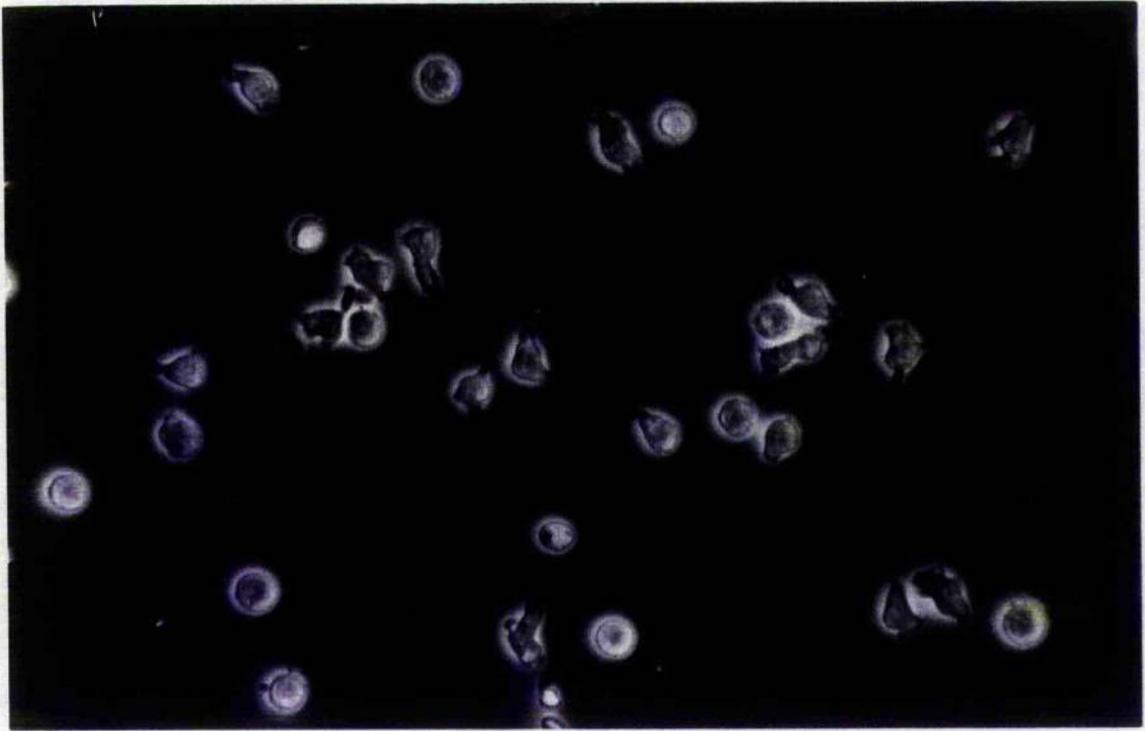


FIG 2.2 (b). Phase-contrast photographs of lymphocytes after 30 min in rheumatoid synovial fluid (at dilution 1: 4) showing that a proportion of cells change to a typical , polarized locomotor shape

2.4.2. Invasion of collagen gels

i. Background

Three dimensional collagen gels have been shown to be a useful substratum for the study of locomotion of leukocytes in vitro including neutrophils (Brown 1982; Islam 1985), monocytes (Brown 1984) and lymphocytes (Haston., *et al* 1982; Schor., *et al* 1983; and Wilkinson 1985a). Several factors have been shown to stimulate lymphocyte locomotion into collagen gels, including colchicine (Wilkinson, 1986); phorbol esters (Wilkinson *et al* 1988) IL-8 (Wilkinson and Newman), IL-2 (Newman and Wilkinson 1994), IL-15 (Wilkinson 1995) and synovial fluid from rheumatoid arthritis patients and other arthritides (Al-Mughales *et al* . submitted for publication).

ii. Assay principle

The principle of the collagen gel assay is similar to that of the micropore filter assay. The leukocytes (lymphocytes or neutrophils) are overlaid on the surface of collagen gels. Then the cells are allowed to invade the 3-dimensional fibrous matrix of the gels in response to a diffusing gradient of attractant incorporated within the gel. After an appropriate time interval the gel can be fixed with glutaraldehyde. The number of cells which invaded and which remained on the upper surface of the collagen gel are counted (see below).

iii. Method of assay

As mentioned above type I collagen was prepared from rat tail tendons by the method of Elsdale and Bard, 1972; and stored in H₂O at pH4. Stock collagen gels were prepared by returning the gel to physiological pH and ionic strength as described by Haston and Wilkinson, 1988. Before gelation, the collagen was pipetted into 24-well dishes (0.5ml per well) and allowed to set. Before the gel set, synovial fluid at optimal dilution was added to the collagen to give a final concentration of collagen of 1.75 mg/ml. After setting, cells (0.25-0.5x10⁶ per gel) were carefully layered onto the upper surface of the gels which were then incubated for 4 hours or overnight at 37°C to allow the cells to invade. The tests were then fixed with 2.5% glutaraldehyde. After washing, the number of cells remaining on the upper surface of the gel and the number of cells which had invaded the gel was counted in 6 fields per gel. The percentage of invading cells was calculated. For phenotyping, cells on the upper surface of the gel were gently removed before fixation with glutaraldehyde into a 10 ml tube and washed 2x with HBSS-HSA. The gel was then digested with collagenase (Shields and Wilkinson 1984; Ratner *et al.*, 1988) at 50µl/ml for 30min at 37°C (Lorne Laboratories LTD, Reading, UK) to recover the cells which had invaded. Collagenase was shown not to affect expression of relevant surface markers (Newman and Wilkinson, 1990). Cells from both the invaded and the non invaded populations were phenotyped for CD45RA and CD45RO by

immunofluorescence as described below.

2.4.3. Phenotyping of locomotor cells

2.4.3. A1. Modified alkaline phosphatase anti-alkaline phosphatase (APAAP)

i. Background

The potential value of alkaline phosphatase as an antibody label was established in 1969 by Avrameas *et al.*, Ternynck and Avrameas 1976. Other experiments were done by Druguet and Pepys in 1977 on human lymphocyte membrane immunoglobulin labelling. In 1978 Mason and Sammons introduced a simplified method for this assay by adding alkaline phosphatase anti-alkaline phosphatase in one step (rather than sequentially). APAAP assays have gained wide acceptance through the last decade, because of their clear appearance in term of staining details. In contrast with immunofluorescence the reaction can reviewed at a later date, 6 months (personal observation) without loss of staining intensity. Furthermore, it has been established that a huge number of CD markers of cell or viral antigens can be recognised by immunoperoxidase staining (Mason and Tylor 1977). In 1992 this assay was modified (Newman and Wilikinson 1992) to phenotype polarized and round lymphocytes by prefixing the cells with 2.5% glutaraldehyde to retain locomotor morphology during staining. This modified

assay was performed for detection of CD3, CD22, CD56, CD45RA and CD45 RO, but CD4, CD8, CD14, CD19 and CD25 were not stained by modified APAAP.

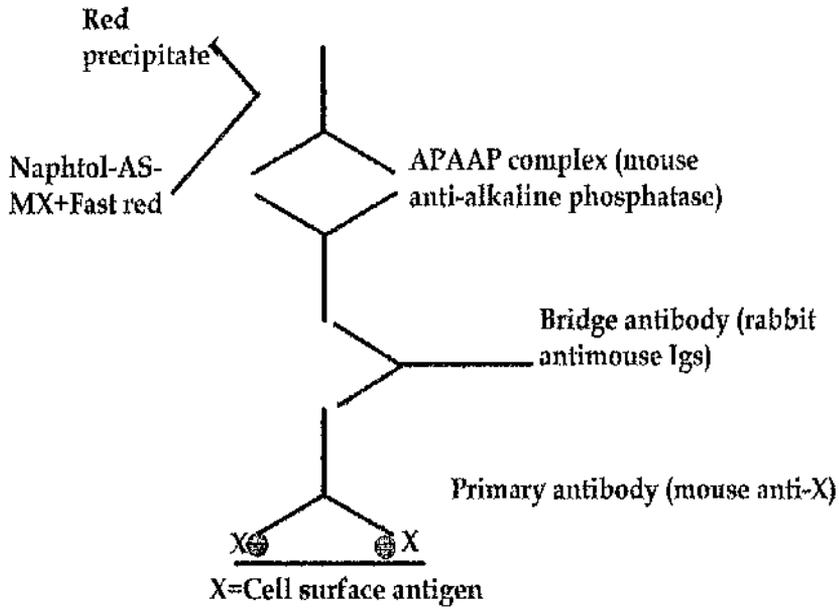
ii. Assay principle

As shown in Fig 2.3 the complex (APAAP) of alkaline phosphatase (AP) and mouse anti-alkaline phosphatase is linked to the primary mouse anti-X antibody by the use of a bridging rabbit anti-mouse immunoglobulin. Developing the phosphatase label, with the substrate Naphto AS-MX and fast red TR produces a red precipitate at the site of the antigen X. Thus in cytopsin preparations or blood smears, lymphocyte differentiation antigens can be identified by the presence of a red cellular staining deposit.

iii. Method of assay

After short term polarisation assays lymphocytes were fixed with an equal amount of 2.5% glutaraldehyde for 15 min at room temperature. Stock 0.05M glycine (Sigma) was diluted to 1/10 in saline and an appropriate amount was added and incubated with the fixed cells for 15 min to quench free aldehyde groups (Sullivan, *et al.*, 1984; Haston, 1987). Cells were then washed 2x in saline and cytocentrifuge slides were prepared (see above). Then 50 μ l of the

FIG 2.3. Principle of alkaline phosphatase anti-alkaline phosphatase (APAAP).



Schematic illustration of monoclonal APAAP procedure. Note that in order to enhance the intensity of labelling, the anti-mouse Ig and APAAP stages may be repeated one or more times (adapted from Cordell *et al* 1984).

diluted unlabelled primary antibodies were added to the slides and incubated for 20-30 minutes at room temperature in a moist chamber . Using a glass Pasteur pipette, slides were briefly rinsed with TBS then washed in the same buffer for 20 min. The excess buffer around the cells was wiped off and then anti-mouse immunoglobulin (AMlg) was added and incubated at room temperature in a moistchamber for 30 min. Slides were briefly rinsed as above and washed for 20 min in the same buffer. APAAP complex was added and incubated for 30 min in a moist chamber. Slides were briefly rinsed as above, washed for 20 min and AMlg and APAAP complex steps were repeated 10 min incubation each. This idea behind repeating steps was to enhance staining intensity. Slides were covered with alkaline phosphate substrate and incubated for 15 min at room temperature and then rinsed with buffer and washed. Counterstain (0.5% w/v aqueous methyl green, BDH) was added to the slides and incubated for 20 min , then the slides were rinsed with distilled water. Slides were dried and mounted with glycerol mountant and examined under the Olympus BH2 microscope at x40 magnification. The proportion of marker positive and marker negative polarised and round cells (Fig 2.4, a, b, c) was scored (500 cells were counted).

FIG 2.4 Lymphocyte incubated with synovial fluid in a 30 min polarization assay and fixed in glutaraldehyde to retain morphology, then phenotyped by APAAP for:

a) CD45RO



S=spherical or round , P=polarized.

b) CD45 RA



c) Negative control (no surface marker).



2.4.3 A2 Immunofluorescence

i. Background

The development of flow cytometry systems capable of sensing electronic and optical signals illuminated by laser or mercury arc lights has made possible simultaneous multiparameter analysis of surface characteristics, cell volume and cell size (Park, and Lanier 1985). The essential goal of immunophenotyping analysis of human lymphocytes is to identify and enumerate those populations that may be associated with particular functional properties and which may be altered in disease states.

ii. Assay principle

When surface antigen present on peripheral blood lymphocytes is bound by specific mouse monoclonal antibody, the cells labelled with this antibody are detected with fluorescent dye directly conjugated to the monoclonal antibody or itself bound to a secondary antibody. The most common fluorescent dye for identifying surface lymphocytes antigens is fluorescein which when excited, emits green light with a wavelength near 490nm. Rhodamine, which emits an orange-red colour with a wavelength near 545nm has been recently replaced by phycoerythrin dyes which emit light when excited at the same wavelength as fluorescein, thus allowing two colour staining analysis with a single light source.

Characteristics such as light scatter and cell volume are used to identify selected leukocytes populations by electronic analysis and then the fluorescent characteristics of each cell in the selected group are measured. The data are displayed as frequency histograms for the selected characteristics (for example number of cells versus fluorescing intensity) or as two dimensional dot plots.

iii. Method of assay

Phenotyping of cells recovered from collagen gels (for which cell shape was irrelevant) was performed by flow cytometry. Appropriate primary unlabelled antibodies were added to cell suspensions and incubated for 30 min on ice, then washed in PBS+0.1% sodium azide (BDH). After washing, the secondary antibody, fluorescein-conjugated F(ab')₂ rabbit anti-mouse immunoglobulin (DAKO) at concentration of 1/100 was added. Then the cells were washed and fixed in an equal volume of 1% paraformaldehyde (BDH). Cell surface markers were then analysed on a Becton Dickinson FACS using the lysisII program. 5,000-10,000 events were recorded.

2.4.4. Enzyme Linked Immunosorbent Assay (ELISA).

i. Background

The use of enzyme-labelled antibodies in quantitative immunoassays was suggested in 1968 (Miles and Hales 1968) and described in 1971 (Engvall and Perlmann 1971; Engvall and Johanson 1971). This was followed by rapid development of many enzyme immunoassays for a wide diversity of antigens and antibodies (Shurrs *et al.*, 1977; Vollar *et al.*, 1978) including assays related to the present study such as detection of antibodies to IL-2, IL-8, IL-15 and MIP-1a in the synovial fluid. IL-15 was measured by **multiple antibody sandwich ELISA** which was developed in our laboratory (see below) and IL-2, IL-8 and MIP-1 alpha were assayed by **single antibody sandwich ELISA** using commercially available kits from R&D (see below).

ii. Principle of multiple antibody sandwich ELISA

In the multiple antibody sandwich ELISA assay a plastic micrometer plate is coated with purified first antibody and incubated with a test sample or antigen overnight. If the synovial fluid contains IL-15, the IL-15 will form a complex with all-15 on the coated plate. After washing unreacted reagent from the plate, the later is incubated with secondary antibody from different species then an antibody-enzyme conjugate was added . The conjugate will react with the Ab-SF-Ab complex. If the synovial fluid dose not contain IL-15 the conjugate will not

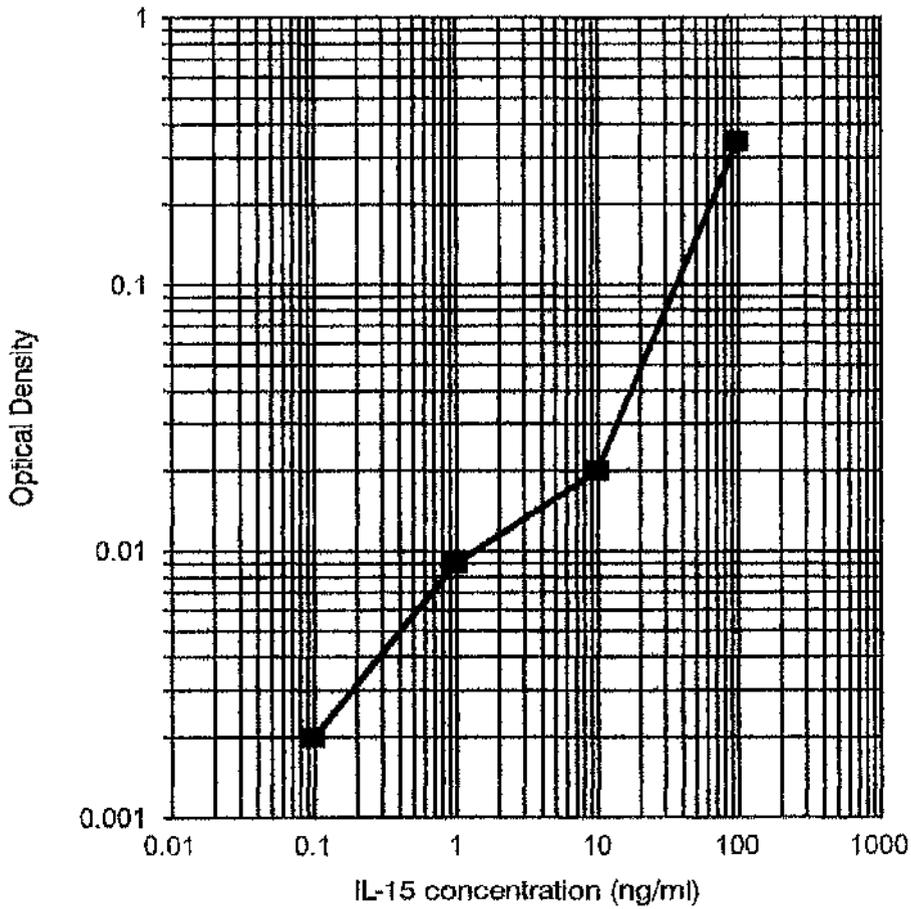
bind to the well plate surface and will be removed by washing. The presence of the enzyme on the plate surface is determined by incubating the washed plate in an appropriate enzyme substrate which leads to the production of a yellow-orange colour which is measured in a spectrophotometer adjusted to a wavelength of 630 test/450 reference.

iii. Method of multiple antibody sandwich ELISA.

Individual wells of a 96 plastic (PVC) plate (Dynatech, Billingshurst, UK) were coated with 50-100 μ l of a predetermined concentration of mouse anti-human monoclonal antibody to IL-15 (M112, Immunex aIL-15 , 5-10 μ g/ml) in coating buffer (0.1M Sodium Bicarbonate pH 8.2). The plate coated with aIL-15 was incubated overnight at 4 $^{\circ}$ C . Then the plate was washed 4x with PBS-Tween (400 μ l/well) for 2 min each time. The prediluted synovial fluid in Hanks was added to all antibody coated wells and incubated for 3h at 37 $^{\circ}$ C . Plates were then washed with PBS/Tween and 1% BSA 4x for 2 min each time. Next , a secondary antibody (100 μ l/well) namely rabbit anti-human polyclonal IL-15) was added to the complex and incubated for 1 h. A third antibody (100 μ l/well) biotin-conjugated goat anti-rabbit IgG (Sigma) diluted 1:1000 in PBS/Tween was added and incubated for 1h at 37 $^{\circ}$ C . The plate was washed 4x in PBS/Tween 20. Subsequently, Extravidin-labelled horseradish peroxidase reagent

(100 μ l/well) was added and then wells incubated for 1h at 37 °C. The plate was washed 4x and paranitrophenyl phosphate substrate (100 μ l/well) was added and incubated 30 min at 37 °C . Then 50 μ l of NaOH was added to all wells to stop the reaction. Increased absorbance due to bound, immunoreactive cytokines was measured by the ELISA reader at a wavelength of 630/450nm (MR500, Dynatech Laboratory Limited Billingshurst). All samples were assayed for IL-15 in duplicate. Results were calculated by taking average of the duplicate readings and the zero standard was subtracted. The concentration of IL-15 in each synovial fluid was calculated from the standard curves (Fig 2.3). The limit detectable dose of IL-15 was between 0.1 and 0.5 ng/ml. This was calculated as the mean optical density of two zero standards (Fig 2.5).

Fig 2.5 IL-15 Standard curve.



The concentration of IL-15 was calculated as follows: first the optical density (OD) value on Y-axis of each sample was determined. Then a horizontal line was extended to the standard curve and at the point of intersection a vertical line was extended to the X-axis and then the concentration of IL-15 in each fluid was calculated. The mean value of 2 OD zero standards ($0.005 \pm 2SD$) was considered as the limit of detection. Results of prediluted samples were multiplied by the dilution factor.

iv. Single antibody sandwich ELISA

This method was used to measure IL-2, IL-8, MIP-1 α and MCP-1 in the synovial fluid. Cytokines were assayed using commercially available kits from R&D (Minneapolis, MN, USA). In brief a microtiter plate (part D2051) was coated with a monoclonal antibody specific for the human IL-2, IL-8, MIP-1 α and MCP-1 and the plates were incubated with 100 μ l of standard and synovial fluid for 2 h at room temperature. Unbound material on the plate was washed 4x for 2min each wash. Then 200 μ l of the polyclonal antibody against cytokines conjugated to horseradish peroxidase was added to the wells and incubated for 2h at room temperature. Finally 200 μ l of the substrate solution was added to each well and incubated for 30 min at room temperature. Increased absorbance due to bound, immunoreactive cytokines was measured by the ELISA reader at 530nm. The cytokine concentration was determined as mentioned above.

2.5. Inhibition of activity of synovial fluid using anti-cytokines specific antibodies.

i Polarization assay

Anti-IL-2, Anti-IL-8, anti-IL-15, anti-MIP-1 α and anti-MCP-1 were used to inhibit the lymphocyte attractant activity of their respective cytokines. First the optimal dilution of antibody sufficient to inhibit the activity of its own cytokine at maximal effective concentration was determined. Since the effective dilution for all antibodies was 1/500, (except anti-MCP-1 which was used at 1/50) this dilution was used for all three. Synovial fluids showing strong activation of lymphocyte locomotion, i.e from the top of the range (see chapter 3., Fig 3.2) were chosen for study. The fluids were preincubated with antibody for 30 min before being tested in polarisation assays as described above. Percent incubation was calculated as:

$$\%inhibition=100-\frac{(\%polarized\ cells\ in\ antibody-treated\ fluid \times 100)}{(\%polarized\ cells\ in\ untreated\ fluid)}$$

where the medium control value has been subtracted from each reading.

ii. Collagen gel assay

This was carried out as mentioned above. Briefly neutralising antibodies (at maximal effective concentration mentioned above) aIL-8, aIL-15, aMIP-1 α , aIL-8+aIL-15 (combined), aIL-8+aMIP-1 α (combined) , aIL-15+aMIP-1 α (combined) and IgG control were incubated with the synovial fluid for 30 min at 37°C. Then the SF-antibody complex was incorporated in a collagen gel. Immediately after setting cells were overlaid on the top of the gel. The gels were then incubated overnight at 37°C. Fixation and counting procedure was as previously described.

2.6 Inhibition of lymphocyte locomotion using anti-rheumatic drugs.

i. Polarization assay

The inhibitory effect of Analgesics, NSAIDs and DMARDs on lymphocyte polarization in response to different samples of synovial fluid was tested in this study. In preliminary experiments dose-response curves for drug-induced inhibition of polarization of human lymphocytes after 24hr of culture in OKT3 (10ng/ml) was determined. Then further dose response data for drug induced-inhibition of polarization were determined using precultured human lymphocytes in 25% FCS in response to different synovial fluids in a 30 min polarization assay. Drugs were incubated with the lymphocytes for 30 min at 37 °C and the

cells were then exposed to the optimal dilution of different synovial fluids in a short term polarization assay as mentioned above.

ii. Collagen gel assay

Drugs that significantly inhibited lymphocyte polarization in response to synovial fluid in polarization assays were then tested in collagen gel assays. These drugs were cyclosporin A, Dexamethasone and prednisolone. The maximal effective concentration of drugs (1-100 μ g/ml) as determined in the polarization assay was chosen for study. The drug treated-lymphocytes (lymphocytes incubated with the drugs for 30 min at 37 °C) were overlaid on the top of collagen gels incorporated with the optimal dilution of synovial fluid. Test tubes were incubated as mentioned above.

2.2.7. Photomicroscopy

Photomicrographs were taken on an Olympus microscope equipped with phase contrast and differential interference contrast optics with x10 or x40 objectives, using an Olympus camera.

2.8. Statistical analysis

Statistical analysis was carried by using student t-test, Mann-whitney U test and Wilcoxon's sum rank test as appropriate. P values < 0.05 were regarded as significant and p values <0.01 as highly significant. Numerical results in Tables and Figures are given as mean \pm SEM unless otherwise stated.

CHAPTER 3

CHEMOATTRACTANT ACTIVITY OF RHEUMATOID SYNOVIAL FLUID FOR HUMAN LYMPHOCYTES AND NEUTROPHILS.

3.1 Chemoattractant activity of synovial fluid for human lymphocytes measured by polarisation and collagen-gel invasion assays

3.1.1 Background

Several studies on proportions of human T and B lymphocytes have reported that T cells are predominant in both synovial tissue (John *et al.*, 1975) and synovial fluid (Sheldon 1974). Recently several investigators have also reported that T lymphocytes play a critical role in rheumatoid arthritis and are thought to contribute to the initiation and sustained activity of the disease (Cush and Lipsky 1988; Cavallo *et al.*, 1994; Iannone, *et al.*, 1994). T cells show a rapid circulatory pattern and although some memory T cells are thought to remain at the site of inflammation a majority of cells traffic through the rheumatoid joint (Kingsley *et al.* 1988; Lasky *et al.*, 1988; Mackay 1991). The migration of such inflammatory cells from blood requires adhesion to vascular endothelium at the inflammatory site, accompanied by locomotor attraction mediated by chemotactic factors released in the lesion which activate the selective migration of defined leukocyte types (Wilkinson, 1987). Activation of locomotion is characterised by rapid morphological changes in the responding cells from a spherical to polarised

shape with a formation of an extended ruffled, anterior veil or lamellipodium (Zigmond 1974). This morphological change is easily measured *in vitro* (Haston and Shields 1985; Wilkinson 1986). As mentioned above the majority of lymphocytes that infiltrate into the joint are T-cells. Many of these are of the memory cell type (Pitzalis *et al.*, 1987). These cells pass through the synovium, and become activated as shown by their expression of T cell activation markers such as adhesion molecules, HLA-DR, the IL-2 receptor α chain (CD25) or CD69 (Iannone, *et al.* 1994). It has been also reported that neutrophils obtained from the peripheral blood of RA patients show reduced locomotion activity *in vitro* (Mowat & Baum 1971) although accumulation of neutrophils within the joint space is a feature of inflammation in rheumatoid arthritis (Ropes & Baurer 1953). There is little information about the locomotor activity of neutrophils from the synovial fluid of rheumatoid arthritis, although their phagocytic activity is reduced as compared to peripheral blood neutrophils (Bodel & Hollingsworth, 1966; Turner, *et al.*, 1973). However it has not formally been demonstrated that synovial fluid attracts neutrophils using assays for polarisation and collagen gel invasion, though such attraction has been studied using the filter assay. The aim of the present study in this chapter was to examine the effect of synovial fluids on the locomotion and polarization of circulating peripheral blood lymphocytes, and to phenotype the responding cells in order to better understand the mechanism of local inflammation in RA. The assays used were the polarization assay (Haston and

Shields 1985) which measures the morphological changes correlated with locomotion and the collagen gel invasion assay (Haston and Wilkinson 1988) which measures locomotor attraction directly. In this the chemoattractant effect of SF for peripheral blood and synovial fluid neutrophil was also examined as well as the chemoattractant effect of FMLP for synovial fluid neutrophils to compare the response of neutrophils with those of T lymphocytes.

3.1.2 RESULTS

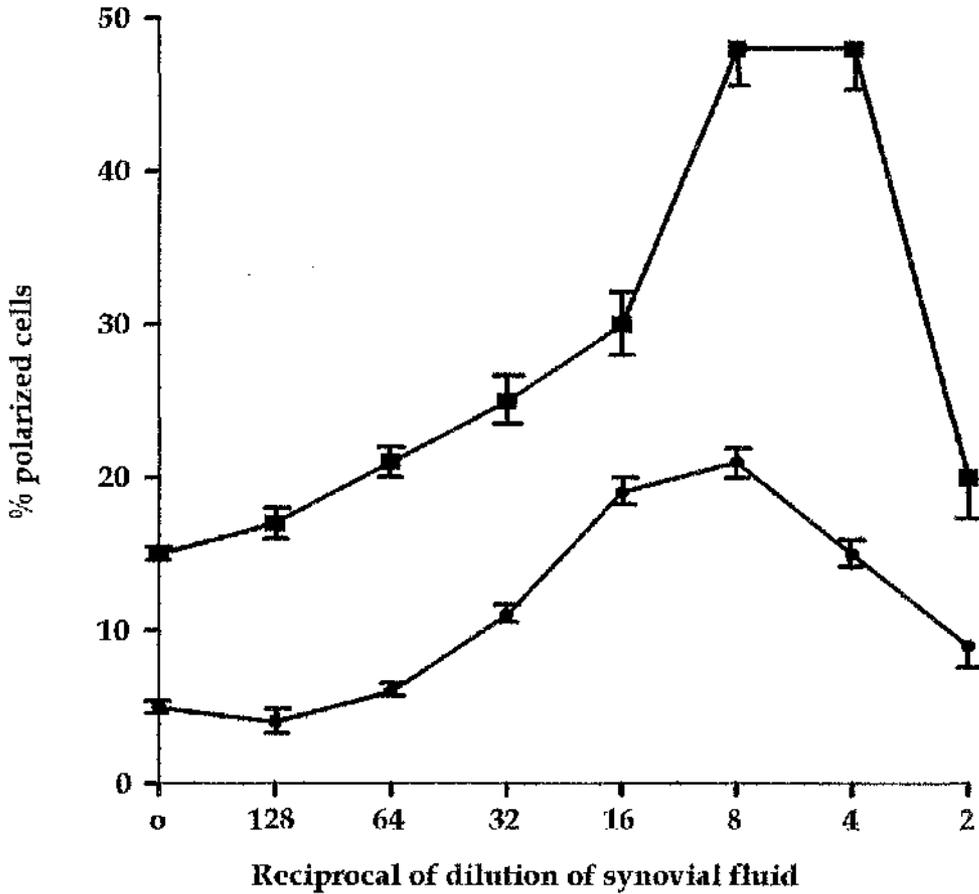
3.1.2A Response of normal blood lymphocytes to synovial fluid.

3.1.2A1. Comparison of responses of cultured and non cultured lymphocytes responses.

In order to prepare lymphocytes for locomotion studies and to check the chemoattractant activity of synovial fluid, first lymphocytes direct from normal blood were tested against several synovial fluids from patients with active rheumatoid arthritis. Synovial fluids were first diluted in HBSS and then incubated with lymphocytes in dose response polarisation assays. The PBMC from normal blood was also tested after overnight culture in FCS. Fig 3.1 shows that lymphocytes cultured overnight in 25% FCS gave higher responses to a representative synovial fluid than lymphocytes direct from blood. As reported earlier overnight culture in FCS increases the locomotor capacity of lymphocytes (Berman 1966; O' Neill and Parrott 1977). FCS also has mitogenic activity (Woodliff and Onesti 1968). However activation of locomotion is seen long before mitosis and is evident with 24 hours.

FIGURE 3.1

Effect of FCS culture on the polarisation response of normal blood lymphocytes to synovial fluid.



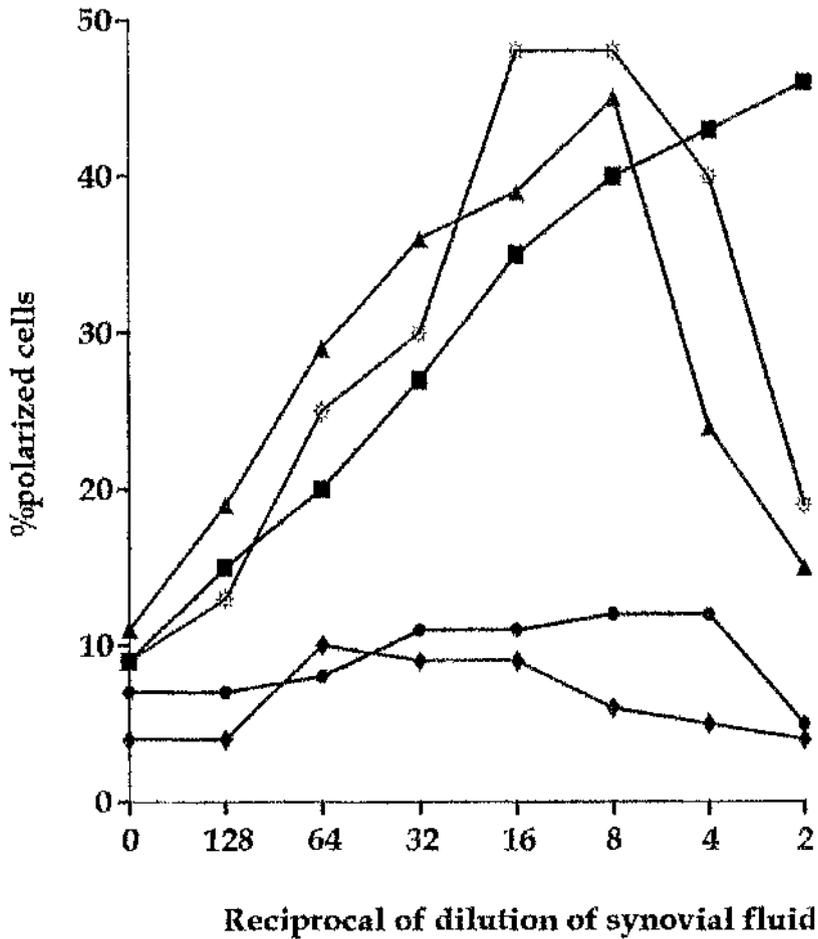
Lymphocytes were incubated with the same synovial fluid after separation from blood or after culture for 24 hours in 25% FCS. Although the background was higher after overnight culture, cells on the day of separation gave low responses (circles) compared with cells cultured overnight (squares). The graph shows means and standard error of three experiments.

3.1.2A2. Polarization dose response of Lymphocytes cultured overnight to synovial fluids.

In these experiments lymphocytes were first cultured overnight in 25% FCS (0.5×10^6 cells/ml) then washed twice in HBSS and tested against different dilutions of synovial fluid from patients with RA, OA and other inflammatory arthritides. First the dose response curve for each fluid was obtained. In most cases, a bell-shaped dose-response curve was obtained with maximum activity at dilutions between 1:2 and 1:16 (Figure 3.2). From these curves an optimal dilution of each fluid at which lymphocyte polarisation was maximal, which was used in subsequent experiments, was determined. There was no relation between the optimal dilution and the amount of activity obtained at that dilution. Some fluids gave very flat curves, others gave good peaks of activity (Figure 3.2). The fluids at optimal dilution were then retested several times in polarisation assays. Figure 3.3 shows that the majority (23/29) of RA joint fluids caused strong polarization of lymphocytes but 6 fluids were negative or slightly higher than the medium control. The response of OA fluids was also tested, but 3 out of 5 showed low activity. Also fluid from other inflammatory arthritides were tested but 3 out of 7 gave low responses.

FIGURE 3.2

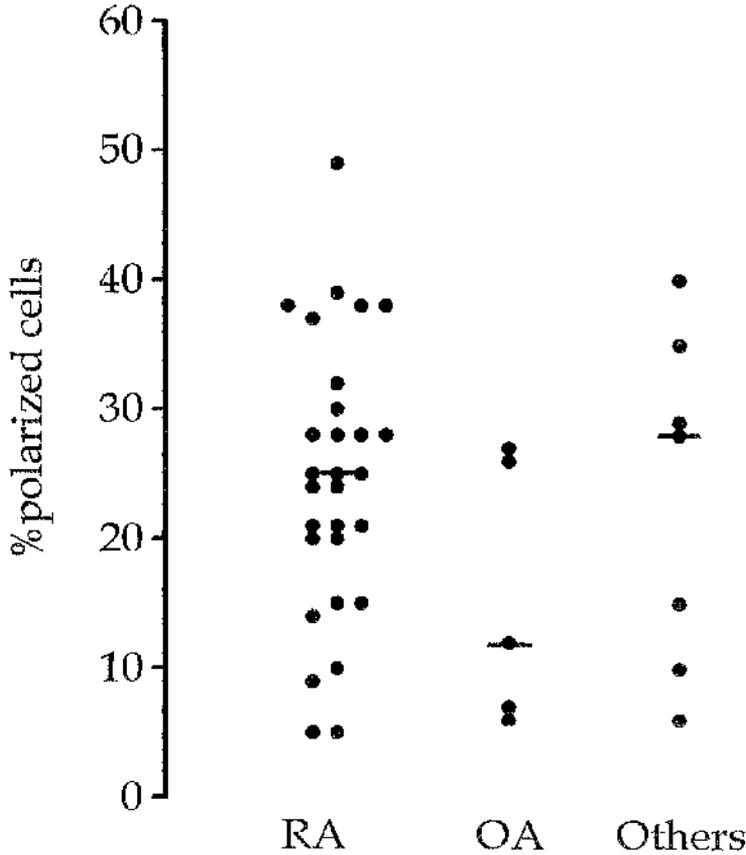
Lymphocyte polarisation dose-response to synovial fluid.



Polarisation dose-response of normal blood lymphocytes to five synovial fluids, three with high activity and two with low activity, selected to show the diverse patterns of response obtained. Optimal concentrations determined from such data were used in subsequent experiments.

FIGURE 3.3

Lymphocyte polarisation in response to optimal dilutions of synovial fluid.



Lymphocytes were tested after overnight culture against an optimal dilution of synovial fluid of 29 patients with rheumatoid arthritis (RA), five patients with osteoarthritis (OA) and seven patients with others inflammatory arthritides (others). Each point represents the mean of six experiments. Bars represent the median for each group. The control value (HBSS-HSA) has been subtracted from the values shown. Results were statistically significant when compared RA to OA ($p < 0.05$ Mann Whitney).

These were from patients with psoriatic arthritis (n=1) , intermittent hydro-arthritis (n=1) and spondylo-arthritis (n=1).

3.1.2A3. Time-course of lymphocyte response to synovial fluid

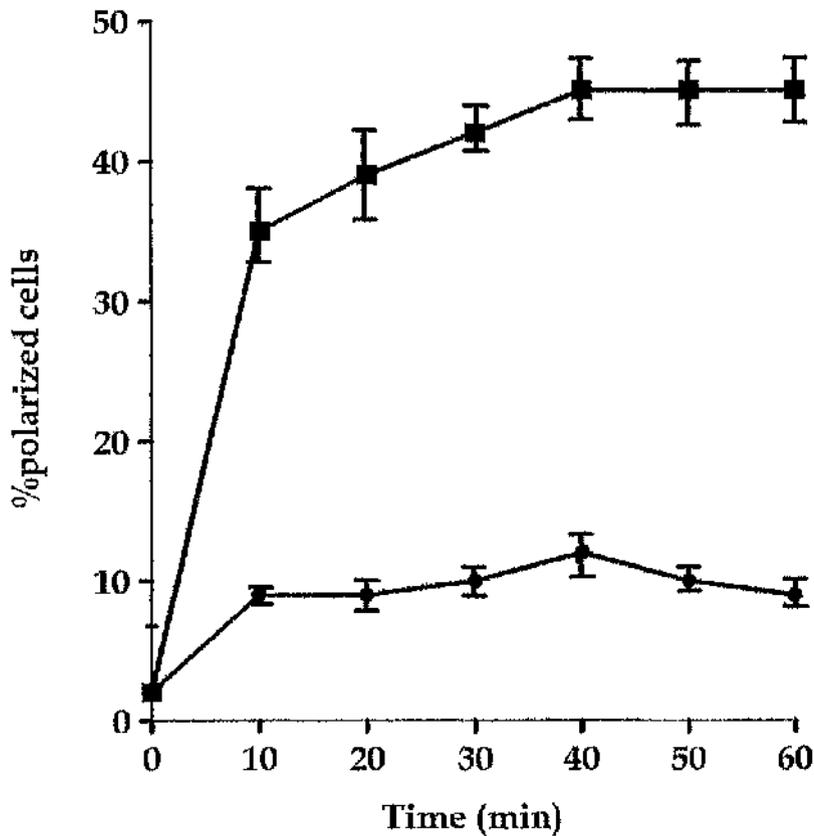
In these experiments synovial fluid at an optimal dilution of 1:4 was added to a cell suspension that had been cultured overnight and the polarisation response was measured at various times from 0 to 60 minutes. Fig 3.4 shows that the polarisation response was at maximum after 10-40 minutes when 45% of the cells were polarised. These results indicated that lymphocytes respond to synovial fluid within a short time of exposure by showing rapid shape changes which were dose and time dependent.

3.1.2A4. Long term effect of synovial fluid on lymphocyte polarisation.

To assess the effect of synovial fluid on lymphocyte polarisation in a long term assay, cells were cultured with synovial fluid at a dilution of 1:8. Fig 3.5 shows that prolonged incubation (24hr) caused a slight increase in lymphocyte polarisation compared with the short term assay. Culture of these cells for 48hr led to the same results. The percentage decreased upon further incubation up to 72hr. This may be due to internalisation of receptors during continuous culture in synovial fluid.

FIGURE 3.4

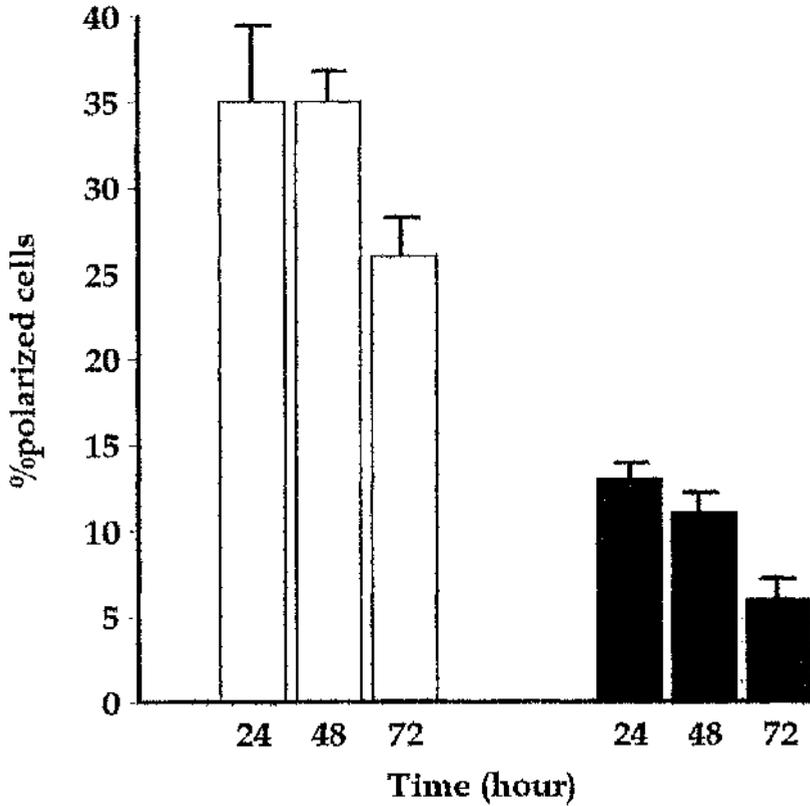
Time course of lymphocytic shape change during 60 min incubation with synovial fluid.



Lymphocytes were incubated with synovial fluid at a dilution of 1:4. Cells were fixed in suspension with 2.5% glutaraldehyde and the proportion of polarised cells was counted as described above. Squares represent the mean polarisation response to synovial fluid and circles represent the medium control (Mean \pm SEM for two experiments).

FIGURE 3.5

Long term effect of synovial fluid on lymphocyte polarisation.



Lymphocytes were cultured with an optimal dilution of a chosen synovial fluid at a dilution of 1:8 for 24, 48 and 72 hours (clear bars). Cells were fixed in 2.5% glutaraldehyde and polarisation was measured as mentioned before. Each column represents the mean \pm standard error of three experiments. The mean proportion of the medium control (black bars) was different at 24, 48 and 72 hours of incubation.

Also at 72hr cell viability was 79% (as measured by trypan blue) compared with 89% at 48hr.

3.1.2A5. Collagen gel assay

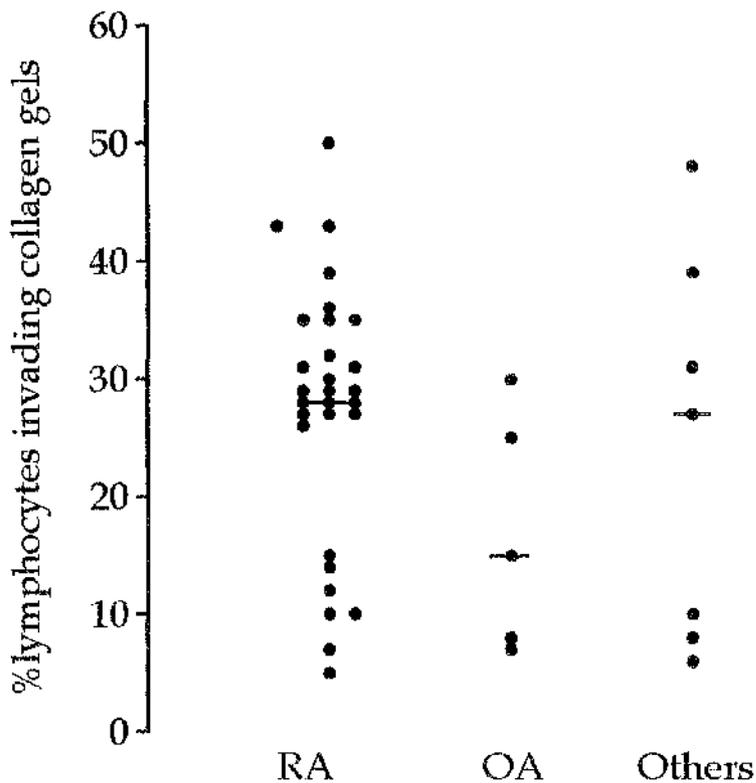
This assay supplements the polarisation assay and provides direct evidence for active locomotion of lymphocytes towards an attractant (Wilkinson 1986). An optimal dilution of each fluid (found in polarisation assays) was incorporated into collagen gels and the proportion of lymphocytes which invaded the gel was measured. Fig 3.6 shows that the majority (21/29) of rheumatoid joint fluids attracted lymphocytes strongly but that there was a small group of fluids which were negative or only slightly higher than the medium control. Too few fluids from other forms of arthritis were tested to draw definitive conclusions, but 3 out of 5 osteoarthritis fluids and also 3 of 5 of other inflammatory arthritides showed low locomotion activity.

3.1.2A6. Correlation between polarisation and collagen gel assays

Figure 3.7 is a scatter graph showing the responses of blood lymphocytes to the joint fluids, each at optimal dilution, using both the polarisation and the collagen gel invasion assay. In most cases the proportion of lymphocytes that invaded the collagen gels closely paralleled the proportion of polarised cells. There was an excellent correlation between the two assays. This provides evidence that polarisation is therefore a reliable correlate of locomotion ($r=0.85$, $P<0.01$).

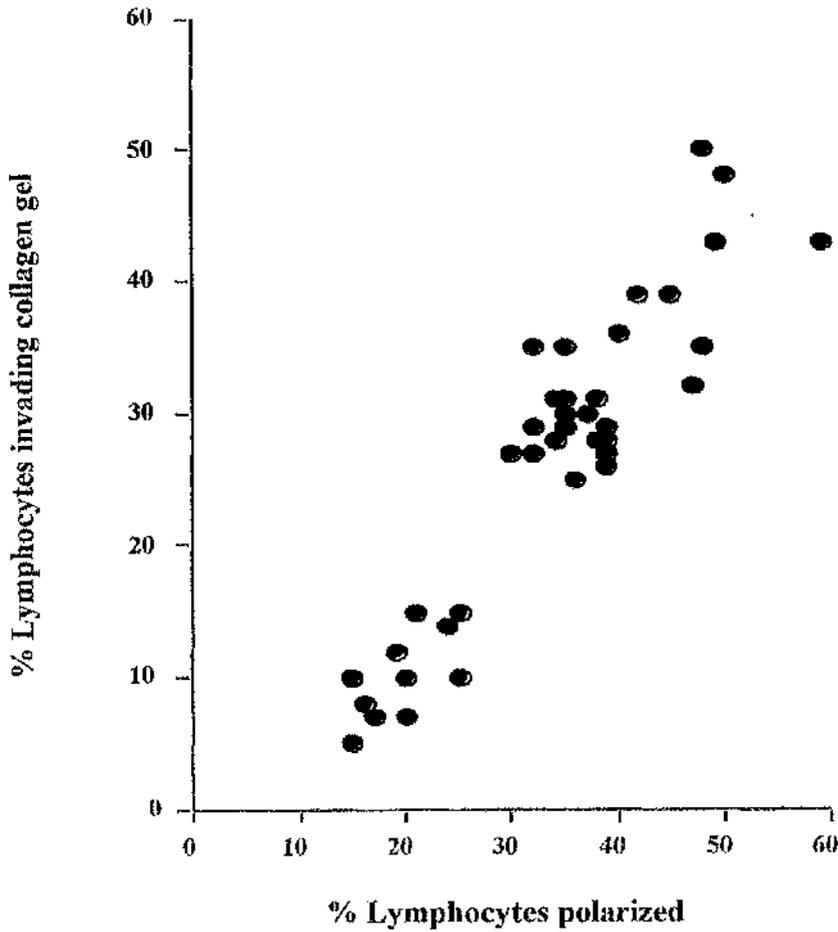
FIGURE 3.6

Lymphocytes invading collagen gel in response to synovial fluid



Lymphocytes were allowed to invade the collagen gels overnight. The collagen gels incorporated optimal dilutions of synovial fluids from 29 RA, five OA and seven patients with others inflammatory arthritides. Each point represents the mean of three experiments. Bars represent the median of lymphocytes invading collagen gel in each group. The negative control value (HBSS-HSA) has been subtracted from the values shown. Results were statistically significant when compared RA to OA ($p < 0.05$; Mann Whitney)

FIG 3.7. Correlation between polarization and collagen gel assays.



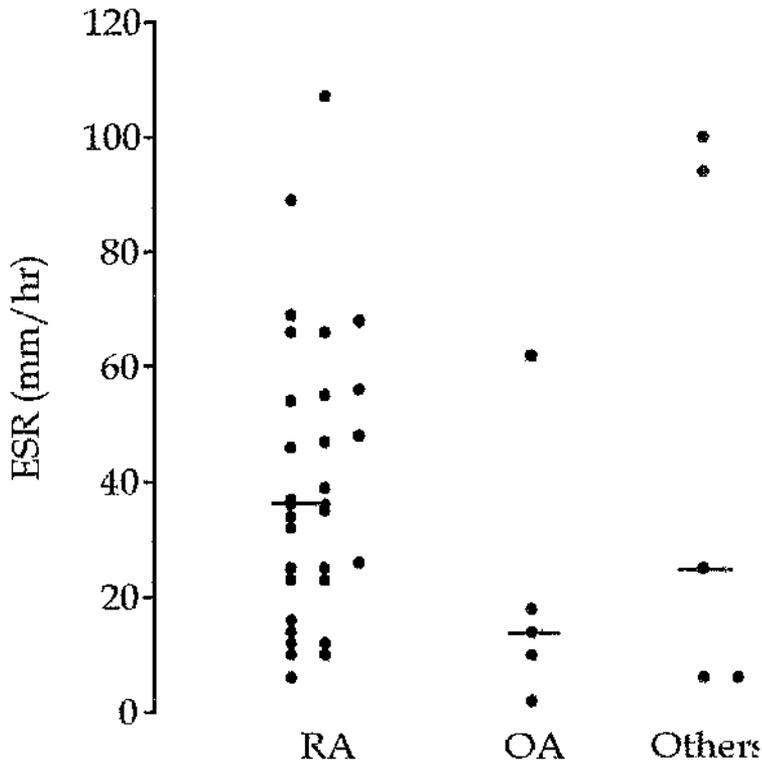
Response of blood lymphocytes to 39 fluids each at its optimal concentration, in a polarization assay (x-axis) and a collagen gel invasion assay (y-axis): each point represents the mean of three experiments.

3.1.3. Correlation between locomotion activity of RA-SF and the clinical parameters of RA-disease activity.

C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were first measured in blood samples taken at the same time as fluid was removed. Although the CRP (Fig 3.8) and ESR (Fig 3.9) tend to be higher in the RA than non RA group, the correlation of these results with the locomotion activity of RA-SF was not statistically significant.

FIGURE 3.9

Measurement of ESR in RA and non RA patients.



ESR in patients with RA (n=31) OA (n=5) and other inflammatory arthritides (n=5) was measured utilising the Westergren technique. Each dot represents the percent value for each patient. Normal value is : M 0-20, F 0-30 mm/h (see reference ; ICSH , 1977). Bars represent median in each group.

3.2. Lymphocytes from synovial tissue

3.2.1. Characteristics of synovial tissue cell suspensions after Ficoll-Hypaque centrifugation.

In these experiments, first it was necessary to assess the characteristic features of the cell suspension purified from the synovial tissue (cell viability, differential count or phenotyping). Table 3.1 shows the differential counts of the cells after Ficoll-Hypaque centrifugation in three patients with active rheumatoid arthritis. The number of mononuclear cells was different from patient to patient varying with the amount, size, and quality of the tissue and varies with the severity of the inflammation. The average viability was 84% (range; 73-92). Differential counts showed lymphocytes to be the predominant cells (Table 3.1). The other cells were macrophage like cells, neutrophils, unclassified cells and dead cells. T lymphocytes of the CD45RO⁺ type were found to be the predominant in all cell suspensions as phenotyped by FACS.

3.2.2. Effect of collagenase on the cell surface markers (control experiments)

In order to phenotype cells it was necessary to investigate whether collagenase (used during synovial tissue digestion for lymphocytes separation) has any effect on the cell count and on cell surface markers.

***TABLE 3.1**

Differential count of synovial tissue cell suspension after Isopaque-Ficoll centrifugation in three patients with RA.

Cells	Patients		
	G.D	N.M	P.E
Total count (x10 ⁶ /ml)	9.6	5.1	7.3
Cell viability (%)	87%	92%	73%
Lymphocytes	89	88	80
Macrophage-like cells	10	9	10
Neutrophils	2	-	1
Unclassified cells	2	2	4
Dead cells	1	1	5

* Cells were separated from the synovial tissue of patients with classical RA (n=3) with joint involvement receiving surgical treatment. The tissue was obtained at synovectomy performed on knee joints (see chapter).

The percentage of viable cells was assessed by the trypan blue exclusion test.

For the differential counts cells were stained with May-Grünwald/Giemsa (200 cells were counted).

Mononuclear cells obtained from normal blood were treated the same way as the synovial tissue. Table 3.2 shows that collagenase treatment caused slight reduction in the cell count but had no effect on cell surface marker expression as examined by FACS for CD3, CD45RA, CD45 RO and CD22.

3.2.3. Locomotion assay

Lymphocytes purified from different samples of synovial tissue were motile and responded by polarisation in response to synovial fluid. Fig 3.10 shows the proportion of polarised cells in response to HBSS and synovial fluid. In contrast to blood cells, which required a period of culture to allow them to acquire locomotor capacity, the synovial tissue cells were active directly after preparation, suggesting that the cells were already activated in vivo.

3.3. Lymphocytes from synovial fluids

Synovial fluid lymphocytes were separated from their own synovial fluid, washed three times in HBSS and tested against that synovial fluid and FCS or against IL-8. In contrast to the blood lymphocytes and the synovial tissue lymphocytes, synovial fluid cells showed almost no response in polarisation assays compared with the medium control. (Figure 3.11).

TABLE 3.2

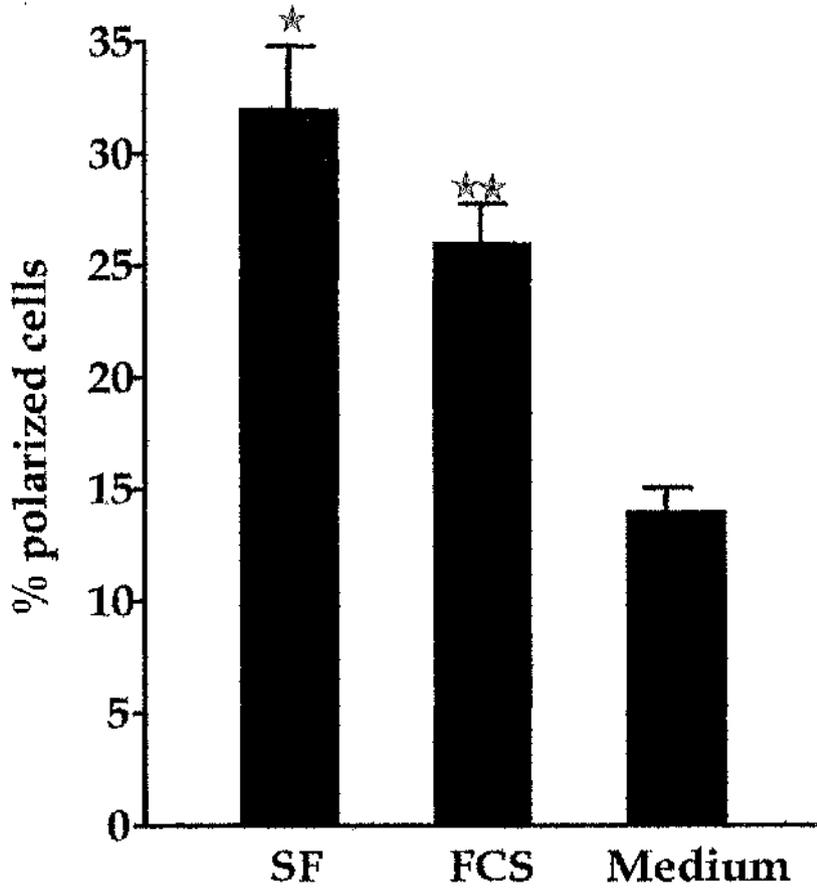
Effect of incubation of mononuclear cells from normal blood with Collagenase on their expression of CD3 CD45RA CD45RO and CD22.

	Cells treated with *collagenase	Cells without collagenase
Total cell count (10^6 /ml)	1.7	1.8
Marker		
CD3	59.2	54.3
CD45RA+	55.6	57.2
CD45RO+	26.4	28.4
CD22+	11.5	9.6

* Collagenase (Worthington Biochemical Corporation, New Jersey, U.S.A) used here was different from that used in the collagen gel invasion assay (BDH, Type 1 collagenase). The type 2 preparations are extracellular enzyme produced by the bacterium *Clostridium histolyticum* and especially used for tissue dissociation.

FIGURE 3.10

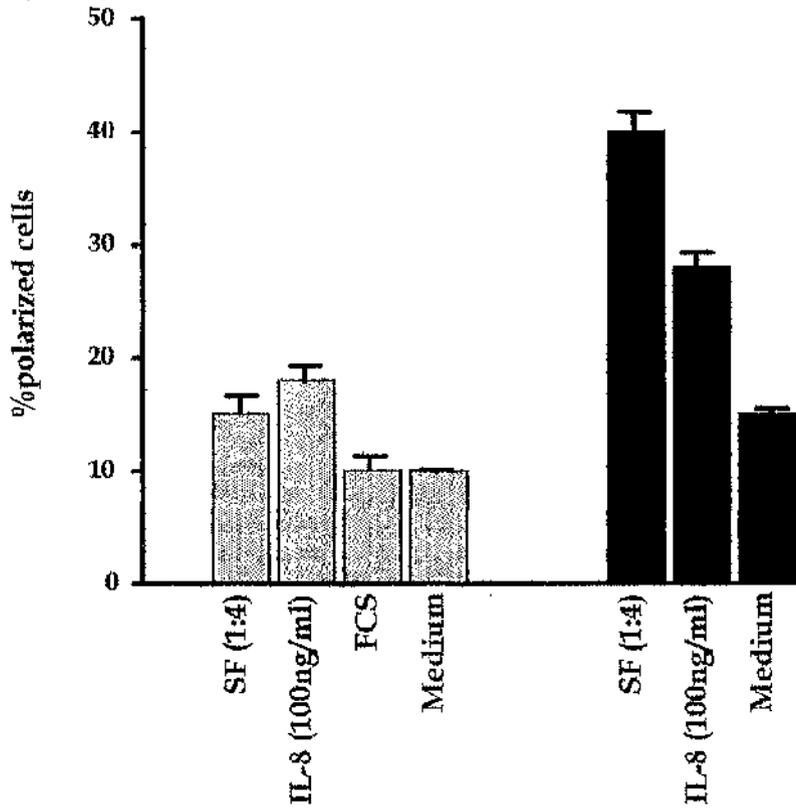
Effect of synovial fluid on the polarisation of synovial tissue lymphocytes



Cells were separated from synovial tissue and then exposed directly (see separation method) to synovial fluid (1:8) in a dose response polarisation assay. Results were statistically significant (* $p=0.0026$; ** $p= 0.0091$) when compared with the medium control. The SF bar represents the mean \pm SD for 3 synovial fluids results in three different experiments.

FIGURE 3.11

Polarisation response of rheumatoid synovial fluid lymphocytes in response to various stimuli.



Synovial fluid lymphocytes were separated from RA synovial fluid (n=10) and washed three times with medium and then exposed to Synovial fluid (SF 1:4), IL-8 (100ng/ml) and FCS (25%). Clear bars show that the polarisation response to stimuli was not statistically significant when compared with the negative control (medium alone). Filled bars show response of normal blood lymphocytes (used as control cells) to SF and IL-8. Results expressed as mean±SEM (n=6 for synovial fluid cells and n=3 for blood cells).

3.4. Cell Phenotyping using APAAP

3.4.1. Control experiments

The blood lymphocytes that polarised in response to different synovial fluids were phenotyped by APAAP staining to see if the response to these fluids was selective for any cell class. Before phenotyping some control experiments were performed to confirm APAAP previous observations using this assay (Newman and Wilkinson 1992). First it was necessary to compare the proportion of polarised cells in suspension and the proportion of the same cells in APAAP-stained cytopsin preparations. Table 3.3 shows that the proportion of polarised cells was similar in both assays. Second to check if the monoclonal APAAP procedure can be used to label a wide range of monoclonal antibodies (such as CD3, CD4, CD8, CD22, CD56, CD45RA and CD45RO) with a degree of clarity and fully comparable to that obtained with the FACS labelling technique. Table 3.4 shows CD3, CD8, CD22, CD56, CD45RO and CD45 RA were stained positively with the same intensity as in FACS preparations. CD4 did not stain positively in the APAAP assay.

***TABLE 3.3**

Comparison of fixed polarised lymphocytes (in response to synovial fluid) in unstained wet and APAAP-stained cytopsin preparations.

Stimulus	EXP1		EXP2		EXP3	
	%lymphocytes polarised (200 cells counted)					
	WP	CP	WP	CP	WP	CP
HBSS-HSA	6.3	9	7.5	4.5	10.5	12
SF1	28	25	32	33	39	49
SF2	49	46	37	35	39	41
SF3	39	32	25	27	37	28
SF4	52	49	30	37	47	57

* Lymphocytes were separated from blood and cultured overnight in FCS.

Cells suspensions from wet preparations were fixed and examined under phase contrast microscopy x40. Cells from the same suspension were fixed cytocentrifuged and stained by APAAP and then examined under phase contrast X40. SF=synovial fluid, WP=wet preparation (unstained cells)

CP=cytopsin preparation (APAAP-stained cells)

TABLE 3.4

Comparison of percent staining intensity using the APAAP and FACS procedures with monoclonal antibodies for CD3, CD4, CD8, CD22, CD56, CD45RA and CD45 RO lymphocytes.

%Marker	EXP1		EXP2		EXP3	
	APAAP	FACS	APAAP	FACS	APAAP	FACS
CD3+	49.2	48	76.8	64.58	76.67	78.67
CD4+	ND	40.63	ND	41.87	ND	39.6
CD8+	25.58	27.9	20.7	22.36	29.3	26.87
CD22+	7.8	3	6.9	8.36	7.3	9.84
CD56+	6.1	5	10.2	7.55	15.3	14.2
CD45RA+	56	51.60	61.3	69.3	49.2	56.7
CD45RO+	27.2	31.64	24.3	22.1	19.2	25.3

* Note that the lymphocytes used for both assays in each experiment were from the same preparation. EXP=Experiment, ND=Not detectable.

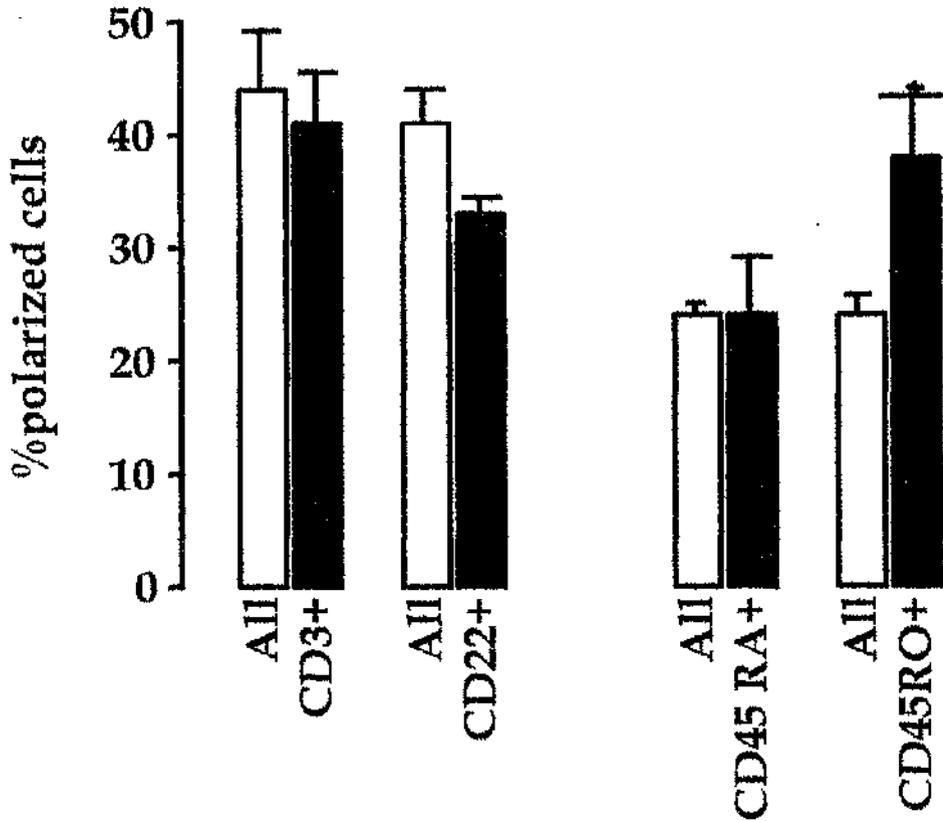
3.4.2. Phenotyping of polarised lymphocytes.

The normal blood lymphocytes polarised in response to synovial fluids were phenotyped using APAAP staining to see if the response to these fluids was selective for any class. Fig 3.12 shows there was no significant selection for or against CD3, and CD22. The polarisation response of CD22+ and CD3+ cells was not statistically significant. CD56+ cells were also studied, but the number of polarised cells in this minority population that it was possible to count was too low to give reliable results.

3.4.3. Phenotype of lymphocyte subsets

Fig 3.12 also shows no significant selection for or against CD45RA+ cells but a higher proportion of CD45RO+ cells was polarized than would be expected from the proportion of polarized cells in the whole lymphocyte population (*p <0.05). These CD45RO+ cells were mostly (>90%) T cells. This suggests that lymphocytes that have recently responded to antigen and which are characteristically CD45RO+ (Smith et al., 1986) are attracted more vigorously by factors in the fluid than antigen-unreactive (naive) cells.

FIGURE 3.12 Phenotyping lymphocytes and lymphocyte subsets using APAAP.



Proportion of CD3+ CD22+ , CD45RA+ and CD45RO+ lymphocytes which polarised after short term exposure to synovial fluid. Cells were incubated with an optimal dilution of different synovial fluids for 45 min and phenotyped by APAAP. Clear bars represent the proportion of polarized cells among all cells, both positive and negative for the marker only. Note that variation in the proportion of total cells polarized between different groups (clear bars) is due to tests for different markers being done on different samples. Differences between the total and the marker positive populations were not significant except for CD45RO+ (* $p < 0.05$). Each bar represents the mean \pm SEM of 12 RA synovial fluids (n=3).

Conclusions

SF attracts lymphocytes and activated cells respond better than unactivated cells. Synovial tissue cells respond immediately but blood cells respond best if activated by overnight culture. Synovial fluid cells are unresponsive cells . Cells of CD45RO phenotype respond better than those of CD45RA phenotype.

3.5 Phenotyping of locomotor cells invading collagen gels recovered after collagen digestion.

Since the polarization assay suggested that CD45RO⁺ locomotor cells were selected for, phenotyping for CD45 isotypes was carried out by FACS analysis on lymphocytes. Two populations were studied. (a) The non-motile cells that remained on top of the gel. (b) The non-motile cells that had migrated into the gel were recovered after collagenase digestion of the gel. Both were stained for CD45RA and CD45RO. The superior locomotor activity of CD45RO⁺ cells over CD45RA⁺ cells was more clear-cut than in the polarization assay and selection against CD45RA was also obvious (Table 3.5)

TABLE 3.5

Distribution of CD45 isotypes in locomotor and non locomotor lymphocytes following invasion of collagen gels incorporating rheumatoid synovial fluid

percent of lymphocytes in population
(Mean±SEM, n=3)

Subset	on upper surface of gel	released from collagenase-digested gel (locomotor cells)
CD45RA+	73.4±4.3*	33.8±3.8
CD45RO+	44.5±6.6*	59.2±1.9

*Note that the total % of CD45+ cells in the non motile population is >100%, suggesting the presence of double-positive cells. Results are statistically significant (P<0.05; for both CD45RA and CD45RO Student t test) when comparing invasive with non invasive cells.

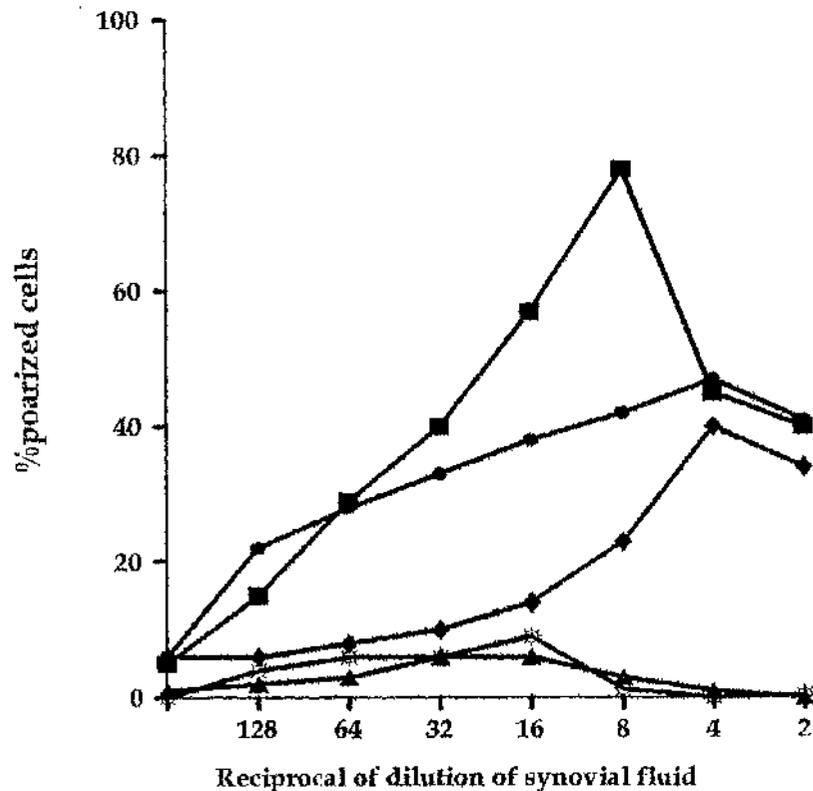
3.6. Chemoattractant activity of synovial fluid for human neutrophils measured by shape change and collagen gel-invasion

3.61A. Response of normal blood neutrophils to synovial fluid

In this study neutrophils from normal blood (>90% pure) were tested with different synovial fluids (from different groups of inflammatory diseases such as RA, OA and other inflammatory arthritides), medium (used as negative control) and in a uniform concentration (10^{-8}) of FMLP (used as a positive control). Neutrophils incubated in HBSS/HSA at 37 °C for 30 minutes remained spherical. Neutrophils tested in a short term polarisation assay in different synovial fluids changed shape from round to polarised. Fig 3.13 shows a bell shaped dose response with maximal activity between 1:4 and 1:8. Then from these curves an optimal dilution was tested many times in a subsequent short term polarisation assays. Synovial fluid from different rheumatoid arthritis patients showed high polarisation activity compared with fluid from osteoarthritis patients and other inflammatory arthritides (Fig 3.14).

FIGURE 3.13

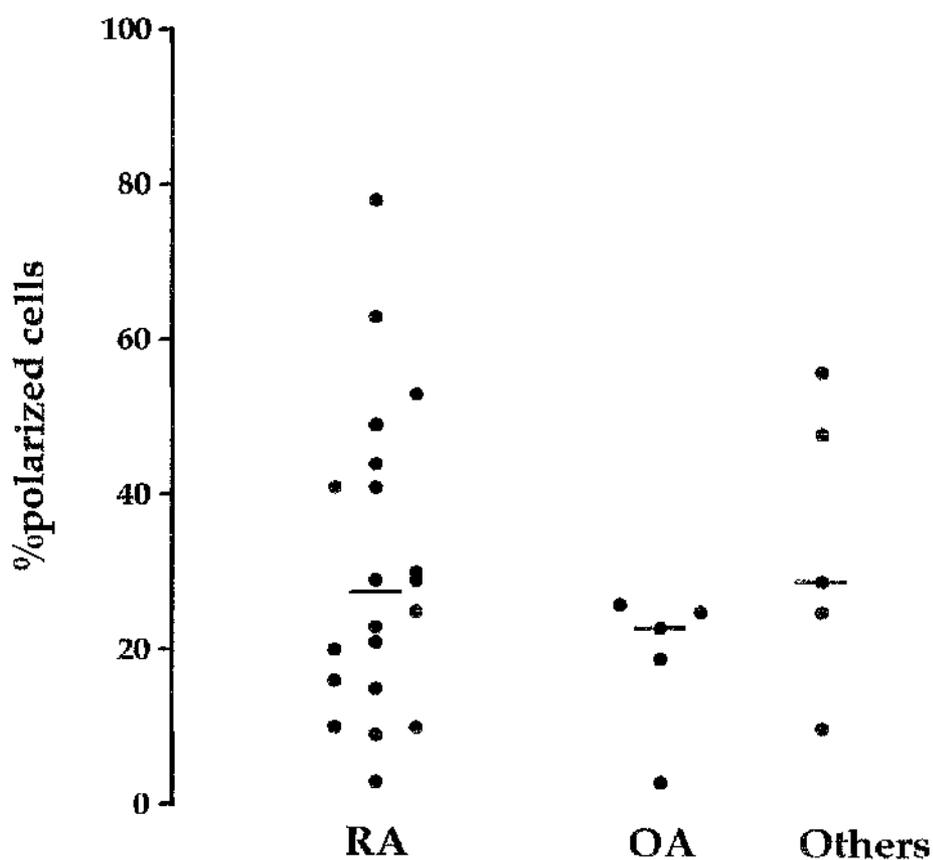
Polarisation dose response of normal blood neutrophils in response to synovial fluid.



Cells were separated from normal blood and then exposed to five synovial fluids, in a dose response polarisation assay. Three fluids with high activity and two with low activity have been selected to show the diverse pattern of activity obtained.

FIGURE 3.14

Neutrophil polarisation in response to various synovial fluids at optimal dilutions.



Cells were tested against an optimal dilution of synovial fluid from RA (n=20) OA (n=5) and other (n=5) inflammatory arthritides (n=5). Each point represents the mean of 3 experiments. Bars represents the median of polarization response in each group. Control value has been subtracted from the values shown. Results statistically were not significant

3.6.1B. Collagen gel invasion

To investigate the locomotion activity of neutrophils in response to synovial fluids, 20 RA and 10 non RA synovial fluids were incorporated into collagen gels and incubated for three hours at 37⁰ C. Fig 3.15 shows that 11/20 RA and 3/10 non RA synovial fluids were capable of attracting neutrophils. These results also correlated with the polarization response mentioned above ($r=0.789$, $p<0.01$).

3.6.2. Neutrophil from synovial fluid

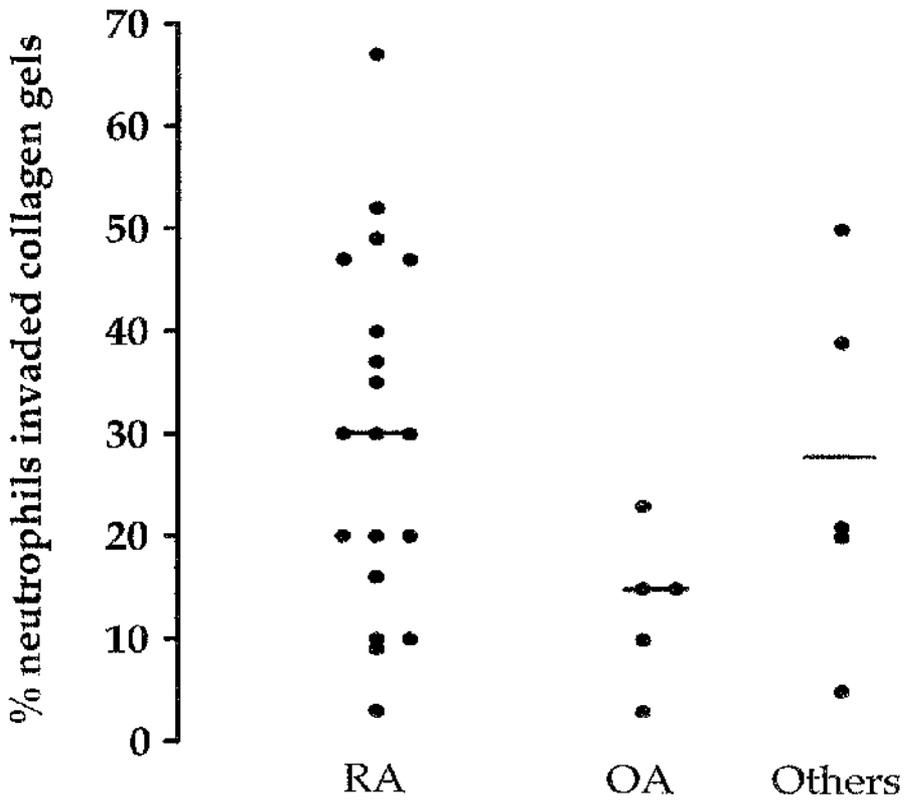
Neutrophils separated from synovial fluid were washed three times in medium, and tested against their own synovial fluid and against other chemotactic factors in a 30 min polarisation assay. Figure 3.16 shows a comparison between the response of normal blood neutrophils and synovial fluid neutrophils to synovial fluid, and FMLP (10^{-8}). Synovial fluid neutrophils showed almost no response.

Conclusions.

Rheumatoid synovial fluid attracts significantly normal blood polymorphonuclear cells. Synovial fluid polymorphonuclear cells showed defective locomotion activity in response to rheumatoid synovial fluid and FMLP compared with the control.

FIGURE 3.15

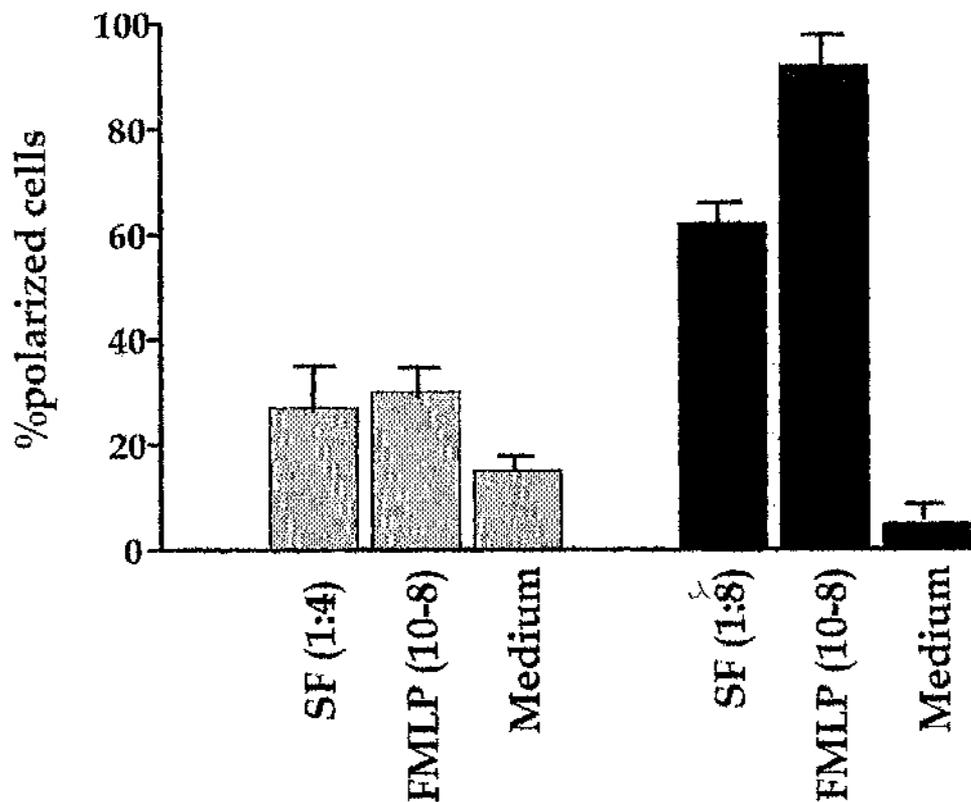
Neutrophil invasion collagen gels in response to synovial fluids.



Synovial fluids were from RA (n=20) OA (n=5) and others patients (n=5) Each point represents the mean of two experiments. Bars represent the median of polarization response. Control value has been subtracted from the values shown. Results were statistically significant when compared RA to OA ($p < 0.05$).

FIGURE 3.16

Polarization of synovial fluid neutrophils in response to different stimuli.



Polarization activity for polymorphonuclear neutrophils of synovial fluids (clear columns) from patients with rheumatoid arthritis (n=10) in response to synovial fluids (n=10) and FMLP as compared with normal human neutrophils (Filled column). Results are expressed as mean \pm SEM (n=3).

3.7 CONCLUSIONS

Rheumatoid Synovial fluid has strong chemotactic activity for human peripheral blood lymphocytes as measured by polarization and collagen gel assays. It has been found that the locomotion activity of these fluids is time dependent and uncorrelated with the inflammatory indicators including ESR and CRP. The data from polarization assays are correlated with collagen gel assays.

It has been also shown that the synovial fluid attracted significantly CD45RO+ cells (90% T cells) compared with insignificant selection against CD45RA, CD3, CD22 and CD56 (as phenotyped by APAAP and FACS). In addition it has been observed that the synovial tissue lymphocytes had strong chemotactic activity in response to synovial fluid compared with cells from synovial fluid which were found to be immotile cells.

Regarding the neutrophils it was concluded that the synovial fluid has strong chemotactic activity for peripheral blood neutrophils, whereas neutrophils from synovial fluids were immotile cells.

3.8. DISCUSSION

3.8.1 Locomotion activity of normal blood lymphocytes in response to synovial fluids.

3.8.1 A1. Comparison of the polarization activities of cells direct from blood and cells cultured overnight.

In the present study normal human lymphocytes directed from blood and cultured overnight (in FCS) were tested against rheumatoid synovial fluid in a short term polarization assay in order to compare the maximal polarization activity of these cells. Lymphocytes cultured overnight in FCS (Berman 1966), gave higher polarization responses (30-49% to rheumatoid synovial fluid (at a dilution of 1:4 and 1:16) than lymphocytes direct from blood (15-19% at dilution also of 1:4 and 1:16). This results are consistent with the suggestion by Parrott and Wilkinson 1981; Wilkinson, 1986 and Wilkinson 1987, that lymphocyte contact during culture with activators including FCS, or anti-CD3, PPD, PHA and allo-MLR (O'Neill and Parrott 1977; Wilkinson 1986, Wilkinson and Higgins 1987) or Con A (Wilkinson *et al.*, 1976), is essential for lymphocytes to acquire locomotor capacity. In other words the locomotion activity of lymphocytes taken from blood is very low, because these cells are not in cell cycle (G0). When they are cultured overnight with activators such as FCS, they acquire locomotor capacity and increase in size and

RNA and protein synthesis. These metabolic changes occur during the G1 phase of cell cycle. Although FCS is reported to be a mitogenic factor, its mitogenic activity is not a critical step for stimulation of locomotion during overnight culture, because locomotion is activated long before the cell begins to synthesize DNA. Increase in the locomotor response is dependent on activation and is enhanced as resting cells move into early G1 (Wilkinson 1986; Wilkinson 1992)

In conclusion to get the maximal locomotor immune response, resting or immotile cells should be cultured with a suitable activator to acquire locomotor capacity and then tested against chemoattractant stimuli.

3.8.1 A2. Short term lymphocyte polarization in response to synovial fluids.

As the previous section determined that the proportion of polarized lymphocytes direct from blood was very low, cells were routinely cultured overnight and then exposed to a wide range of rheumatoid and non rheumatoid synovial fluids. There were differences of both optimal dilution and the level of polarization activity found in these fluids. The majority of the rheumatoid synovial fluids gave a bell-shaped dose response curve with a maximal activity at dilutions of between 1:2 and 1:16. The maximal proportion of polarized lymphocytes was 50-60%. In contrast synovial fluids from OA gave negative or slightly higher responses than the normal control. This is not surprising since as will be discussed in chapter four, OA synovial fluid contains very low concentrations of cytokine chemoattractants. In addition several

synovial fluids from other inflammatory arthritides such as polymyalgia rheumatica and psoriatic arthritis gave higher responses with 49-51% of polarized lymphocytes. These fluids had got sufficient levels of chemoattractants (>40ng/ml) to produce this maximal polarization activity. There was no relation between the optimal dilution and the amount of activity obtained at that dilution. However as will be discussed in the next chapter the polarization activity of these fluids is most likely to be due to a combination of chemoattractants. The maximal polarization activity was time dependent. The maximal percentage of lymphocytes was 35-45% after 10-30 min incubation. Further incubation of these cells at 40, 50 and 60 minutes did not result in any further increase.

3.8.1 A3. Lymphocytes invading collagen gels in response to synovial fluids.

In rheumatoid synovial tissues in vivo migrating lymphocytes must interact with extracellular matrix, and collagen is the major component of extracellular matrix of vertebrates (Linsenmayer 1981; Kuhn and Ghanville 1980). To simulate migration or locomotion of lymphocytes as it occurs in vivo at synovial compartments, Type 1 collagen gel is a suitable matrix in vitro (Elsdale and Bard 1972). This method has got several advantages including allowing cells to be visualised in situ in the gels, and digestion of collagen gel matrix (Shields, *et al.*, 1984; Schor 1980) allows the invading cells to be recovered and phenotyped. In this section lymphocytes were allowed to invade the collagen gel incorporated with the optimal dilution of synovial

fluids. It was found that the results of this assay correlated with the polarization assay mentioned above. This data are consistent with the observations of Wilkinson (1986) who reported that collagen gel assay supplements the polarization assay and provides direct evidence for active locomotion of lymphocytes towards chemoattractants.

3.8.2. Locomotion activity of synovial tissue lymphocytes

During short term polarisation assays it was observed that lymphocytes separated directly from synovial tissue respond successfully without any pre-activation with any stimulators, compared with normal blood cells mentioned above (which need overnight culture to increase their locomotion capacity). This is interesting as these cells (synovial tissue lymphocytes) may acquire their locomotion capacity during ongoing local inflammatory processes (i.e recognition of unknown antigen somewhere in the synovial tissue, or may be these cells are activated in the circulation and then migrate to the joint tissue via specific homing receptors. Whether these cells are activated in a joint by a yet unidentified antigen or are in fact selected to migrate from lymphoid tissue to the site in the inflamed joint needs further investigations.

3.8.3. Locomotion activity of rheumatoid synovial fluid lymphocytes

During separation of these cells from the synovial fluid it was noticed that cell viability in the majority of rheumatoid arthritis synovial fluids was not affected, and several other studies from different laboratories reported that these cells can proliferate in response to various stimuli (Hemler, *et al.*, 1986; Gaston *et al.*, 1990).

During short term polarization assays (in responses to active rheumatoid synovial fluid or other chemotactic factors such as IL-8), the polarization activity of rheumatoid synovial fluid lymphocytes was impaired (i.e the synovial fluid lymphocytes are not locomotor or non motile), compared with the control. This is interesting but it is difficult to explain this unresponsiveness. The following suggestions are possible (a) These cells might have completed their locomotor function *in vivo* and ceased their movement because they have internalised their chemotactic receptors but can continue other activities including cytokine production (b) As rheumatoid arthritis is considered as an autoimmune phenomenon autoantibodies or immune complexes produced *in vivo* (including autoantibodies against cytoplasmic proteins) might affect the functional activity of the chemotactic receptors of these cells, (c) chemotactic inhibitors (in synovial fluid) may selectively block the activity of particular chemotactic factor receptors. Agents that raise cyclic AMP levels in cells, e.g IL-1, prostaglandin, are able to do this and may be present in the fluids.

It has been reported that during binding of chemotactic receptors to leukocytes the intracellular concentration of calcium and cAMP rises (Bouceck and Synderman 1976; Simchowitiz *et al.*, 1980). The former is reported to be necessary for many cellular functions, whereas the latter seems to inhibits leukocyte activities (Pike and Synderman 1984). There may be inhibitors (in the synovial fluid) to cAMP-phosphodiesterase which in turn raises cAMP, which might contribute to the termination of the cells response to any chemotactic receptors (i.e render these cells immotile). These chemotactic inhibitors may be considered as immunoregulatory molecules for the inflammatory immune response in rheumatoid arthritis.

3.8.4. Phenotyping of polarized lymphocytes and lymphocytes subsets

The proportion of CD3+, CD22+, CD45RA+ and CD56+ polarized cells that were polarized reflected the proportion of these phenotypes in the whole population. However the proportion of polarized CD45RO+ (95% are T cells) was higher than would be expected suggesting that this phenotype is associated with locomotion. The significant selection of CD45RO+ cells suggests cells that have recently responded to antigen are more likely to respond to chemotactic factors in synovial fluid than naive cells. In collagen gel assays it was also observed that CD45RO+ cells were more motile than CD45RA+cells. In addition the acquisition of locomotor capacity is associated with cell growth (Wilkinson 1986) and CD45RO+

cells are larger in area and volume than CD45RA cells after activation (Newman and Wilkinson 1994).

It has been reported that cytokines such as RANTES are selectively chemotactic factors for CD45RO+ lymphocytes using polycarbonate filters (Schall *et al.*, 1990). We did not include RANTES in our assays, though this cytokine may be present in the synovial fluid. We have not obtained good chemotactic activity for T cells using RANTES. As will be discussed in the next section, our results do not support a simple correlation between levels of single cytokines and migration of lymphocytes into the inflammatory sites.

3.8.5 Locomotion activity of neutrophils

It has been reported that neutrophil migration into an inflammatory site is a self-amplifying phenomenon. Specific granules contain a protease that cleaves complement component C5 to release the strong chemotactic fragment C5a (Matzner *et al.*, 1983). Chemotactic factors are released by the first few neutrophils which arrive at a site of inflammation, attracting more neutrophils. It has been also suggested that the locomotion response causes tissue damage in the inflammatory site including the rheumatoid synovium. If this response uncontrolled it could lead to a serious consequences.

Neutrophil chemotaxis in rheumatoid arthritis had been studied using the leading front technique or raft technique (Hanlon *et al.*, 1980; Goddard, *et al.*, 1984). These

studies concluded that neutrophils from RA patients (cells isolated from RA blood) showed reduced chemotaxis, and this defect in locomotion was reported to be due to several factors including neutrophil chemotactic inhibitors (i.e anti-complement fragments or unidentified factors) either in RA blood or rheumatoid synovial fluid.

In this study polarization and collagen gel assays were used to do further neutrophil locomotion studies. It has been observed that cells from normal blood have shown strong chemotactic activity in response to a wide range of rheumatoid and non rheumatoid synovial fluid. This is not surprising as I will discuss later that synovial fluid contains a high concentration of neutrophil chemotactic factors including IL-8, but as will be discussed in the next chapter, IL-8 is not correlated with the neutrophil accumulation in the fluid. This data suggest that the chemoattraction activity of synovial fluids for neutrophils is due to combined chemotactic factors including IL-8 and C5a or other as yet unidentified factors.

On the other hand synovial fluid neutrophils incubated with their own synovial fluid or FMLP in a short term polarization assays showed a significantly reduced locomotion activity compared with controls. This indicated that these cells may be in the same situation as lymphocytes separated from synovial fluid (see above). In addition it has been observed here that the cell viability of neutrophils in some fluids was very low compared with the cell viability of synovial fluid lymphocytes from the same fluids. This indicates that synovial fluid can be considered as a grave for some inflammatory cells.

CHAPTER 4:

CYTOKINES IN SYNOVIAL FLUID:

THE PRESENCE OF LYMPHOCYTE CHEMOATTRACTANTS

4.1 INTRODUCTION

As the previous chapter showed that synovial fluid has a chemotactic activity for human leukocytes, cytokines present in SF may be relevant to its chemoattraction activity and to the accumulation of lymphocytes and other inflammatory cells in the synovia. The activity of IL-2 (Kornfeld, *et al.*, 1985; Wilkinson and Newman 1994), IL-8 (Wilkinson and Newman 1992), MIP-1 α (Taub *et al.*, 1993; Wilkinson and Newman 1994) and MCP-1 in stimulating of lymphocyte locomotion and chemotaxis has been reported. Recently IL-15 was shown to be a lymphocyte chemoattractant (Wilkinson and Liew 1995) and also, may play an important role in T cell migration and activation in rheumatoid synovial tissue (McInnes *et al.*, 1996).

In order to quantify the cytokine levels in SF, various commercially available immunoassays based on monoclonal antibodies were used. To measure the IL-15

levels, a sensitive and specific double sandwich ELISA assay had to be developed in our laboratory.

By measuring chemoattractant cytokine levels in SF from patients with RA, OA and other inflammatory arthritides, it was possible to compare differences in chemoattractant cytokine distribution in different joint diseases. It was also possible to assess the correlation between the leukocyte count and the chemoattractant levels in SF. Finally it was important to investigate the role of these chemoattractants as mediators of SF locomotor activity by study of the inhibitory effect of anti-cytokine- specific antibodies (anti-IL-8, anti-IL-15, anti-MIP-1 α and anti-MCP-1) on SF-induced lymphocyte polarization and collagen gel invasion.

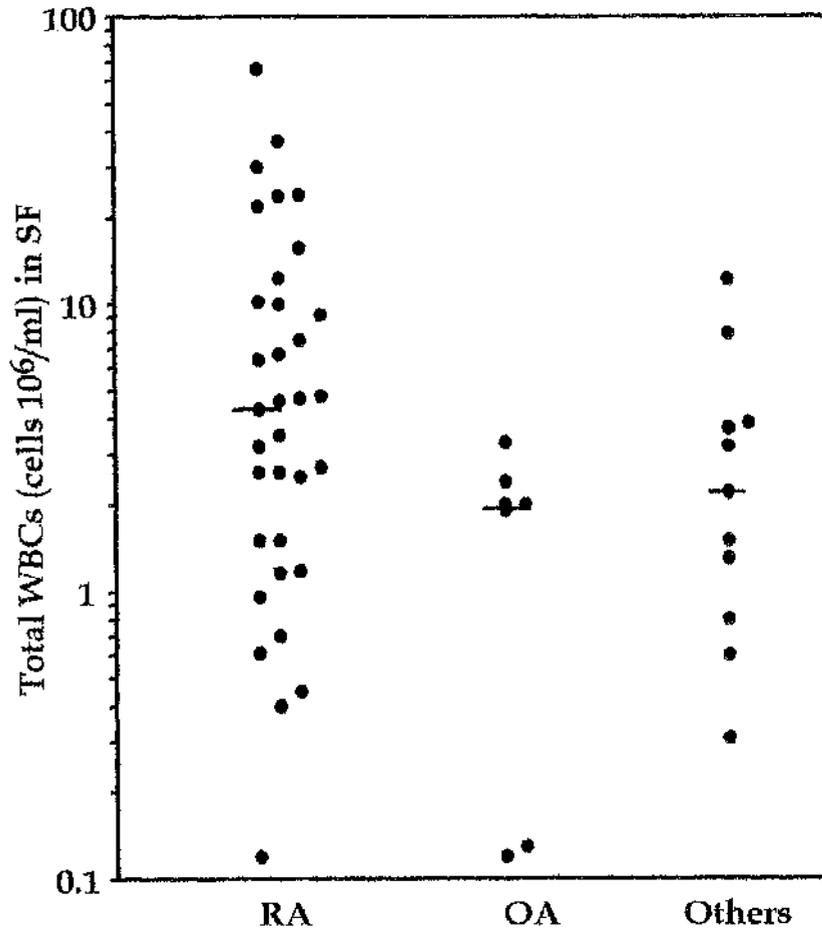
4.2 RESULTS

4.2.1 Preliminary Experiments (leukocyte counts in inflammatory synovial fluids)

As mentioned in chapter 2, synovial fluids collected from different inflammatory arthritides were initially spun and the cells in the pellets were washed. The total WBCs, were counted and preparations were stained for differential counts. As shown in figure 4.1 the synovial fluid total white blood cell (TWBCs) count was significantly higher in rheumatoid arthritis patients than in osteoarthritis ($p < 0.02$) and also significantly higher than in other inflammatory arthritides ($p < 0.05$). In addition synovial fluid from patients with rheumatoid arthritis contained more lymphocytes (fig 4.2) than OA fluids ($P < 0.04$) or fluid from other inflammatory diseases ($P < 0.05$). Neutrophil counts were also obtained (fig 4.3). RA-SF contained a significantly higher numbers of neutrophils when compared to OA ($P = 0.0446$) and other inflammatory arthritides ($p < 0.05$).

FIGURE: 4.1

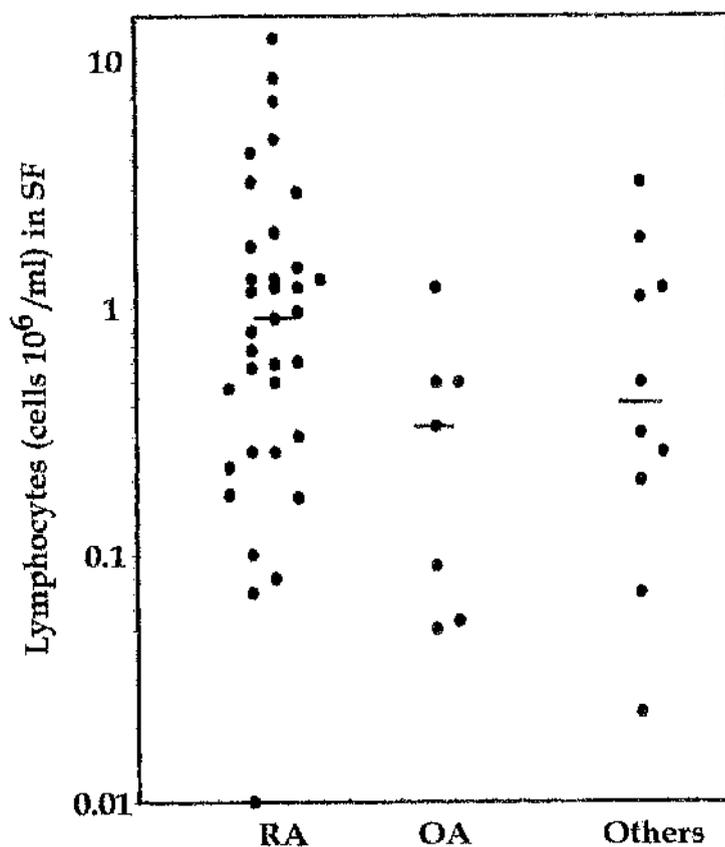
Total Leukocyte count in Synovial fluids



Total WBC count in SF of patients with RA (n=34) OA (n=6) and other inflammatory arthritides (n=11). All results are expressed as individual data. Medians are indicated by horizontal bars. For more details see tables 4.1, 4.2 and 4.3.

FIGURE: 4.2

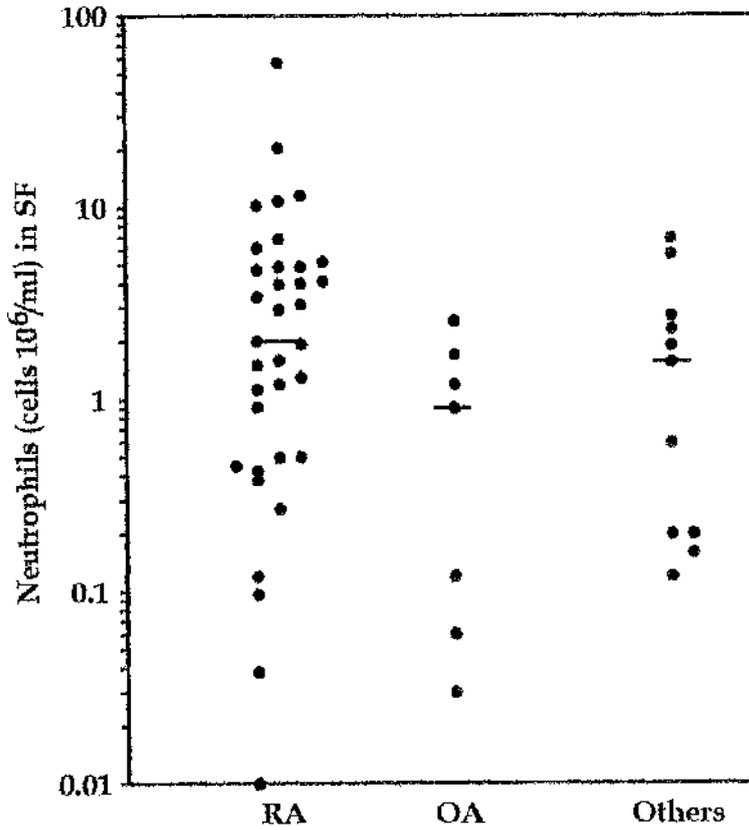
Lymphocyte Count in Synovial fluids



Lymphocyte count in SF of patients with RA (n=34) OA (n=6) and other inflammatory arthritides (n=11). All results are expressed as individual data. Medians are indicated by horizontal bars. For more details see tables 4.1, 4.2 and 4.3.

FIGURE: 4.3

Neutrophil count in synovial fluids.



Neutrophils count in SF of patients with RA (n=35) OA (n=6) and other inflammatory arthritides (n=11). All results are expressed as individual data. Medians are indicated by horizontal bars. For details see table 4.1, 4.2 and 4.3.

Table: 4.1 leukocyte counts in RA-SF*

<i>Code</i>	<i>Total WBCs (10⁶/ml)</i>	<i>Lymphocytes (10⁶/ml)</i>	<i>Neutrophils (10⁶/ml)</i>
M.B	0.5	0.1	0.5
M.S	6.7	0.8	4.9
W.M	0.4	0.3	0.1
S.S	12.3	1.8	4.1
J.C	0.1	0.2	0.2
D.R	0.7	0.2	0.5
R.L	2.7	0.6	1.9
G.W	4.7	0.5	4.1
J.G	65.5	4.2	56.9
C.M	21.7	2.1	6.8
C.W	15.7	1.2	10.2
M.M.A	4.3	0.3	3.9
W.C	24.0	12.0	10.8
M.J	0.7	0.1	0.1
R.M	0.2	0.1	0.1
M.M	1.2	0.6	0.5
H.M	1.5	0.6	1.3
S.K	9.7	4.8	3.4
R.E	30.0	8.4	20.4
A.M	1.2	0.7	0.4
D.G	2.6	1.5	0.9
E.M	4.8	1.3	2.9
H.Y	1.5	0.2	1.3
M.G	7.5	0.6	6.1
J.A	2.6	0.1	1.2
M.N	6.4	4.7	1.2
J.K	3.2	1.6	1.3
I.S	2.5	1.5	0.9
L.M	3.5	2.0	1.2
E.P	4.6	3.0	1.3
R.M	9.2	4.9	2.9
M.E.Z	10.2	5.2	3.2
G.H	23.8	11.5	6.8
B.L	0.1	0.5	0.3
I.F	1.2	0.7	0.1

*Total cell count in RA-SF (n=35) was determined (for details see method in chapter 2) as described above and is expressed as cells/ml SF (total leukocyte number). The proportion of those cells that are lymphocytes and neutrophils is given.

Table : 4.2. leukocyte counts in OA-SF*

<i>Code</i>	<i>Total WBCs (cells x10⁶/ml)</i>	<i>Lymphocytes (cells x10⁶/ml)</i>	<i>Neutrophils (cells x10⁶/ml)</i>
H.Me	0.1	0.1	0.1
D.SC	0.2	0.1	0.1
E.Me	0.1	0.1	0.1
A.SO	3.3	0.3	2.5
D.BA	2.4	0.5	1.7
A.AL	2.1	1.2	0.9
E.CA	1.9	0.5	1.2

Table: 4.3. Leukocyte counts in other inflammatory arthritides*.

<i>Code</i>	<i>Provisional diagnosis</i>	<i>Total WBCs (cells x10⁶/ml)</i>	<i>Lymphocytes (cells x10⁶/ml)</i>	<i>Neutrophils (cells x10⁶/ml)</i>
D.SM	SPA	2.2	0.3	1.6
C.CA	SPA	12.2	3.2	6.9
R.MC	SPA	3.2	0.5	2.7
F.BW	PMR	1.3	1.1	0.2
M.GR	PART	3.7	1.3	1.9
D.SH	AS	0.3	0.1	0.21
S.T	IMHA	0.8	0.2	0.6
W.G	PSA	0.2	0.1	0.1
B.T	PSA	7.9	1.9	5.7
W.SP	PSA	3.4	1.2	2.3
M.TH	PSA	0.6	0.3	0.2

Total cell count in OA-SF (n=7 ,table 4.5) and other inflammatory arthritides (n=11,table 4.6) was determined (for details see method in chapter 2) as described above and is expressed as cells/ml SF (total leukocyte number). The proportion of those cells that are lymphocytes and neutrophils is shown. OA=osteoarthritis, SPA=seronegative spondyloarthritis, PMR= polymyalgia rheumatica, AS=ankylosing spondylitis, IMHA=intermittent hydroarthrosis , PSA=psoriatic arthritis, PART=pyrophosphate arthropathy.

4.2.2A. Detection of chemoattractant cytokines in SF

4.2.2A1. Interleukin-2 (IL-2)

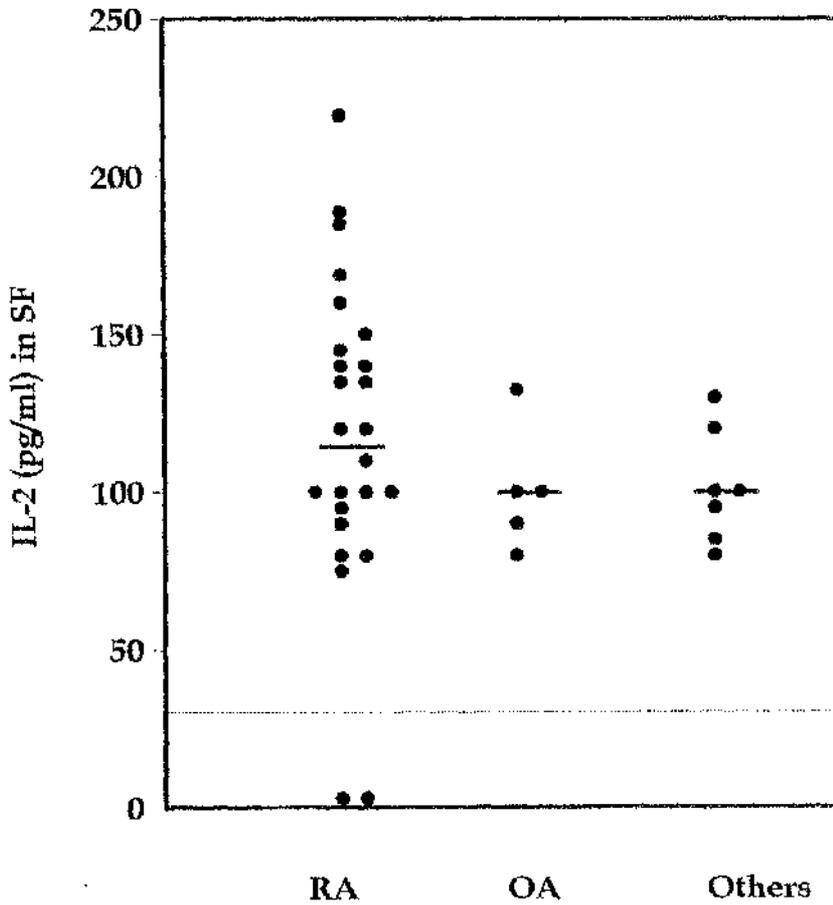
The levels of IL-2 in the synovial fluid of patients with RA, OA and other inflammatory arthritides were measured. As shown in figure 4.4, low amounts of IL-2 were detected in patients with RA, (n=29) OA (n= 5) and patients with other inflammatory arthritides (n=7). IL-2 levels were below 250 pg/ml in all fluids. Although the IL-2 levels was significantly higher in RA compared with OA and other inflammatory arthritides the concentrations shown in Fig 4.4 are well below the level (10-100ng/ml) at which pure IL-2 acts as a T lymphocyte attractant (Wilkinson and Newman 1994).

4.2.2A2. Interleukin 8 (IL-8).

To confirm whether IL-8 is present in the synovial fluids of patients with RA, synovial fluids from RA (n=28) were tested as well as fluid from OA (n=5) and other arthritides (n=9). IL-8 was detected in the synovial fluids of most patients with rheumatoid arthritis (Fig 4.5). The concentration of IL-8 in the synovial fluids from patients with seropositive rheumatoid arthritis

FIGURE: 4.4

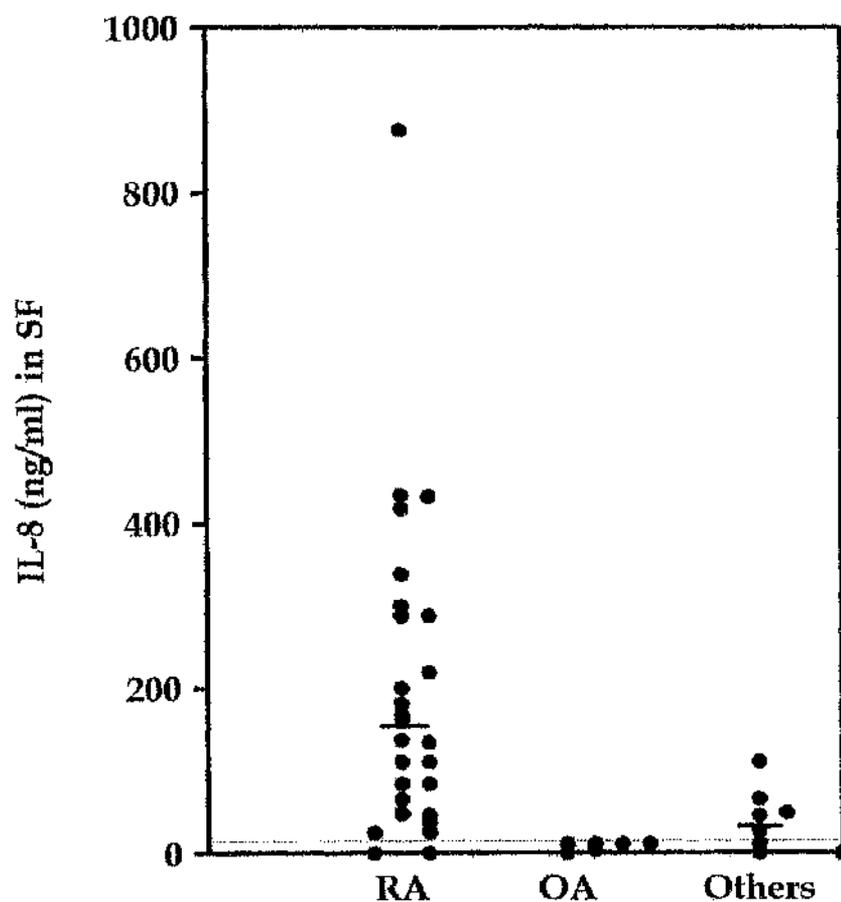
Levels of IL-2 in synovial fluids.



Concentration of IL-2 in synovial fluids obtained from patients with RA (n=29) OA (n=5) and other inflammatory arthritides (n=7) was determined using ELISA. All results are expressed as individual data, Medians are indicated by horizontal bars. Broken line represents the limit of detection. For details see tables 4.4, 4.5 and 4.6.

FIGURE: 4.5

Levels of IL-8 in synovial fluids.



Concentration of IL-8 in synovial fluids obtained from patients with RA (n=29) OA (n=5) and other inflammatory arthritides (n=7) was determined using ELISA. All results are expressed as individual data. Medians are indicated by horizontal bars. Broken line represents the limit of detection. For more details see tables 4.4, 4.5 and 4.6.

was higher than in the two groups of OA and other arthritides. There was a very wide variation in IL-8 levels between different fluids. Most contained substantial quantities of IL-8 (for details see table 4.4) though, in two patients with RA, one patient with OA and two patients with other inflammatory arthritides, IL-8 was not detectable. T lymphocytes from normal peripheral blood (Wilkinson and Newman 1992) respond optimally to IL-8 at 100 ng/ml (and many rheumatoid joint fluids contained IL-8 at levels close to this). Although the clinical parameters of disease activity (ESR and CRP) tended to be higher in RA patients than in OA or non RA/OA there was no correlation between these parameters and the concentration of IL-8 in the synovial fluids. The concentration of IL-8 in the synovial fluids from seropositive RA patients was comparable to concentrations previously shown (Wilkinson and Newman 1992) to induce chemoattractant activity for lymphocytes or neutrophils.

4.2.2. A3. Levels of interleukin-15 (IL-15)

The IL-15 concentration in synovial fluid from patients with different rheumatic disease was determined. As shown in fig 4.6 (for details see tables 4.4, 4.5 and 4.6) IL-15 was detected at nanogram level in most cases of rheumatoid arthritis tested. On the other hand very low levels of IL-15 were found in OA synovial

fluids. IL-15 was also detected at high levels in some cases of other inflammatory arthritides.

4.2.2A4. Levels of macrophage inflammatory protein-alpha (MIP-1 α).

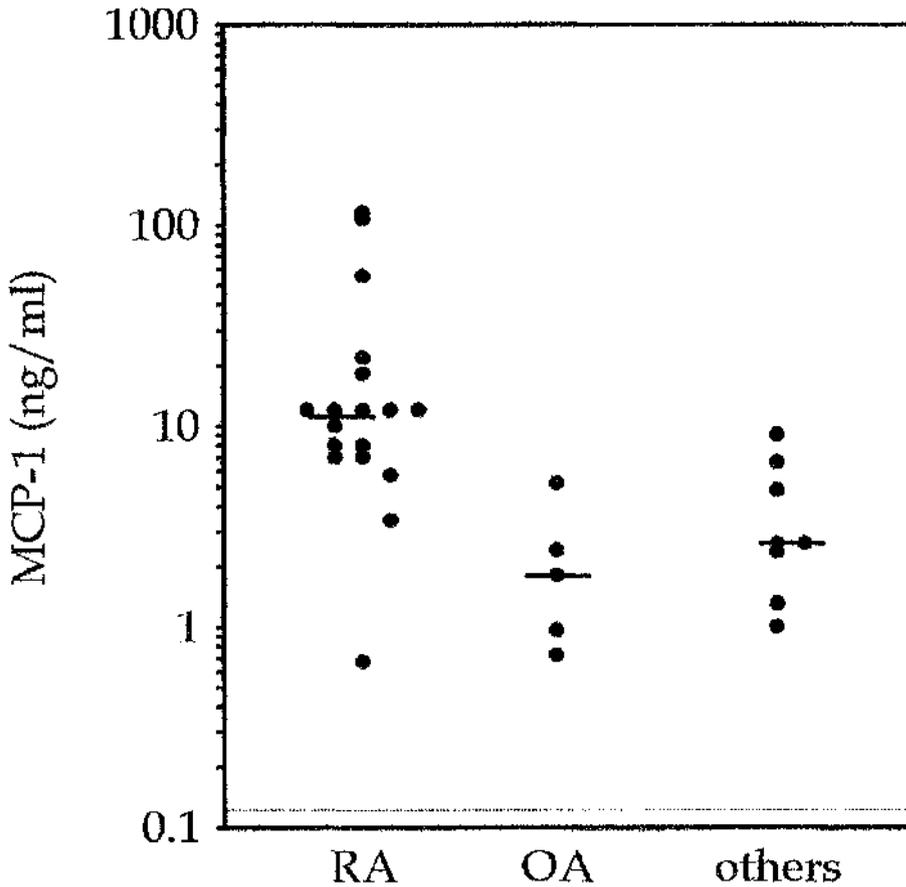
MIP-1 alpha levels was detected in synovial fluid using ELISA (Fig 4.7). The patients with RA (n=30) had MIP-1 α in most fluids, 7/30 at a level above the level at which pure MIP-1 α acts as a T lymphocyte attractant (Wilkinson and Newman 1994). MIP-1 α alpha was also detected in small quantities in OA (n=6) and other inflammatory arthritides (n=8).

4.2.2.A5. Levels of macrophage chemotactic protein-1 (MCP-1)

MCP-1 levels were determined in synovial fluid of patients with RA (n=18) OA (n=5) and other inflammatory arthritides (Fig 4.8). Almost all RA fluids had MCP-1, (17/18) above the level at which pure MCP-1 level acts as T lymphocyte attractant (1-10ng/ml) (Wilkinson.P.C. unpublished observations). MCP-1 was also detected in 3/5 OA and in five patients with other inflammatory arthritides.

FIGURE. 4.8

Level of MCP-1 in synovial fluids



Concentration of MCP-1 in synovial fluids obtained from patients with RA (n=18), OA (n=5) and other inflammatory arthritides (n=6) was determined using ELISA. All results are expressed as individual data, Medians are indicated by horizontal bars. Broken line represents the limit of detection. For details see table 4.4, 4.5 and 4.6.

Table: 4.4. Levels of chemoattractants in SF of patients with RA.

<i>Code</i>	<i>IL-2 (pg/ml)</i>	<i>IL-8 (ng/ml)</i>	<i>IL-15 (ng/ml)</i>	<i>MIP-1α (ng/ml)</i>	<i>MCP-1 (ng/ml)</i>
M.B	90	0.0	NA	0.2	NA
M.S	120	47.8	17	0.9	NA
D.M	95	287.2	78	0.1	107
W.M	145	26.5	110	0.1	12
S.S	120	0.0	58	0.1	22
J.C	150	84.4	NA	0.2	NA
D.R	189	417.8	8	32.3	0.67
R.L	102	181.2	74	83	55
G.W	185	200.3	17	0.1	NA
J.G	135	161.7	41	0.1	5.7
C.M	169	46.0	27	32.3	NA
C.W	100	65.0	36	0.2	NA
M.EZ	135	432.0	31	1.2	NA
W.C	0.0	876.0	16	1.5	10.2
MJ	100	84.4	86	0.2	18.3
R.M	75	287.2	120.0	0.1	8.2
M.M.A	80	24.6	3.5	3.8	6.6
H.M	95	110	57.0	3.4	21.9
S.K	110	137	NA	0.8	0.0
R.E	120	134	2.5	2.5	116
A.M	2.9	434	36.0	34.6	3.4
D.G	140	167	65.0	8.6	0.0
MO.E	160	219	65.0	48.7	11.9
H.Y	100	110	NA	253.0	11.6
M.GI	216	299	51.0	19.6	11.7
JA	80	338	82.0	230	33.2
BL	2.9	NA	NA	NA	NA
LF	85	NA	11.0	1.9	NA
M.MO	NA	NA	89.0	NA	6.0
MN	NA	36.7	NA	2.8	11.8
JK	NA	25.0	NA	NA	8.2
IS	NA	NA	NA	5.8	NA
LM	NA	NA	NA	1.9	NA

Levels of chemoattractants (IL-2, IL-8, IL-15, MIP-1 alpha and MCP-1) in synovial fluid of patient with rheumatoid arthritis. NA= not available. Note that the concentration of IL-2 was in pg/ml.

***Table. 4.5. Levels of chemoattractants in synovial fluid of patients with OA.**

<i>Code</i>	<i>IL-2 (pg/ml)</i>	<i>IL-8 (ng/ml)</i>	<i>IL-15 (ng/ml)</i>	<i>MIP-1 α (ng/ml)</i>	<i>MCP-1 (ng/ml)</i>
H.Me	0.2	11	NA	1.6	2.8
D.SC	100	NA	2.0	2.0	NA
E.Me	132	11	3.0	2.7	2.4
A.SO	100	11	3.0	1.7	0.1
D.BA	80	11	1.0	0.9	1.3
A.LA	NA	3.0	NA	2.7	NA
E.CA	NA	0.0	2.0	NA	2.4

***Table. 4.6. Levels of chemoattractants in SF of patients with other inflammatory arthritides.**

<i>Code</i>	<i>Diagnosis</i>	<i>IL-2 (pg/ml)</i>	<i>IL-8 (ng/ml)</i>	<i>IL-15 (ng/ml)</i>	<i>MIP-1 α (ng/ml)</i>	<i>MCP-1 (ng/ml)</i>
D.SM	SPA	100	65	19	0.1	5.2
F.BW	PMR	80	110	40	4.0	1.0
M.GR	PART	NA	11	2.0	5.0	2.6
D.SH	AS	120	0.0	24	0.1	0.7
S.T	IMHA	95	0.0	1.0	0.0	1.8
W.G	PSA	100	110	1.0	0.1	NA
B.T	PSA	NA	26.4	NA	2.0	4.8
W.SP	PSA	130	48.5	13.0	12	6.6
M.TH	PSA	85	44.9	5.0	2.0	NA

Levels of chemoattractants (IL-2, IL-8, IL15 and MIP-1alpha) in patients with OA and other inflammatory arthritides. OA=osteoarthritis, SPA= seronegative spondyloarthritis, PMR=polymyalgia rheumatica, PART= pyrophosphate arthropathy, AS=ankylosing spondylitis, IMHA= intermittent hydroarthrosis, and PSA= psoriatic arthritis. Note that the level of IL-2 was in pg/ml.

4.3. Correlation between Chemoattractant levels and accumulation of inflammatory cells in synovial fluids.

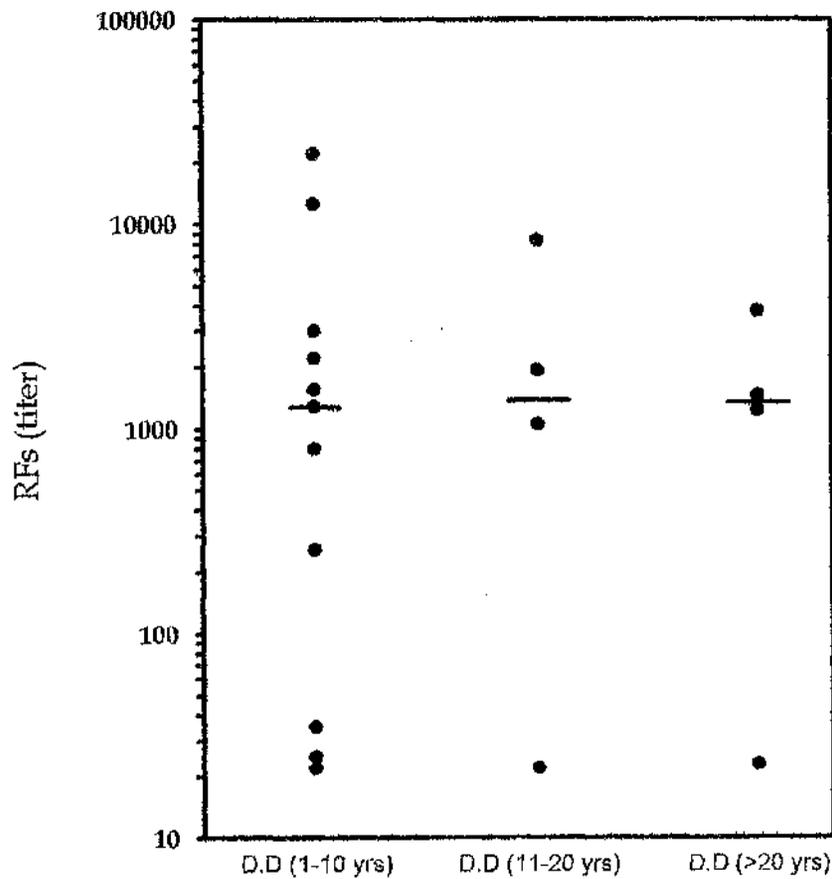
To compare levels of IL-2, IL-8, IL-15 and MIP-1 α with accumulation of inflammatory cells in synovial fluid, I calculated the correlation coefficient between levels of the chemoattractants and counts of inflammatory cells and also compared levels of each cytokines with those of the other cytokines. As shown in table (4.7a and 4.7b), levels of cytokines in SF did not correlate with each other except IL-8 and MIP-1 α ($p < 0.01$), IL-15 and MCP-1 ($p < 0.01$), MCP-1 and TWBC ($p < 0.05$). There was no correlation between levels of any synovial fluid chemoattractants (table 4.7) and accumulation of inflammatory cells in any of the three patient groups (RA, OA and others).

4.4. Correlation between SF IL-8 and serum RF

It was reported that mononuclear cells from RA patients spontaneously release IL-8 and that production is markedly enhanced by RF-containing immune complexes (Sietz, *et al* 1991). In the present study rheumatoid factor was measured in 17 patients with seropositive RA (Figure 4.9). There was no correlation between the titer of serum-RF and the concentration of IL-8 in the synovial fluid.

FIGURE 4.9

Level of rheumatoid factors in serum of patients with RA.



Level of rheumatoid factors in the serum of rheumatoid arthritis patients with disease duration (D.D) 1-10 years (n=11), 11-20 (n=4) and >20 years (n=4) were determined utilizing Immunoturbidometry. All results were expressed as individual data, medians are indicated by horizontal bars.

Table: 4.7 Summary of correlation coefficient of chemoattractants levels with inflammatory cell accumulation in synovial fluids.

<i>IL-2</i> (pg/ml)	<i>IL-8</i> (ng/ml)	<i>IL-15</i> (ng/ml)	<i>MIP-1α</i> (ng/ml)	<i>MCP-1</i> (ng/ml)	<i>TWBCs</i> (10 ⁶ /ml)	<i>Lymph</i> (10 ⁶ /ml)	<i>Neutro</i> (10 ⁶ /ml)	<i>r</i>	<i>P*</i>
X	X							-0.181	NS
X		X						0.163	NS
X			X					0.060	NS
X						X		0.164	NS
	X	X						0.127	NS
	X		X					0.372	0.01
	X				X			0.267	NS
	X					X		0.220	NS
	X						X	0.189	NS
		X	X					0.050	NS
		X			X			-0.031	NS
		X				X		0.136	NS
			X		X			0.044	NS
			X			X		0.161	NS
			X				X	0.027	NS
X				X				0.089	NS
	X			X				-0.029	NS
		X		X				0.505	0.01
			X	X				0.034	NS
				X	X			0.443	0.05
				X		X		-0.095	NS
				X			X	-0.014	NS

* Calculated by Spearman rank correlation test.

NS=not significant.

Table: 4.7b. Summary of correlation coefficient of chemoattractants levels with inflammatory cell accumulation in synovial fluids.

IL-2 (pg/ml)	IL-8 (ng/ml)	IL-15 (ng/ml)	MIP-1 α (ng/ml)	MCP-1 (ng/ml)	TWBCs (10 ⁶ /ml)	Lymph (10 ⁶ /ml)	Neutro (10 ⁶ /ml)	r	P*
X	X							-0.181	NS
X		X						0.163	NS
X			X					0.060	NS
X						X		0.164	NS
	X	X						0.127	NS
	X		X					0.372	<u>0.01</u>
	X				X			0.267	NS
	X					X		0.220	NS
	X						X	0.189	NS
		X	X					0.050	NS
		X			X			-0.031	NS
		X				X		0.136	NS
			X		X			0.044	NS
			X			X		0.161	NS
X			X				X	0.027	NS
	X			X				0.089	NS
		X		X				-0.029	NS
			X	X				0.505	<u>0.01</u>
				X				0.034	NS
				X	X			0.443	<u>0.05</u>
				X		X		-0.095	NS
				X			X	-0.014	NS

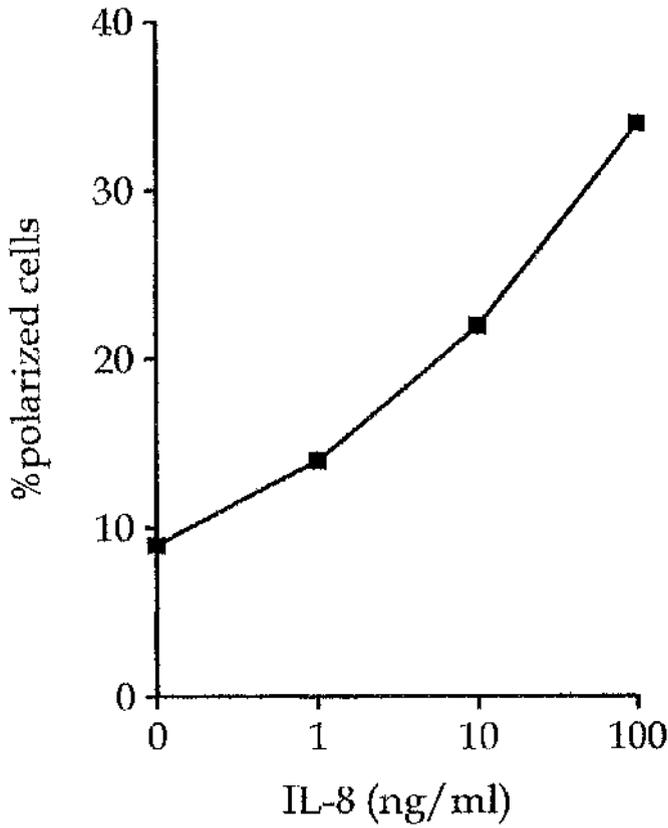
* Calculated by Spearman rank correlation test. The P values above indicate the attained confidence level for individual pairs of variables or correlates. However, if we apply the 95% confidence criteria to the table taken as a whole, using Bonferoni's inequality, we find that only one of the correlation's may be regarded as statistically significant namely that between IL-15 and MCP-1 (P=0.0085)

NS=not significant.

4.5. Chemoattractant activity of cytokines for rheumatoid human lymphocytes

As discussed earlier the synovial fluid has several chemoattractants with a concentration above the level at which they can act as T lymphocyte attractants. A further study was carried out to investigate the locomotion activity of pure cytokines using lymphocytes from RA synovial tissue and RA peripheral blood. As there were difficulties in collecting enough materials (RA Synovial tissue and RA peripheral blood) the locomotor response of RA synovial tissue T lymphocytes and peripheral blood T lymphocytes to IL-8 and IL-15 only was investigated. As shown in Figure 4.10 T lymphocytes from **synovial tissue** responded significantly to IL-8 (100ng/ml) compared with the medium control (HBSS). A dose response assay was also done to test polarization of RA lymphocytes to IL-15. T lymphocytes from **active RA peripheral blood** show (Fig 4.11) a similar polarization dose response to IL-15 as blood from normal subjects (Wilkinson and Liew 1995). RA Lymphocytes also were allowed to invade collagen gels in response to IL-15 (100ng/ml) and to optimal dilutions of synovial fluid (1:8). Figure 4.12 shows the locomotion activity of these cells compared with the cells from normal peripheral blood (used as a positive control).

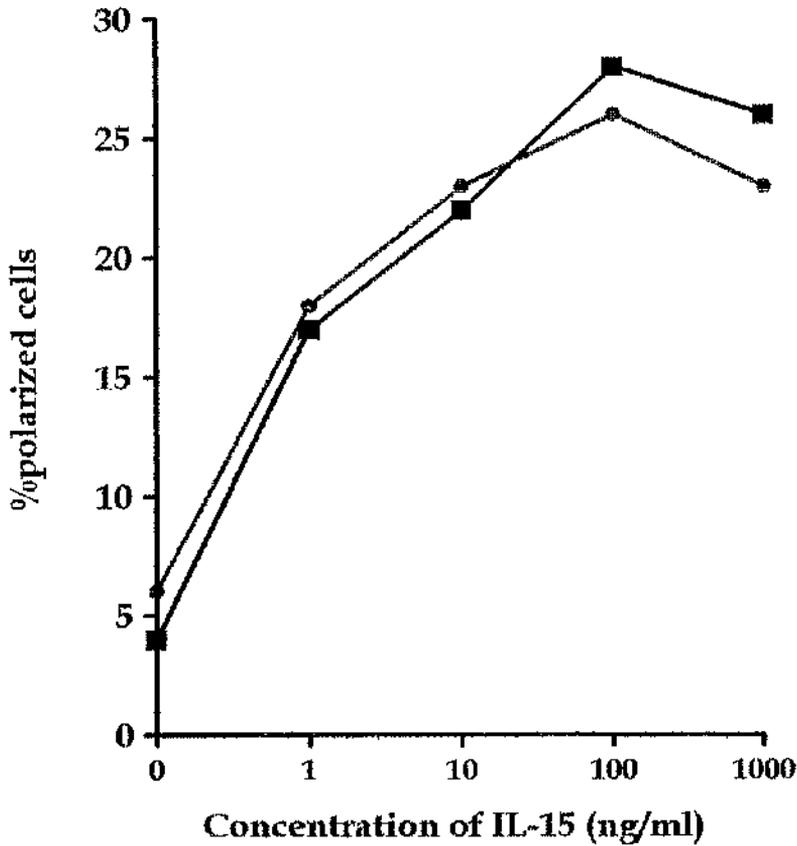
FIG 4.10 . Effect of IL-8 on the polarization of synovial tissue lymphocytes



Dose response of synovial tissue lymphocytes to IL-8 in a polarisation assay. Cells were tested directly after separation against IL-8 at different concentrations.

FIGURE: 4.11

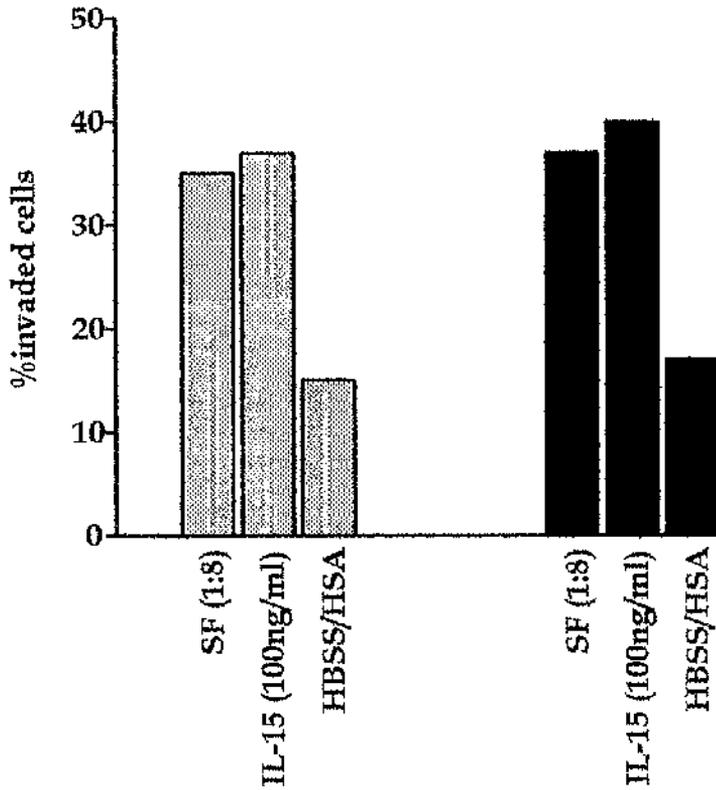
Polarisation of T cells from RA and normal blood in response to IL-15.



Lymphocytes were separated from peripheral blood and incubated with IL-15 in a dose response polarisation assay. Squares represent the polarisation response of cells from active RA patients and circles represent the polarisation response of cells from normal blood.

FIGURE 4.12

Invasion by RA and normal blood lymphocytes in response to IL-15 and synovial fluid.



Lymphocytes were allowed to invade collagen overnight in response to IL-15 (100ng/ml) and SF (1:8). The percentage of invaded lymphocytes was measured as mentioned above. The Clear bars represent the results with RA cells and filled bars represent the results with normal blood cells (used as control)

4.6. Inhibition of activity of synovial fluids with cytokine-specific antibodies

4.6.1 Preliminary experiments

To examine the inhibitory activity of anti-cytokine antibodies, seven rheumatoid RA synovial fluids (Table 4.8 A and 4.8 B) were chosen for study. Four specific cytokine-antibodies (anti-IL-8, anti-IL-15, anti-MIP-1a, and anti-MCP-1) were used for study of their inhibitory effect. The effective dilution of all antibodies was determined as 1/500 or higher (P.C. Wilkinson, personal communication) except anti-MCP-1 which was used at 1/40 or 1/50 (Fig 4.13).

4.6.2 Inhibitory effect of antibodies to individual cytokines

Studies using aIL-8, aIL-15, anti-MIP-1 α and anti-MCP-1 showed that no antibody against any individual cytokine (see below), used alone, had significant inhibitory activity on the polarization response of lymphocytes to synovial fluid even though the antibody was used at a concentration which had previously been shown to block the activity of pure cytokine at levels present in the synovial fluid.

Table 4.8 A: Levels of chemoattractants in SF of RA patients (used for anti-cytokine antibodies inhibitory study).

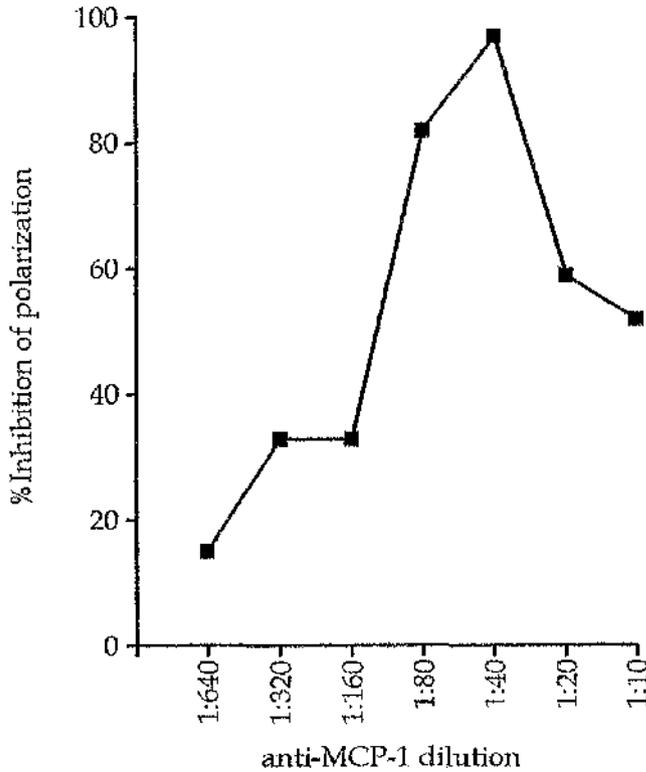
<i>Code</i>	<i>IL-8 (ng/ml)</i>	<i>IL-15 (ng/ml)</i>	<i>MIP-1 α (ng/ml)</i>	<i>MCP-1 (ng/ml)</i>
D.G	167	65	8.6	0.0
A.J	338	82	230	33.2
R.L	181	417	74	55
E.MO	219	65	48.7	11.9
H.Y	110	NA	253	11.6
D.M	287	78	0.1	107
M.S	48.7	17	0.9	NA

Table 4.8 B: Clinical descriptions of RA patients (used for anti-cytokine antibodies inhibitory study).

<i>Code</i>	<i>D.D</i>	<i>Age years+Sex</i>	<i>Overall severity+Erosion</i>	<i>Drugs</i>
D.G	7	35 (F)	Mod+none	Voltarol+Gold
A.J	23	53 (M)	Mild+Eros (++)	Indocid+SASP
R.L	16	51 (M)	V.seve+Eros (++)	Feldene+Cyclophosphamide
H.Y	6	88 (F)	V.Seve	Sulindac+acupan
E.M	10	59 (F)	Seve+Eros (++)	Ibuprofen+Gold
D.M	23	59 (F)	Seve+Eros (++)	Voltarol
M.S	40	74 (F)	Mild+Eros (++)	Indocid

These tables show the chemoattractants levels and the clinical descriptions of fluids from RA patients used in the inhibition of lymphocyte attractant activity of synovial fluid using anticytokine specific antibodies (see above). For study of inhibitory effect of combined **anti-IL8+anti-IL-15** 6 RA fluids were used (D.G, A.J, R.L H.Y, E.M and M.S). H.Y, A.J, and E.M were used for **anti-IL-8+anti-MIP-1 α** or combined **anti-IL-15 and anti-MIP-1 α** . D.M, A.J, and E.M were used for **anti-MCP-1** combined with other anti-cytokine antibodies. D.D=disease duration, Eros=erosion, V.seve=very severe

FIG 4.13: Inhibitory effect of anti-MCP-1 against MCP-1 (1ng/ml)

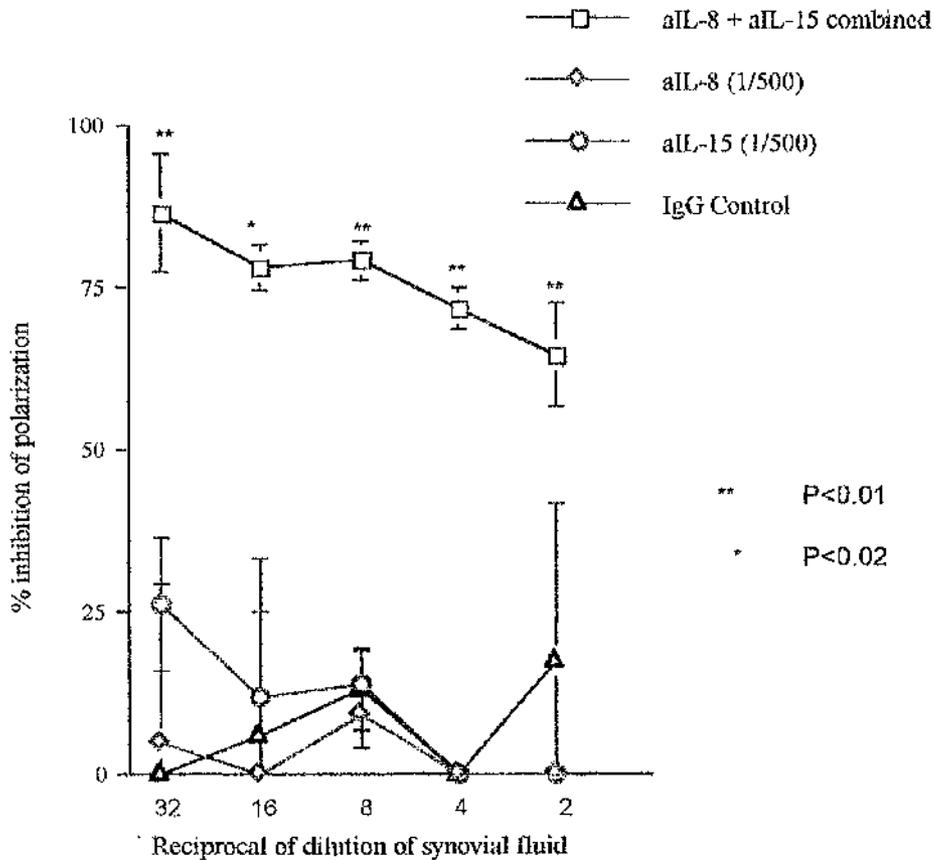


This figure represents the inhibition of the response of blood lymphocytes to MCP-1 (used at 1ng/ml, Wilkinson, P.C unpublished observations) in the presence of anti-MCP-1 at various dilutions. Note that at a dilution of 1/40, anti-MCP-1 inhibited >97% of MCP-1 polarization activity. This dilution (1/40) was determined as the effective dilution of anti-MCP-1. Actual values for proportion of polarized cells in MCP-1 (1ng/ml) and Hanks-HSA were 27% and 14% respectively.

4.6.3 Inhibitory effect of combinations of antibodies to cytokines

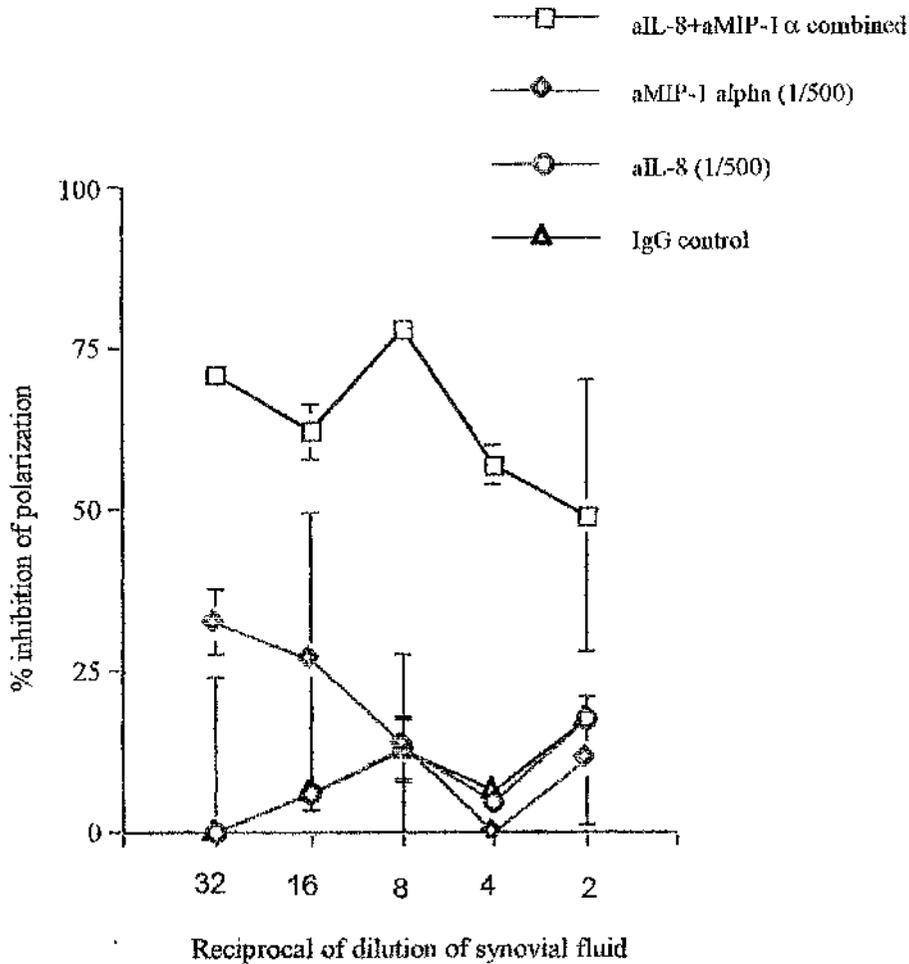
As shown in Fig 4.14 preincubation of fluids from 5 RA patients (Table 4.8) with a combination of anti-IL-8+anti-IL-15 substantially inhibited the attractant effect of synovial fluid ($p<0.05$ - $p<0.01$) for lymphocytes as judged by polarization. Anti-IL-8 or anti-IL-15 alone had no inhibitory effect on SF chemoattraction activity. These results indicated that the IL-8 and IL-15 present in the fluids (see Table 4.8A) may play a major role in lymphocytes chemoattraction into the synovial compartments of rheumatoid arthritis. The inhibitory effect of a combination of anti-IL-8 and anti-MIP-1 α was also studied. In these experiments preincubation of fluid from 3 RA patients with the combination of anti-IL-8 and a MIP-1 α also showed significant inhibitory activity of polarization of lymphocytes in response to synovial fluid (Fig 4.15). These results shows that IL-8 and MIP-1 alpha (Table 4.8) may contribute to the lymphocyte attractant effect of synovial fluid. In addition the inhibitory effect of combinations of anti-IL-15 and anti-MIP-1 α was studied. When combinations of these antibodies were used, consistent inhibitory effects were not seen (Fig 4.16). The inhibitory effect of anti-MCP-1 together with other cytokines was investigated.

Fig 4.14 : Inhibition of the response of polarized lymphocytes to SF in the presence of combination of anti-IL-8 and anti-IL-15 antibodies.



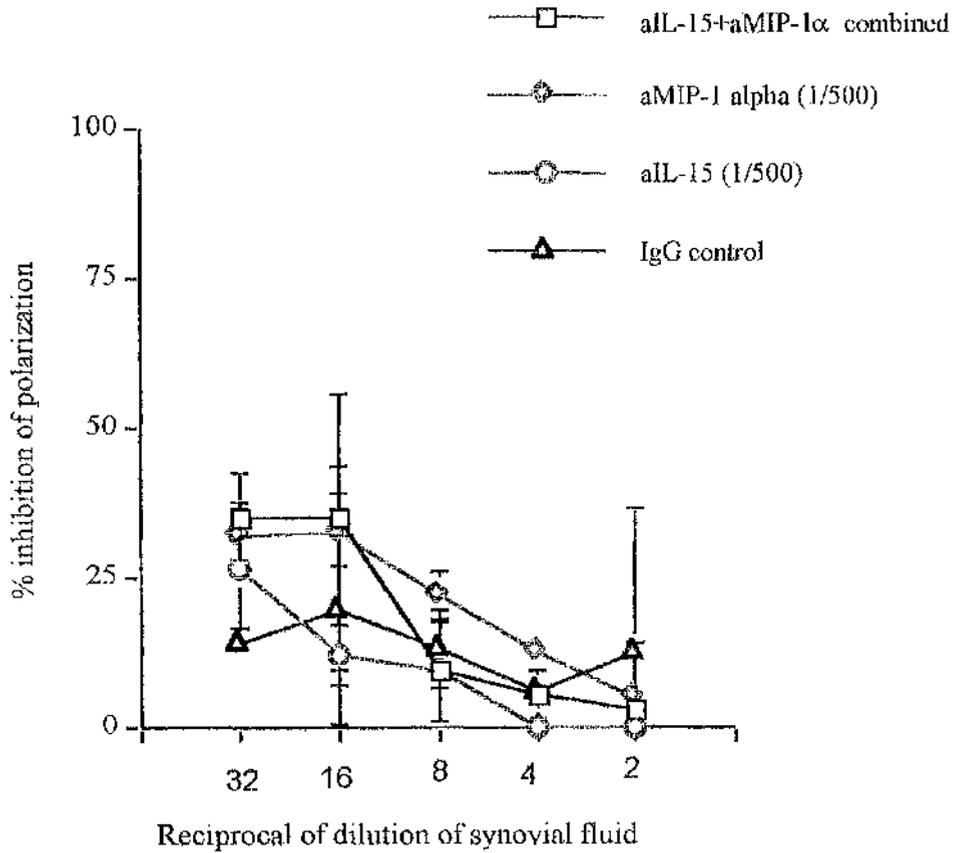
Inhibition of lymphocyte polarization to RA synovial fluids (n=5) in the presence of anti-IL-8, anti-IL-15 or a combination of the two antibodies. Each curve represents the Mean±SEM (n=3). P values were compared with the IgG control.

FIGURE 4.15 Inhibition of the response of polarized lymphocytes to SF in the presence of a combination of anti-IL-8 and anti-MIP-1 α .



Inhibition of lymphocyte polarization to RA synovial fluids (n=4) in the presence of anti-IL-8, anti-MIP-1 α or a combination of the two antibodies. Results represents the Mean \pm SEM (n=3). Inhibition by the combined antibodies aIL-8+aMIP-1 α was statistically significant when compared with IgG control (P<0.01 for fluid at dilution of 1:8 or higher; not significant for fluid at 1:2 or 1:4).

FIG 4.16: Inhibition of the response of polarized lymphocytes to SF in the presence of a combination of anti-IL-15 and anti-MIP-1 α .



Inhibition of lymphocyte polarization to RA synovial fluids (n=3) in the presence of anti-IL-15 and anti-MIP-1 α or a combination of the two antibodies. Results represents the Mean \pm SEM (n=3). Inhibition by the combined antibodies aIL-15 and aMIP-1 α was not statistically significant when compared with IgG control.

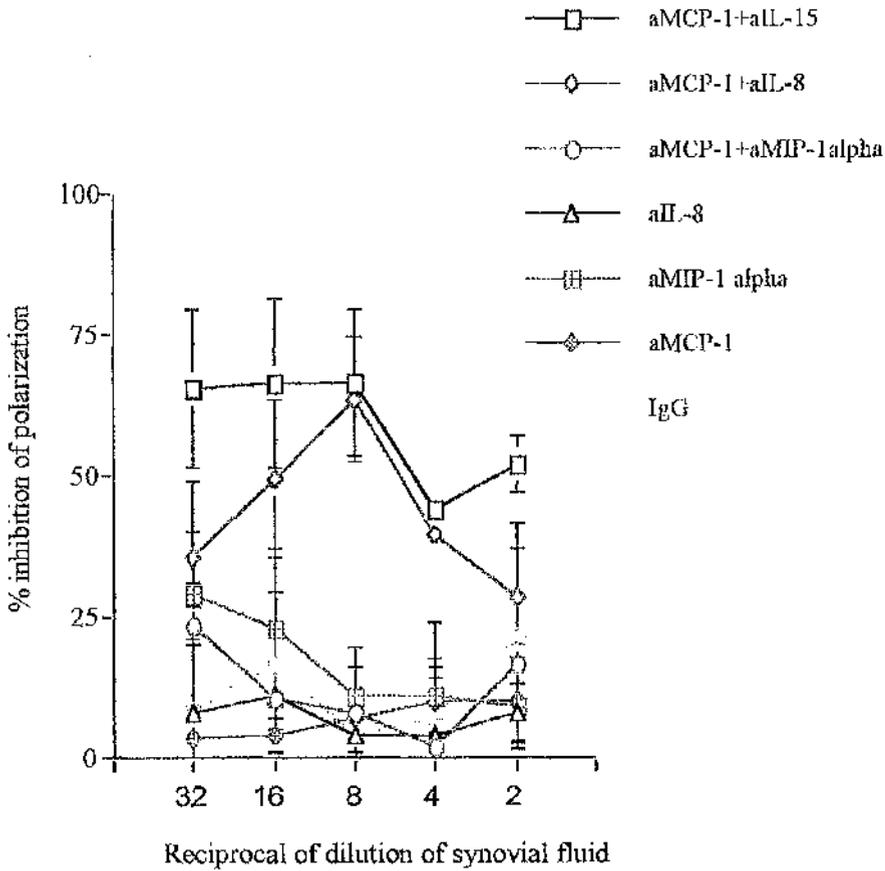
As with the combinations of antibodies described above, the combination of anti-MCP-1 with anti-IL-15, or anti-IL-8 also caused significant inhibition of lymphocyte polarization to synovial fluid compared with the IgG control (Fig 4.17). This result indicated that MCP-1 present in the fluid (Table 4.8 A) may play a role in lymphocyte locomotion in rheumatoid arthritis.

Inhibition with combinations of three antibodies (anti-IL-8, anti-IL-15 and anti-MIP-1 α) or with four antibodies mentioned above together was still stronger but was still below 100% (Fig 4.18). This indicates that other cytokines (or non cytokines attractants) may contribute to the chemoattractant activity of these fluids for lymphocytes.

4.6.4 Inhibition of lymphocytes invasion of collagen gels containing synovial fluid by anti-cytokine antibodies.

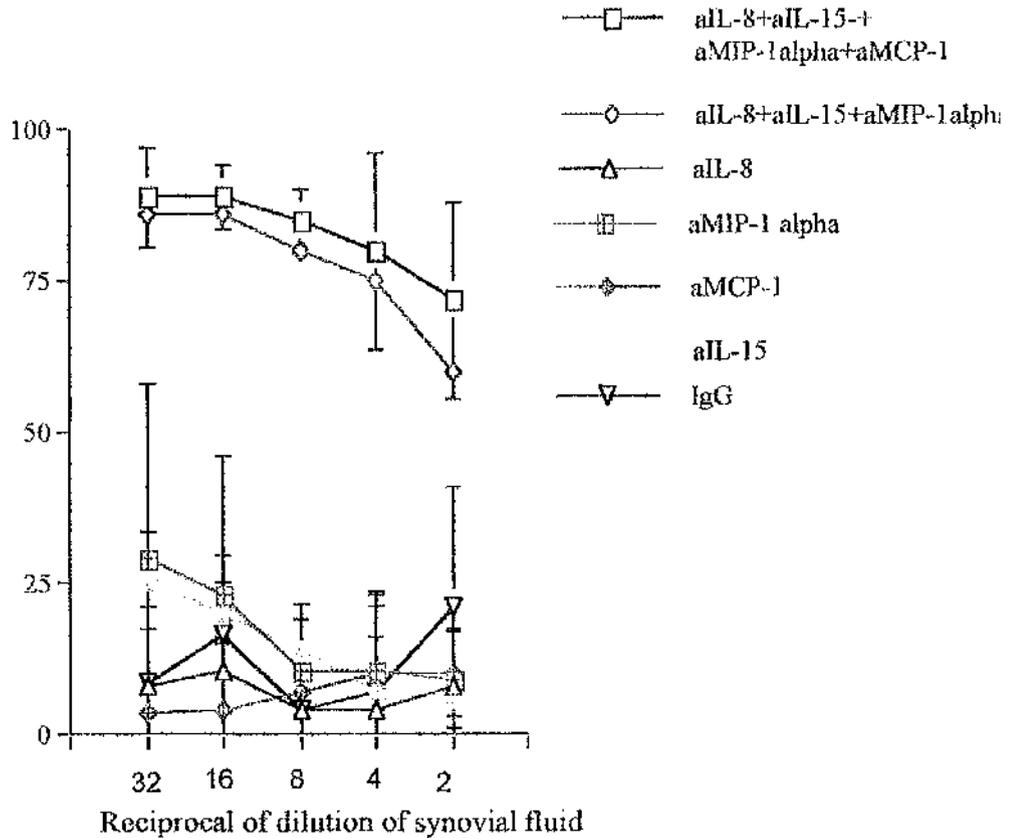
As shown in table 4.9 a combination of anti-IL-8 and anti-IL-15 significantly inhibits lymphocyte migration in collagen gels induced by RA fluids ($p < 0.001$). A combination of anti-IL-8 and anti-MIP-1 alpha antibodies also significantly prevented the locomotion of lymphocytes into collagen gel induced by the same fluids ($p < 0.05$, Table 4.10).

FIG 4.17 Inhibition of the response of polarized lymphocytes to SF in the presence of a combination of anti-MCP-1+IL-15, and anti-MCP-1 and anti-IL-8 or anti-MCP-1+anti-MIP-1 alpha.



Inhibition of lymphocyte polarization to RA synovial fluids (n=3) in the presence of anti-MCP-1+anti-IL-15, and anti-MCP-1+aIL-8 or anti-MCP-1+anti-MIP-1 α . Results represents the mean \pm SEM (n=3). Inhibition by the anti-MCP-1 together with aIL-15 or aIL-8 was statistically significant ($P < 0.05$ for fluid at dilution of 1:8 or higher) when compared with the IgG control.

FIG 4.18 Inhibition of the response of polarized lymphocytes to SF in the presence of four or three antibodies together.



Inhibition of lymphocyte polarization to RA synovial fluids ($n=3$) in the presence of combined four antibodies (aIL-8+aIL-15+anti-MCP-1+anti-MIP-1 α) or in the presence of three antibodies together (anti-MIP-1+ α IL-15+aIL-8). Results represents the mean \pm SEM ($n=3$). The inhibition by four or three antibodies was stronger and highly significant when compared with the IgG control ($p<0.05-0.001$ for fluid at dilution of 1:2 or higher) but still under 100%.

Table 4.9 Inhibition of lymphocyte invasion of collagen gels by anti-IL-8 and anti-IL-15 antibodies.

Treatment of synovial fluid	% cells invading collagen gel mean±SEM	% inhibition mean±SEM
No treatment	29.5±1.2	-
IgG (1/500)	26.6±1.0	9.7±1.0
anti-IL-8 (1/500)	28.0±0.7	5.0±2.4
anti-IL-15 (1/500)	27.1±2.3	8.4±6.3
anti-IL-8+anti-IL-15	11.4±2.2	60.6±8.8

Table 4.10 Inhibition of lymphocyte invasion of collagen gel by anti-IL-8 and anti-MIP-1 α antibodies.

Treatment of synovial fluid	% cells invading collagen gel mean \pm SEM	% inhibition mean \pm SEM
No treatment	34.1 \pm 1.8	-
IgG (1/500)	33 \pm 1.4	3.2 \pm 0.85
anti-IL-8 (1/500)	32.5 \pm 1.5	4.7 \pm 0.50
anti-MIP-1 α (1/500)	33 \pm 2.0	3.2 \pm 0.90
anti-IL-8+anti- MIP-1 α	21.50 \pm 2.7	36.5 \pm 1.8

4.7 CONCLUSIONS

Chemotactic cytokines are present in the synovial fluid in RA. The concentrations of IL-15, IL-8, MIP-1 α and MCP-1 are high enough to attract lymphocytes into the joint. The concentration of IL-2 is too low to do so. The chemoattractant activity of the joint fluid cannot be accounted for by any single cytokine, because no single anti-cytokine monoclonal antibody produces significant inhibition of the activity of the joint fluid. However combination of antibodies do produce significant inhibition activity for lymphocytes.

In comparison to RA, the level of chemotactic cytokines in OA fluid are low and these fluid are less potent in attracting lymphocytes.

4.8. DISCUSSION

4.8.1. Lymphocyte chemoattractants and lymphocyte accumulation

IL-8 is reported to be a lymphocyte chemotactic factor for lymphocytes (Wilkinson and Newman 1992), but the proportion of responding locomotor lymphocytes is low compared with the very high proportion of blood neutrophils observed to be responding to IL-8. In the present study, it was found that there was no correlation between IL-8 level and lymphocyte accumulation in the synovial fluid. This is expected since as will be discussed later, lymphocyte accumulation in synovial fluid probably results from the action of several chemoattractants.

The infiltration or the accumulation of lymphocytes or lymphocyte subsets within the synovial compartments has been well investigated. The locomotion or migration of these cells into the joints or into the joint fluid is believed to be due to the activity of chemotactic factors. Although this study demonstrated that there are several chemoattractants in the synovial fluid, it has been found some of these factors were present at concentrations too low to induce the locomotion activity of lymphocytes. For example the level of IL-2 was found to be less than 1ng/ml in all patients tested either from active or non active RA. This dose is not enough to induce lymphocyte locomotion activity compared with that found by Wilkinson and Newman 1994 (10-100ng/ml). This data is consistent with

other studies showing that the IL-2 production in rheumatoid arthritis was deficient (Comb *et al.*, 1985; Kitas *et al.*, 1988).

The explanation for the IL-2 defect production in RA still as yet not clear, but IL-2 may consumed in the RA inflamed joints or there might be other potential mechanisms including inhibition of production by suppressor cells, or intrinsic abnormalities of the IL-2 producing T lymphocyte population (Combe *et al.*, 1985). In addition other scientists suggested that the defect in IL-2 production may be due to factors produced by activated monocytes/ macrophages such as prostaglandin and hydrogen peroxide which may inhibit lymphocyte functions which in turn lead to the defect in IL-2 production (Kitas *et al.*, 1988).

In this study IL-15 was found (in fluids mostly from patients with RA in a dose level (>20ng/ml) that can induce locomotion activity of lymphocytes. These data are consistent with those observed by Wilkinson and Liew (1995) that > 20 ng/ml of pure IL-15 can attract peripheral blood lymphocytes *in vitro*. The presence of IL-15 in the synovial fluid, and synovial tissue (McInnes *et al.*, 1996) may provide an important alternative mechanism for chemoattraction and activation of T cells in the synovial compartments in the absence of IL-2. IL-15 has a similar action to IL-2 as an activator of T lymphocytes. Activation of T cell by IL-15 is blocked by treating cells with antibody to the β chain of the IL-2 receptor (Grabstein *et al.*, 1994) and the β chain of the IL-2 receptor is required

for activation of locomotion by both IL-2 (Wilkinson and Newman 1994) and IL-15 (Wilkinson and Liew 1995). The discovery of IL-15 may provide an explanation for the IL-2 knockout mice make nearly-normal immune response (Kundig *et al.*, 1993) since it can substitute for IL-2 and may do so in the rheumatoid joint.

This study also demonstrated that other lymphocyte chemoattractants including MIP-1 α and MCP-1 were present in the majority of RA patients and several patients with other inflammatory arthritides in a dose high enough to induce lymphocyte locomotion. These data are consistent with other studies showing that MIP-1 α and MCP-1 are produced at the site of the inflammatory joints (see chapter 1). MIP-1 α and MCP-1 are inflammatory chemokines which contribute to lymphocyte accumulation in the joint as suggested by inhibition by their specific antibodies

The finding that adding antibody singly (including anti-IL-8, anti-IL-15, anti-MIP-1 α and anti-MCP-1) to the synovial fluids has no effect on the locomotion activity of these fluids, but combined antibodies can eliminate between 55-89% of the synovial fluid chemoattraction activity (both polarization and collagen gel migration), indicates that the locomotion activity of lymphocytes in rheumatoid synovial compartments cannot be attributed to any single cytokine. Moreover the accumulation of lymphocyte within the synovial fluid was not correlated with the

level of any chemotactic factor in the fluids. It is probable that there are as yet unidentified lymphocyte chemotactic factors to be investigated.

It can be concluded that rheumatoid synovial fluids contain several of lymphocyte chemoattractants at sufficient concentration to induce lymphocyte locomotion. In contrast synovial fluids from OA contain small amounts of such factors and the accumulation of both neutrophils and lymphocytes was low.

4.8.2. Chemoattractants and lymphocytes (RA peripheral blood and synovial tissues)

It has been suggested that lymphocytes from RA patients (peripheral blood) have a functional defect in response to several stimulators including P.H.A and Con A (Silverman *et al.*, 1976). This study shows that the locomotion activity of RA lymphocytes (peripheral blood) is not impaired as judged by their normal locomotion response to IL-15 and synovial fluids compared with cells from normal blood. In order to draw a definitive conclusion regarding the normal locomotion of these cells a large study is required and , other chemotactic factors need to be tested.

Investigation of the other hand the locomotion activity of synovial tissue lymphocytes in response to the above mentioned chemoattractants was an important one of the important aim of the present study, but unfortunately there

was only limited material for such investigations. Lymphocytes separated from the few synovial tissues available from patients with active RA showed locomotor responses to IL-8. This suggested that these cells have been already activated in vivo, and they can respond to chemoattractants without needing further activation.

4.8.3 IL-8 level and accumulation of neutrophils.

As stated earlier the accumulation of inflammatory cells in the rheumatoid joint or rheumatoid synovial fluid is a histological feature of acute and chronic inflammation. For example in case of neutrophils it has been found (present study) that in most patients with active rheumatoid arthritis, neutrophil accumulation was very high in the synovial fluids. This data confirms the previous observations reported by other laboratories (Endo *et al.*, 1991; Brennan *et al.*, 1990). The neutrophil accumulation in the fluids was suggested to result from attraction by chemotactic factors such as IL-8, C5a, platelet activating factor or leukotriene B4 (Sietz *et al.*, 1992). In the present study, IL-8 was found to be abundant in the synovial fluid of most patients with active RA and in several patients with other inflammatory diseases (see Table.4.6). The concentration of IL-8 was not correlated with neutrophil accumulation. These data suggested that IL-8 acts as contributory factor for such accumulation, but

several molecules structurally related to IL-8 (gro gene products) were shown to have neutrophil chemotactic activity equivalent to IL-8 (Brennan *et al.*, 1990). These factors are reported to be produced by fibroblasts and endothelial cells following stimulation with IL-1 or TNF- α and such factors may be expressed during the inflammatory immune response in RA and may contribute to the chemotactic activity of the synovial fluid (Brennan *et al.*, 1990).

Other investigators (Seitz *et al.*, 1992; Rampart, *et al.*, 1992) reported that the high-potency of rheumatoid factor-containing immune complexes triggers IL-8 generation by peripheral blood and synovial fluid mononuclear cells. The presence of such immune complexes in the rheumatoid joints generates IL-8 locally (Rampart *et al.*, 1992). However the correlation between RF and IL-8 was not significant (present study) and the production of IL-8 in the synovial environment may predominately be due to other stimuli including, IL-1 and TNF- α which have been observed to be stronger inducers of IL-8 *in vitro* and reported to be in abundance in the synovial fluids (Seitz *et al.*, 1992).

CHAPTER 5:
EFFECTS OF ANTI-RHEUMATIC DRUGS ON LOCOMOTOR
ACTIVITY OF LYMPHOCYTES IN RESPONSE TO SYNOVIAL
FLUID

5.1 Background

The rapid development in understanding of mechanisms of leukocyte locomotion has led to interest in the possibility of inhibiting white cell migration in vivo. Although not necessarily as part of their mode of therapeutic action, various anti-inflammatory drugs are widely used to inhibit the circulation, migration, activation and locomotion of leukocytes. The corticosteroids cause circulating lymphopenia in the blood (Fauci and Dale 1974; Yu, *et al.*, 1974; Clark *et al.*, 1977; Cooper, *et al.*, 1977), which appears to be due to the retention and selective redistribution of recirculating T lymphocytes (Yu, *et al.*, 1977) and increased migration into the bone marrow (Cox and Ford 1982). Corticosteroids also downregulate cytokine production (see chapter 1) by mononuclear cells with a consequent inhibition of lymphocyte migration (Pitzalis 1995) and proliferation. In addition it has been observed that corticosteroids inhibit lymphocyte-endothelial cell (EC) binding through the inhibition of LFA-1 and

CD2 expression following lymphocyte activation (Pitzalis *et al.*, 1995; Pitzalis and Panayi 1993).

Cyclosporin A appears to have some therapeutic efficacy in RA (Tugwell *et al.*, 1995). Cyclosporin A was reported to interfere with the early stage of T cell activation (see chapter 1) and consequently reduces lymphocyte migration and locomotion. It inhibits cytokine production including production of IL-2 and IL-6, cytokines which play a major role in acute inflammatory immune responses (Crilly *et al.*, 1995).

Taken together this suggests that drugs that interfere with lymphocyte inflammatory activity may be effective clinically (Carlos and Harlan 1990). In this chapter study of the effect of drugs on inhibition of lymphocyte locomotion in vitro is described. Since during the G1 phase of cell cycle, lymphocytes acquire locomotor capacity, activated lymphocytes with locomotor capacity may therefore be recruited from the blood into the inflammatory lesion, e.g into the synovial tissue of rheumatoid joints. Such locomotor activation can be inhibited in vitro by culture of lymphocytes in the presence of cyclosporin A or FK506 (Wilkinson and Higgins 1987 b, Wilkinson and Watson 1990), both of which are drugs which inhibit entry of lymphocytes into G1. In this section, experiments

are described in which the inhibitory effect of antirheumatic drugs such as NSAIDs, DMARDS and corticosteroids on the locomotor activity of lymphocytes in response to SI^1 was investigated.

5.2 RESULTS

5.2.1A. Preliminary experiments

5.2.1A1. Effect of preincubation with anti-rheumatic drugs on lymphocyte viability.

To determine the effect of preincubation with different anti-rheumatic drugs on lymphocytes viability, 1×10^6 cells/ml were cultured (in HBSS-HSA for 24 h at 37 °C) with graded doses (1000µg/ml-1µg/ml) of different drugs used in the present study. After the incubation period, cell viability (as defined by trypan dye exclusion and confirmed by examining cell morphology under phase-contrast microscopy) for the dose of 100µg/ml was above 79% (range 79-83%) for CSA, Rapamycin; Primaquine, cyclophosphamide and corticosteroids and above 82% (82-87%) for (DMARDs including Gold, D-penicillamine) and above 85% for NSAIDs (range 85-89%) including aspirin, Ibuprofen and indomethacin. On the other hand there was no loss of cell viability at a dose of 10 µg/ml or less of all drugs tested in the present study. Table 5.1 shows the results using a dose of 10µg/ml of various drugs as an example. The cell viability was above 87% (range 87-96%). However there was cell damage (as determined by phase-contrast

microscopy) and a significant loss of cell viability for the graded doses of 500µg/ml or higher. In the course of this study graded doses of 100µg/ml or less were chosen as in vitro physiological doses and used to investigate the inhibitory effect of these drugs on lymphocyte polarization and locomotion.

5.2.1A2. Effect of preincubation with anti-rheumatic drugs on the lymphocyte response to chemoattractants.

In these experiments lymphocytes were cultured in 25% FCS for 24 hr at 37 °C in the presence of 10 µg/ml of Aspirin, Gold sodium thiomalate , Cyclosporin A, rapamycin, dexamethasone, prednisone and prednisolone. After the incubation period cells were separated and washed 3 times in HBSS-HSA and tested for their ability to respond to synovial fluid. Table 5.2 shows that the doses mentioned above are not toxic since the lymphocytes can respond strongly to synovial fluid (after washing out the drugs) compared with the control.

Table 5.1 Effect of drug preincubation on lymphocyte viability

Treatment	cell viability (%)
Aspirin	96%
Ibuprofen	93%
Indomethacin	89%
D-penicillamine	87%
Gold (sodium thiomalate)	89%
Primaquine (8-aminoquinoline)	87%
Cyclophosphamide	89%
Rapamycin	87%
Cyclosporin A	87%
Dexamethasone	89%
Prednisone	92%
Prednisolone	91%

* Lymphocytes were in cultured HBSS-IISA in the presence of drugs at a dose of 10 µg/ml . The percentage of viable cells was assessed by the trypan blue exclusion test. Percent of lymphocytes viability cultured without drugs which werer viable was 96%.

Table 5.2 Effect of preincubation with drugs on the lymphocyte polarization response.

<i>Treatment (Cells preincubated in drugs+FCS 25%)</i>	<i>% Polarized cells SF1 (1:4)</i>	<i>%polarized cells SF2 (1:8)</i>
Aspirin	46	31
Gold sodium thiomalate	39	31
Cyclosporin A	43	30
Rapamycin	38	28
Cyclophosphamide	42	31
Dexamethasone	45	33
Prednisone	39	32
Prednisolone	38	28
** Lymphocytes preincubated in 25% FCS alone	43	34

* Cells were cultured in 25% FCS in the presence of drugs (10 µg/ml) and subjected to washing 3-4 times in HBSS-HSA and then exposed to different synovial fluids (at dilutions of 1:4 and 1:8) in a short term polarization assay.

** lymphocytes were cultured in 25% FCS without drugs washed three times in medium, and exposed to the synovial fluid. The Percent of lymphocyte polarized in HBSS-HSA (as a negative control for all experiments) was 11%.

5.3. Inhibitory effect of drugs on the lymphocyte response to synovial fluid.

The inhibitory effect of several anti-rheumatic drugs on the ability of blood lymphocytes to respond to synovial fluids (Table 5.3) in polarization and locomotion assay were studied as follows.

5.3.1. Inhibition of SF-induced lymphocyte polarization in the presence of NSAIDs and DMARDs (short term effect).

Lymphocytes were cultured for 24hrs in FCS washed and preincubated for 1h with the drugs (100ng to 100µg/ml), and then incubated with an optimal dilution (1:8) of RA-SF in a dose response polarization assay. As shown in fig 5.1 there was little inhibitory effect on lymphocyte polarization in the presence of indomethacin, aspirin or Ibuprofen. Preincubation of the same cells with the DMARDs (gold, D-penicillamine and gold+D-penicillamine combined fig 5.2) or 8-aminoquinoline and cyclophosphamide (Fig 5.3) also had an insignificant effect.

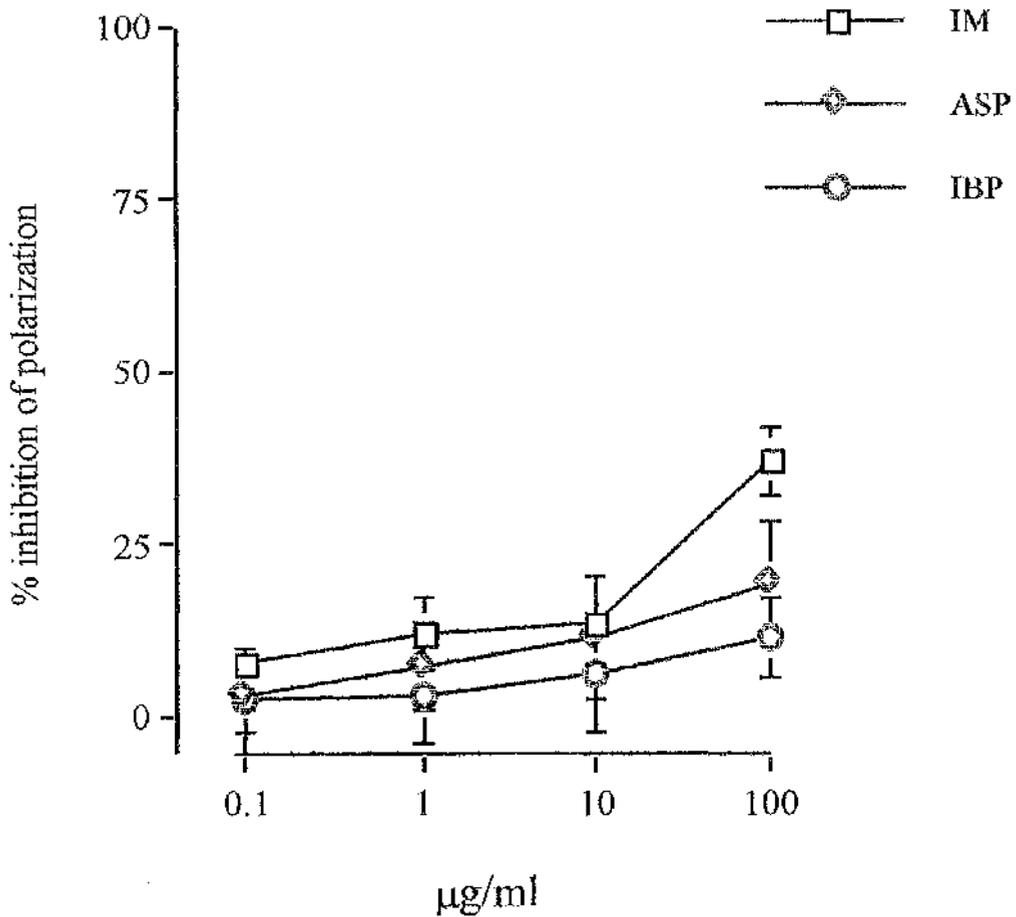
Table 5.3. Clinical descriptions of RA patients used for anti-rheumatic drugs inhibitory study.

<i>Code</i>	<i>D.D</i>	<i>Age years+Sex</i>	<i>Overall severity+Erosion</i>	<i>best dilution</i>
R.M	4	82 (F)	seve+Eros (+++)	1:16
M.EZ	4	70 (F)	Seve+Eros (++)	1:8
C.J	16	51 (M)	V.seve+Eros (++)	1:8
D.M	23	59 (F)	Seve+Eros (++)	1:2
M.S	40	74 (F)	Mild+Eros (++)	1:4
M.G	8	57 (F)	Mod+Eros (++)	1:8
H.Y	6	88 (F)	V.Seve	1:4

This table shows clinical descriptions of RA patients whose synovial fluids were studied for inhibition of lymphocyte polarization using anti-rheumatic drugs (see above).

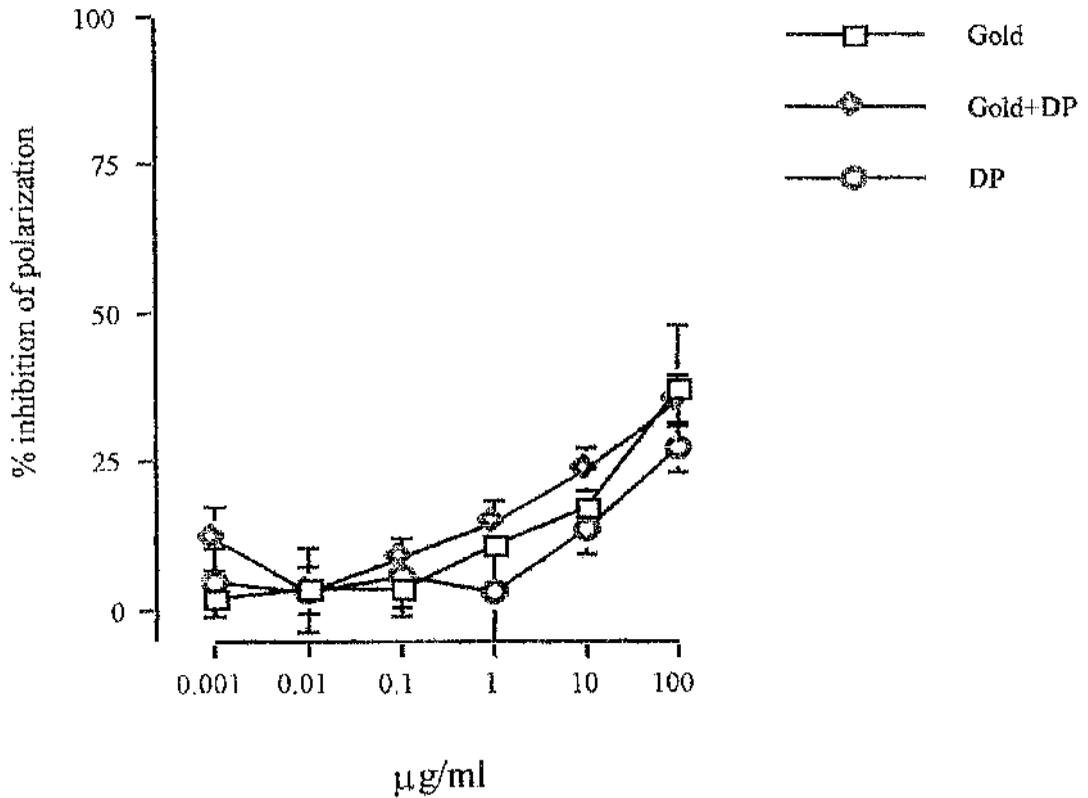
For study of inhibitory effect of NSAIDS 3 RA fluid were used (R.M, M.EZ and M.S). For DMARDS and immunosuppressive drugs all other RA fluids mentioned above were used in different experiments. D.D=disease duration in years, Eros=erosion, V.seve=vcry severe.

Fig 5.1. Inhibition of the response of lymphocytes to SF in the presence of analgesics and NSAIDs.



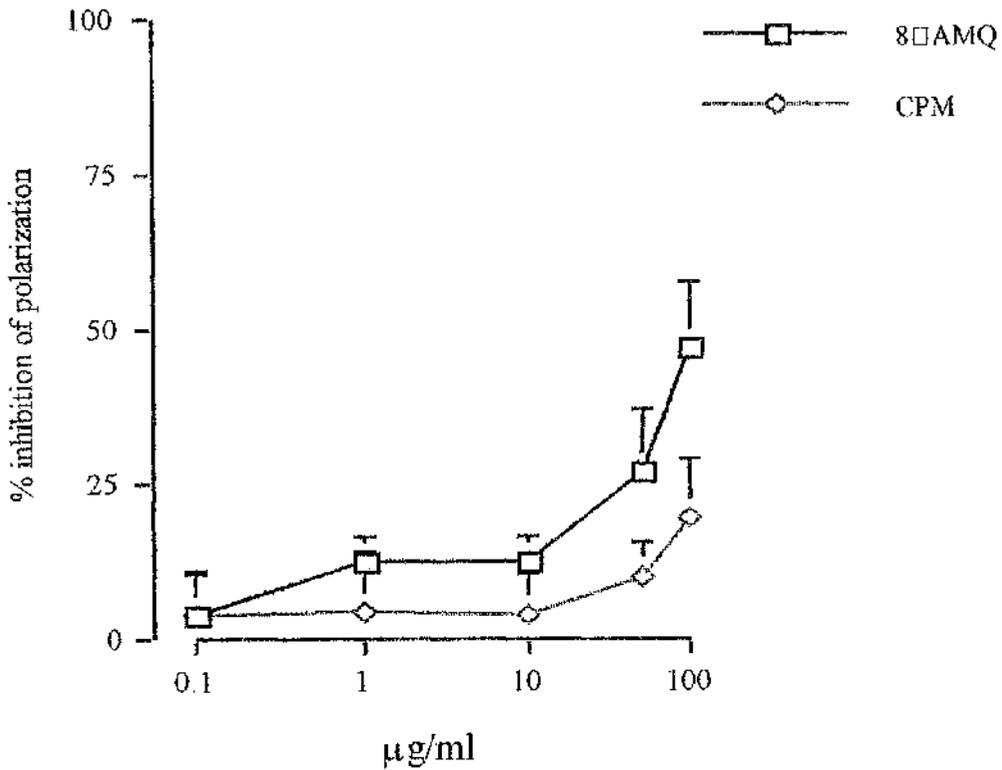
Dose response curves for indomethacin (IM), Aspirin (ASP) and Ibuprofen (IBP) as inhibitors of polarization of lymphocytes in response to SF. Each curve represents the mean \pm SEM (n=2). The y axis shows percentage inhibition : actual values for proportion of polarized cells in SF and Hanks-HSA without drugs were 38% and 18.6%. Results were not statistically significant.

FIG 5.2. Inhibition of the response of lymphocytes to SF in the presence of disease modifying antirheumatic drugs.



Dose response curves for Gold alone, Gold+D-penicillamine (DP) combined and D-penicillamine alone as inhibitors of polarization of lymphocytes in response to SF. Each curve represents the mean \pm SEM (n=3). The y axis shows percentage inhibition : actual values for proportion of polarized cells in SF and Hanks-HSA without drugs were 35% and 15.2% respectively. Results were not statistically significant.

FIG 5.3. Inhibition of the response of lymphocytes to SF in the presence of 8-aminoquinoline and cyclophosphamide.



Dose response curves for 8-aminoquinoline (8-AMQ) and cyclophosphamide (CPM) as inhibitors of polarization of lymphocytes in response to SF. Each curve represents the mean \pm SEM (n=2). The y axis shows percentage inhibition : actual values for proportion of polarized cells in SF and Hanks-HSA without drugs were 42.3% and 11.6% respectively. Results were insignificant.

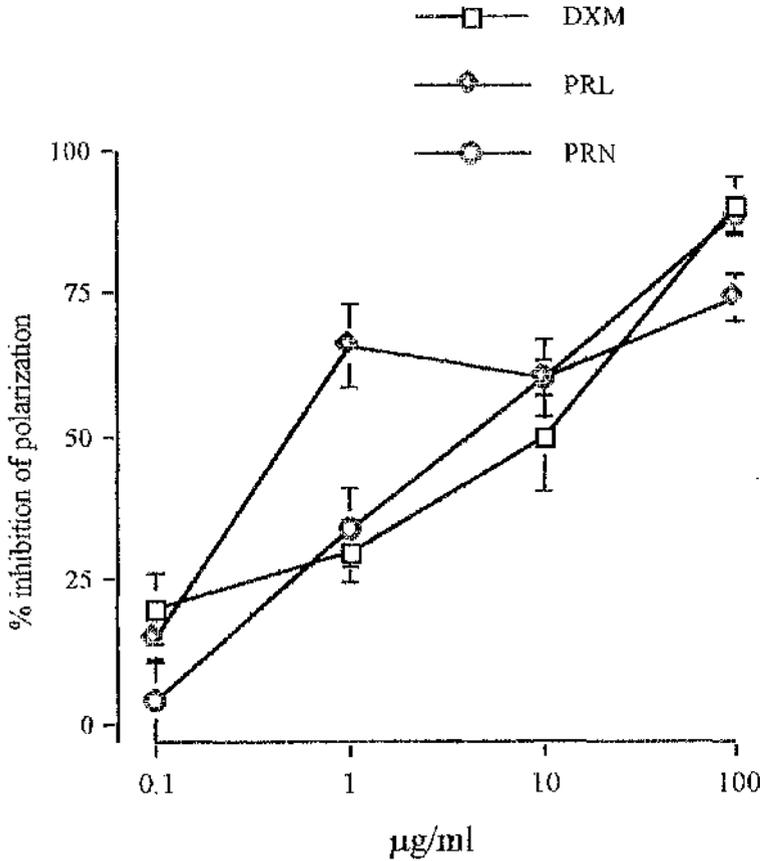
5.3.2. Inhibition of SF-induced lymphocyte locomotion in the presence of corticosteroids (short term effect).

There was a significant inhibition of polarization in the presence of corticosteroids (Fig 5.4) such as dexamethasone (10 $\mu\text{g/ml}$ $P < 0.025$), prednisolone (1 $\mu\text{g/ml}$ $P < 0.01$) and prednisone (1 $\mu\text{g/ml}$ $P < 0.05$). As shown in table 5.4 dexamethasone, prednisolone and prednisone significantly inhibit lymphocyte migration in collagen gels induced by RA fluids ($p < 0.01$ for 10 $\mu\text{g/ml}$ DXM, 1 $\mu\text{g/ml}$ prednisolone and prednisone Table 5.4).

5.3.3. Inhibition of lymphocyte locomotion in the presence of cyclosporin A and rapamycin.

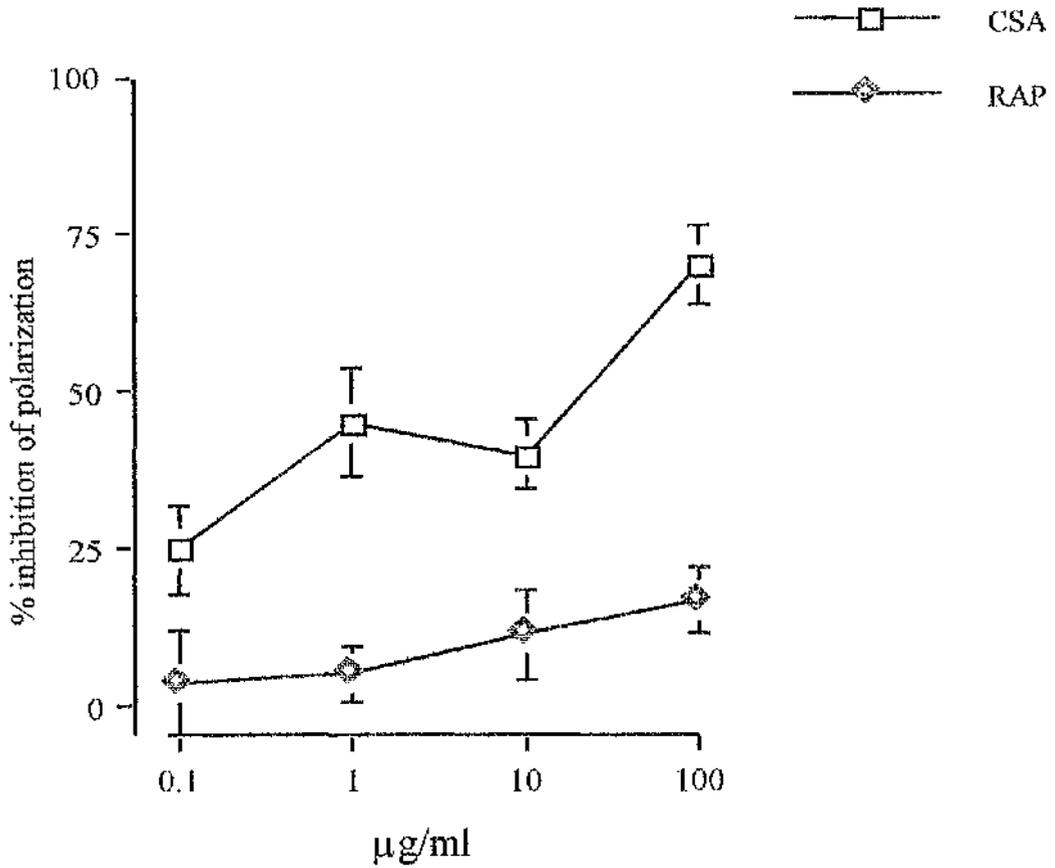
Cyclosporin A and rapamycin were defined as immunosuppressive natural products with a common ability to inhibit T lymphocyte activation by interfering with intracellular signalling mechanisms (Sigal and Dumont 1992). There was a significant inhibition of lymphocyte polarization in the presence of cyclosporin A (1 $\mu\text{g/ml}$ $p < 0.01$) but no inhibitory effect was seen in the presence of rapamycin (Fig 5.5).

FIG 5.4. Inhibition of the response of lymphocytes to SF in the presence of corticosteroids.



Dose response curves for dexamethsone (DXM), prednisolone (PRL) and prednisone (PRN) induced inhibition of polarization of lymphocytes in response to SF. Each curve represents the mean \pm SEM (n=3). The y axis shows percentage inhibition : actual values for proportion of polarized cells in SF and Hanks-HSA without drugs were 49.3% and 9.6% respectively. Results were statistically significant . DXM (10 μ g/ml p=0.025), PRN (1 μ g/ml p<0.05) and PRL (1 μ g/ml p<0.01).

FIG 5.5. Inhibition of the response of lymphocytes to SF in the presence of CSA and rapamycin.



Dose response curves for cyclosporin A (CSA) and Rapamycin (RAP) induced inhibition of polarization of lymphocytes in response to SF. Each curve represents the mean \pm SEM ($n=3$). The y axis shows percentage inhibition : actual values for proportion of polarized cells in SF and Hanks-HSA without drugs were 38.5% and 9% respectively. Results were statistically significant for CSA (1µg/ml, $p=0.012$).

Cyclosporin A also significantly inhibited locomotion of lymphocytes in collagen gels induced by the same fluid ($p < 0.05$, $1\mu\text{g/ml}$ Table 5.4).

5.4. Long term inhibitory effect of SF-induced lymphocytes polarization by anti-rheumatic drugs.

Table 5.5 a shows that drugs including, dexamethasone, prednisone prednisolone and CSA at graded doses between $10\mu\text{g/ml}$ - $0.1\mu\text{g/ml}$ have a significant inhibitory effect on the lymphocyte polarization activity during overnight culture with the synovial fluid at a dilution of 1:8. On the other hand rapamycin has little inhibitory effect. However Table (5.5 b) and Table (5.5 c) show that the DMARDs and NSAIDs have insignificant effects on the lymphocyte polarization activity in response to SF during overnight culture compared with the control.

Table 5.4. Effects of corticosteroids and cyclosporin A on invasion by of lymphocytes of collagen gels containing synovial fluid.

Cells treated in	% cells invading collagen gel \pm SEM	inhibition of invasion \pm SEM
Hanks-HSA	6.0 \pm 2.3	-
*SF alone	33.5 \pm 5.0	-
SF+ DXM (100 μ g/ml)	9.5 \pm 2.7	71.6 \pm 2.6
SF+ DXM (10 μ g/ml)	19.5 \pm 3.9	41.8 \pm 1.9
SF+ PRL (1 μ g/ml)	21.2 \pm 1.5	36.7 \pm 3.00
SF+ PRN (1 μ g/ml)	20.5 \pm 2.7	38.8 \pm 1.2
SF+ CSA (1 μ g/ml)	23.7 \pm 3.8	29.3 \pm 5.9

DXM=dexamethasone

PRL=prednisolone

CSA=cyclosporin A

* 3 RA synovial fluids (SF) were tested at 1:8 dilution.

TABLE 5.5 a . Effect of Corticosteroids, CSA and rapamycin on the polarization activity of lymphocytes during overnight culture.

	% mean polarized cells for two experiments±SEM				
	DXM	PRN	PRL	CSA	RAP
SF (1:8) + 10µg/ml*	11±1.0	13.3±1.3	14.8±0.75	18.8±0.75	29±1.0
SF (1:8) + 1µg/ml**	18±1.0	23.8±0.75	23.8±0.75	20±1.0	29.7±2.6
SF (1:8) + 0.1 µg/ml	30±1.0	29.5±1.5	32.5±1.3	34.8±1.8	31.6±1.4
SF alone (no drugs)		35±0.5			
Hank's-HSA alone (no drugs)		5.6±1.2			

DXM=dexamethasone, PRN=prednisone, PRL=prednisolone, CSA=cyclosporin A and RAP=rapamycin. Results were statistically significant for DXM , PRL, PRN and CSA at a dose of 10 µg/ml (*p <0.01) and 1µg/ml (**p <0.05).

Table 5.5 b. Effect of DMARDs on the polarization activity of lymphocytes during overnight culture.

	%polarized cells in a single experiment		
	GLD	D-PLM	8-AMQL
SF (1:8) + 10µg/ml	26	35	27
SF (1:8) + 1µg/ml	35	33	29
SF (1:8) + 0.1 µg/ml	30	35	31
SF alone (1:8)	31%		
Hanks-IISA alone	6%		

GLD=Gold (sodium thiomalate), D-PLM=D-penicillamine and 8-AMQL=8-aminoquinoline.

Table 5.5 c. Effect of NSAIDS on the polarization activity of lymphocytes during overnight culture.

	%polarized cells in a single experiment		
	ASP	IBP	INDM
SF (1:8) + 10 μ g/ml	30	32	27
SF (1:8) + 1 μ g/ml	35	36	31
SF (1:8) + 0.1 μ g/ml	39	39	35
*SF alone	38%		
Hanks-HSA alone	6%		

ASP=Aspirin , IBP=Ibuprofen and INDM=indomethacin

5.6 Conclusions

NSAIDs, (including Aspirin, Ibuprofen and Indomethacin) DMARDs (Gold sodium thiomalate and D penicillamine and anti-malarial drugs or primaquine (8-aminoquinoline) and cyclophosphamide had no inhibitory effect on lymphocyte locomotion. On the other hand CsA and Glucocorticosteroids had a significant inhibitory activity on the lymphocyte locomotion in response to synovial fluids.

Therefore lymphocyte locomotion in vitro cannot be controlled by NSAIDs and DMARDs, but it can be controlled by CsA and Glucocorticosteroids.

As synovial fluid-activated locomotion in vitro provides a model for chemoattractant-activated locomotion and since the locomotion is inhibited by glucocorticosteroids and CsA, and the significant inhibitory doses used in vitro were comparable to the whole blood concentration (0.5-1 μ g/ml) (Van *et al.*, 1989) it may be reasonable to suggest that part of the action of these drugs is to inhibit the recruitment or locomotion activity of lymphocytes into inflammatory lesions including those in rheumatoid joints.

5.6. DISCUSSION

5.5.1 Anti-rheumatic drugs and lymphocyte locomotion

The data presented here investigated the locomotor inhibitory effect of NSAIDs, DMARDs, cytotoxic and corticosteroids drugs. NSAIDs including Aspirin, Ibuprofen and indomethacin had no effect (either at high or low doses) on lymphocyte locomotion. This suggests that such drugs have no effect on induction of the G1 phase of the cell growth necessary for non motile lymphocytes to acquire locomotor capacity and also had no effect on the locomotor mechanism itself in already motile cells. NSAIDs including aspirin may suppress P.H.A-induced lymphocyte transformation (Opelz and Terasaki 1973). However this activity is related to late stages of the growth cycle (DNA synthesis) after locomotor activation has occurred (Opelz and Terasaki 1973). Other studies reported that Aspirin influenced the migration of neutrophils and monocytes by directly affecting the surface of granulocytes and decreasing their ability to enter the extravascular space. It has been also demonstrated that NSAIDS including indomethacin, increase the number of lymphocytes in the synovium (Bahreman & Ralph 1991). This might not be due to a direct effect of Indomethacin on the metabolic function of these cells but to reduction of prostaglandin synthesis induced by these drugs (prostaglandin as stated earlier is

one of the important inhibitors of lymphocyte locomotion and also lymphocyte proliferation). Other drugs such as Ibuprofen have been reported to inhibit the migration and aggregation of inflammatory cells, mainly neutrophils, as well as the release of lysosomal enzymes (Konstan *et al.*, 1995).

DMARDs (including Gold sodium thiomalate (GST), D-penicillamine, combined gold+ D penicillamine, 8-aminoquinoline) and other cytotoxic drugs (including cyclophosphamide) had little inhibitory effect on lymphocytes locomotion, suggested that these drugs cannot suppress the progression of cell growth from G0 into G1 or arrest the metabolic activity of G1 itself (ie. these drugs do not affect the G1 phase of the cell growth and exert their action on the late stage of DNA synthesis). These data are also consistent with the suggestion that DMARDs including Gold compounds suppress rheumatoid synovitis by reducing the number of high endothelial small blood vessels available for emigration of lymphocytes (Bahreman and Ralph 1991).

The finding that CSA (present work) had an inhibitory effect on lymphocyte locomotion in response to chemoattractant synovial fluid at a dose range between 1-10 μ g/ml confirmed previous work by Wilkinson and Higgins (1987) who reported that CSA inhibits lymphocyte locomotion in response to anti-CD3 antibody. Anti-CD3 activation raises the intracellular free Ca²⁺ (intracellular

calcium mediates lymphocyte activation) and stimulates Ca^{2+} flux-dependent Na^+/H^+ exchange. Unpublished studies by Matthews and colleagues (Yamanouchi Research Institute Oxford) suggest that Na^+/H^+ exchange may be an important event in lymphocyte polarization.

CSA has been reported to block the activation of lymphocytes early in the G1 phase of growth (Sigal and Dumant 1992). Its major activity appears to be an inhibition of IL-2 gene expression. This can be explained as follows: The induction of IL-2 gene transcription is mediated by a regulatory region which contains a number of nuclear proteins common to other promoters including NF-AT (nuclear factor of activated T cells) OCT-1 and AP-1 (Paliogianni *et al.*, 1993). NF-AT is reported to be absent in resting or unstimulated T lymphocytes and appears after 10-20 min of T cell activation and continues to increase until it reaches a peak of less than 36h (Granelli-Piperno & Nolan 1991).

It has been suggested that when CsA binds to intracellular proteins called cyclophilins, it become activated and then blocks the T cell activation mediated by NF-AT, (the binding site for this protein is to sensitive to CSA) either through interference with its synthesis or transport into the nucleus or due to inhibition of its functional activity (Paliogianni *et al.*, 1993; Borel 1994; Fan and Rainsford 1994). Regarding the OCT-1 it has been observed that this molecule cannot be inhibited by CsA or glucocorticosteroids (Paliogianni *et al.*, 1993). On the other

the other hand as will be discussed below it has been observed that AP-1 activity cannot be inhibited by CsA but by glucocorticosteroids. In addition the CsA-cyclophilin complex is reported to inhibit the calcium protein phosphatase, calcineurin and protein kinase-mediated transactivation of the IL-2 promoter (Fan and Rainsford 1994 Paliogianni *et al.*, 1993).

In contrast rapamycin had no inhibitory effect on lymphocyte locomotion. This not surprising as this drug is considered as an anti-proliferative agent (i.e it exerts its activity during DNA synthesis at a late stage of cell division) and not an anti-locomotive agent (i.e inhibiting RNA and protein synthesis) (Sigal and Dumant 1992).

The finding that Glucocorticosteroids (including dexamethasone, prednisone and prednisolone) significantly inhibit lymphocyte locomotion at low dose (figure 5.4 and Table 5.4 and Table 5.5) indicates that these drugs block the progression of cells either from G0 into G1 or block the progression of cell during G1 its self during cell growth. However this significant inhibition may be consistent with principle of the mechanism of action of these drugs which is reported to inhibit IL-2 gene expression (mentioned earlier to be the critical early event during T lymphocyte activation). This observation is confirmed by the addition of IL-2 to cultures of T cells stimulated in the presence of

glucocorticosteroids which overcomes the inhibition of T cell activation (Jeffrey *et al.*, 1992). The inhibitory effect of these drugs is reported to be through glucocorticosteroid receptors (GRs) which interact with specific DNA response elements in the T cells (Jeffery *et al.*, 1992). These receptors exist in the nucleus as high molecular weight molecules and there are about 3000-100000 receptors per cell and they have high affinity for the glucocorticosteroids (Flower and Dale 1994; Evans 1988). It has been reported that glucocorticosteroids (through their GR) including dexamethasone inhibit or interfere with activity of the transcription factor AP-1 by direct protein-protein interaction (Jeffrey *et al.*, 1992; Paliogianni *et al.*, 1993). Although it can be suggested that CsA and glucocorticosteroids act synergistically (i.e. CsA is a potent inhibitor for NF-AT and glucocorticosteroids are a potent inhibitors for AP-1) the inhibitory effect of CsA does not require the presence of other sites of the IL-2 promoter. A synergy is supported by other observations (Winkelstein 1991) that antigen stimulation *in vivo* induces monocyte to release IL-1 (which induces T cells to synthesis IL-2) and also induces responsive T cells to express the receptor for IL-2. Glucocorticosteroids are reported (Winkelstein 1991) to act primarily to inhibit IL-1 (synthesis and release) and cyclosporin suppresses IL-2 production.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE RESEARCH

The inflammatory infiltrate in rheumatoid lesions is heterogeneous but macrophages and T lymphocytes are usually prominent. The precise role of these cells is controversial and will probably remain so until the nature of the antigen (s) responsible for the lesions is determined. The present study found that chemoattractants including IL-8, IL-15, MIP-1 α and MCP-1 are present in substantial quantities in rheumatoid synovial fluid, their levels being higher in RA than in OA. In addition IL-8, IL-15 and MCP-1 were found in abundance in a few patients with other inflammatory arthritides including polymyalgia rheumatica, psoriatic arthritis and ankylosing spondylitis. As stated earlier all four cytokines are produced by macrophages in large amounts. The fifth cytokine, IL-2, has lymphocyte attractant activity similar to IL-15 (Wilkinson and Newman 1994), but is present in quantities much too low to be playing a major role in chemoattraction. Thus it seems possible that macrophages are the important cells for releasing T lymphocyte attractants and that these cells may play a major role in T lymphocyte recruitment into the joint. Synoviocytes may also be able to synthesis IL-15 (McInnes *et al.*, 1996). Macrophages in rheumatoid lesions have been reported to make IL-8 (Koch *et al.*, 1991) and other work from this laboratory (McInnes, *et al.*, 1996) shows that they also make IL-15. No single cytokine is dominant as an attractant and mixtures of anti-cytokine antibodies are needed to inhibit the attractant effect of synovial fluid. IL-8, IL-15, MCP-1 and MIP-1 α are all present in many of

present in many of the fluids in concentrations sufficient for any one alone to attract lymphocytes. It is therefore not surprising that blocking with antibody to single cytokines is insufficient to block the activity of the fluid. This is in contrast to the previous observation that in PBMC cultured with aCD3 or PPD for 48h, the activation of lymphocyte locomotion was almost entirely due to IL-8 produced by monocytes in monocyte-lymphocyte clusters (Wilkinson and Newman 1992). Almost no IL-15 was produced under those conditions (Wilkinson unpublished observations). However, that was a short-term *in vitro* study and it is possible that maturation of monocytes to macrophages is required for IL-15 production.

On the other hand the T lymphocytes found in rheumatoid lesions are activated as judged by expression of CD69, CD25 and MHC Class II (Iannone *et al.*, 1994). The capacity of T lymphocytes to respond by locomotion to soluble attractants is dependent on activation and is expressed as resting cells move into early G₁ (Wilkinson 1986). This is why blood T cells are cultured overnight to obtain optimal locomotor responses. Synovial tissue lymphocytes, which are already activated, do not require such culture. In normal human blood and lymphoid tissues, a variable, but usually a minority, population of T cells is CD45RO⁺ and these are probably cells that have become activated by recent contact with antigen (Bell *et al.*, 1992). The proportion of CD45RO⁺ T cells in rheumatoid lesions is high (Pitzalis *et al.*, 1987), and CD45RO⁺ cells adhere preferentially to peripheral (non-lymphoid) vascular endothelium (Pitzalis *et al.*, 1988). CD45RO⁺ cells, as would be

expected, are more motile than CD45RA (Newman and Wilkinson 1993), and these cells, when tested from blood, showed preferential locomotor responses to rheumatoid synovial fluids, particularly in the collagen gel assay, which has the advantage over most locomotion assays that the locomotor cells can be rescued and phenotyped. The better selection for CD45RO⁺ and against CD45RA⁺ cells in the collagen gel invasion assay than in the polarization assay may be because CD45RO⁺ cells are both more adherent and more motile. The collagen gel assay requires both locomotion and adhesion, but polarization measures locomotion independently of adhesion. The hypothesis relating the above findings to the ingress of lymphocytes into inflammatory lesions is as follows. Unprimed, recirculating lymphocytes (CD45RO negative) make contact with, and respond to, the as-yet undefined antigens responsible for rheumatoid lesions. This takes place on the surfaces of accessory cells either in lymphoid tissue or, since rheumatoid lesions contain high endothelium which recirculating cells are able to cross, within the joint itself. These cells become activated and therefore capable of making locomotor responses to inflammatory chemoattractants. When they detach from the lymphocyte-accessory cell cluster and re-enter the bloodstream, those which have acquired an activated phenotype (CD45RO⁺, CD25/CD122⁺) no longer recirculate but can attach to, and migrate across, inflammatory endothelia (Mackay, 1991). These cells accumulate selectively in the joint attracted by cytokines produced by macrophages and other cells such as synoviocytes in the lesions..

Lymphocytes from synovial fluid were unresponsive to all stimuli in locomotion assays despite the fact that these cells are viable and capable of proliferation. Possibly prolonged exposure to locomotor inhibitory factors in the fluid, for example factors that elevate intracellular cyclic AMP, may have render these cells immotile.

In addition in this study I have used the polarization and collagen gel assay to measure polymorphonuclear cell (PMN) locomotion and have demonstrated a significant locomotor response of PMN (isolated from normal blood) to rheumatoid synovial fluid and several fluids from other inflammatory arthritides. This finding may suggest that IL-8 abundance in these fluids may cause the attraction of these cells, but the insignificant correlation between the accumulation of cells and IL-8 in the fluids indicates that such accumulation may be due to combined chemotactic factors. This results is in agreement with the finding of Brenman et al., 1990. In contrast PMN isolated from the synovial fluids showed significant reduction in chemotaxis. These results suggest that these cells internalized their receptors or may be prior ingestion of immune complexes (in case of PMN; either in RA peripheral blood or RA synovial fluid and RA synovial tissue) causes inhibition of their locomotion activity. In addition several PMN inhibitory factors reported in the synovial fluids may selectively render these cells immotile or these may be a PMN locomotion defect due to autoantibodies against their cytoplasmic proteins.

Since anti-inflammatory drugs (including CsA) which are used for the treatment of RA are reported to inhibit lymphocyte chemotaxis (Wilkinson

and Higgins 1987). It was important to consider the role of anti-rheumatic drugs in the locomotion activity of inflammatory cells including lymphocytes. Two observations are reported. First treatment of these cells in vitro with NSAIDs (including aspirin, ibuprofen and indomethacin) and DMARDs (including gold, D-penicillamine, and primaquine) or other cytotoxic drugs (including cyclophosphamide and rapamycin) showed no inhibitory effect on the locomotion activity of these cells. This suggested that the mechanisms of action of these drugs may be anti-proliferative (i.e they exert their inhibitory effect at a late stage of cell growth) or selectively suppress the release or the activity of inflammatory mediators (produced by the inflammatory cells) rather than suppression of cell motility. For example it has been observed that some of these drugs including cyclophosphamide cross-link DNA strands which results in decreasing the lymphocyte proliferative response in vitro (Winkelstein 1991). Second treatment the lymphocytes with CsA and Glucocorticosteroids (including dexamethasone, prednisone and prednisolone) showed a significant inhibitory effect. These drugs exert their inhibitory action at an early stage of cell growth at a time when the locomotor phenotype in T cells begins to be expressed.

Future Research

Despite a considerable amount of work using a variety of assay systems, the following questions remain about the role of lymphocyte locomotion in RA and the manipulation of locomotion of these cells. First, the locomotor activity of cells isolated from various RA synovial tissues and other inflammatory arthritides needs further investigation and phenotyping of the responsive cells to well known chemoattractants and rheumatoid synovial fluid. A full comparison of lymphocytes from RA and other inflammatory arthritides with normal controls would be useful. Second, inhibitory factors in the synovial fluid are as yet unidentified and their role is not clear. In addition the site of action of these inhibitors is also unclear (i.e whether they act on the lymphocytes or neutrophil themselves or on the chemotactic factors). Third despite the measurements of above mentioned cytokines, other chemoattractants in the synovial fluid need further investigation including RANTES, MIP- β (our assays showed inconsistent results with these cytokines) and other as yet unidentified chemotactic factors.

Finally as long as the antigen which cause the activation of inflammation in rheumatoid arthritis is not identified it will be difficult to understand and control the locomotion of lymphocytes and other cells in rheumatoid arthritis and other inflammatory arthritides.

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