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INVESTIGATIONS INTO THE IMMUNOPATHOLOGY OF
INFLAMMATORY ARTHRITIS

by

FIONA MARION FLORENCE MCQUEEN
MBChB, FRACP

A thesis submitted for the degree of Doctor of Medicine of the
University of Auckland, 1996.
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ABSTRACT

This volume contains an introduction to the topic of inflammatory arthritis, followed by Chapter 2 which is a Materials and Methods section and includes experimental protocols used for the original research detailed in Chapters 3 to 6 (Sections 2.1 - 2.4). Chapters 3 to 6 are each followed by their own Discussion section and References in much the same form as was submitted for publication. The thesis is concluded by a general discussion of the topic and the relevance of this research.
RESEARCH AIMS

The overall aim of this research has been to better define the immunological processes underlying the development and maintenance of chronic synovitis in inflammatory arthritis. Two forms of inflammatory arthritis, rheumatoid arthritis and psoriatic arthritis, have been studied separately and in many instances compared. This research has included the investigation of cytokine biology and its relation to clinical indicators of disease activity, the identification of activated cell types in synovial fluid and peripheral blood, the investigation of lymphocyte responses to antigens and the identification of adhesion molecules displayed by these cells.

Specific Aims

1. To investigate the clinical activity of rheumatoid arthritis and relation of interleukin-1β levels to disease activity in premenopausal women through the menstrual cycle.

2. To examine lymphocyte responses to streptococcal antigens in peripheral blood and synovial fluid of patients with rheumatoid and psoriatic arthritis. To characterise responding cells using flow cytometry and determine the specificity of antigen responses.

3. To investigate the cytolytic activity of NK cells from peripheral blood and synovial fluid of patients with psoriatic arthritis and observe the influence of cytokines and prostaglandins on NK function.

4. To examine the surface expression of the β7 integrin, HML-1, on NK and T cells from peripheral blood and synovial fluid of patients with rheumatoid and psoriatic arthritis. To investigate the influence of cytokines and prostaglandins on expression of this molecule.
Much of the original work presented or referred to in this thesis has been published:


**ABBREVIATIONS**

(in order of appearance in the text)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>PsA</td>
<td>Psoriatic arthritis</td>
</tr>
<tr>
<td>PIP</td>
<td>proximal interphalangeal</td>
</tr>
<tr>
<td>MCP</td>
<td>metacarpophalangeal</td>
</tr>
<tr>
<td>MTP</td>
<td>metatarsophalangeal</td>
</tr>
<tr>
<td>DIP</td>
<td>distal interphalangeal</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigens</td>
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<tr>
<td>CD</td>
<td>cluster designation</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>3HVR</td>
<td>third hypervariable region</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor- α</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic (mouse)</td>
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<tr>
<td>γδ T</td>
<td>gamma-delta T (cells)</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>Fc</td>
<td>fraction crystalline of immunoglobulin</td>
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<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>ADCC</td>
<td>antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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</table>
HML-1  human mucosal lymphocyte adhesion molecule-1
EDTA  Ethylenediamine tetra-acetic acid
µg  microgram
ml  milliliter
C  Celsius
ELISA  Enzyme-linked immunosorbent assay
pg  picogram
kGy  kilo Gray
PBS  phosphate buffered saline
Strep 1  A Lancefield Group B β haemolytic streptococcus isolated from a patient with gutatte psoriasis
Strep 2  A Lancefield Group A β haemolytic streptococcus isolated from a patient with rheumatic fever
SCW  streptococcal cell wall
RNase  ribonuclease
³HTdr  tritiated thymidine
µCi  micro-Curie
SD  standard deviation
SI  stimulation index
FITC  Fluorescein isothiocyanate
FACS  fluorescence activated cell sorter
Δcpm  change in counts per minute
PE  phycoerythrin
mab  monoclonal antibodies
Fab  fraction antibody of immunoglobulin
PB  peripheral blood
SF synovial fluid
FCS fetal calf serum
$^{51}$Cr sodium $^{51}$chromate
CO$_2$ carbon dioxide
PGE$_2$ prostaglandin E2
PBL peripheral blood lymphocytes
SFL synovial fluid lymphocytes
ARA American Rheumatism Association (now American College of Rheumatology)
MF mid-follicular
ML mid-luteal
LL late-luteal
Hg mercury
IDA index of disease activity
pmol picomoles
nmol nanomoles
Hb haemoglobin
SLE systemic lupus erythematusus
Fe iron
Zn zinc
kD kiloDalton
hsp heat shock proteins
PASI Psoriasis Activity and Severity index
SAARDs slow acting anti-rheumatic drugs
NSAIDs non-steroidal antiinflammatory drugs
SAS statistical analysis system
E : T effector : target
<table>
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<th>Acronym</th>
<th>Definition</th>
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<td>IEL</td>
<td>intra-epithelial lymphocytes</td>
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<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>LFA-1</td>
<td>lymphocyte function-related antigen</td>
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<tr>
<td>CRP</td>
<td>c-reactive protein</td>
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<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>AP-MT</td>
<td>acetone-precipitable fraction of mycobacterium tuberculosis</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
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<td>TGFβ</td>
<td>transforming growth factor-β</td>
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CHAPTER ONE

INTRODUCTION

1.1 Clinical features

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are both classified as forms of inflammatory arthritis as they are characterised by inflammation and proliferation of the synovial membrane within the joint space. The production of chemical mediators of inflammation and degradative enzymes by hypertrophied synovium (pannus) results in breakdown of underlying cartilage and bone with the radiological correlates of joint space narrowing and bony erosions (1). While there are many similarities between these conditions (and indeed PsA was not recognised as a separate entity until the mid-1960s) (2) distinctions can be made between them on clinical and radiological grounds.

The distribution of arthritis in RA favours symmetrical involvement of PIP, MCP and carpal joints in the upper limbs and the MTP joints of the feet. As disease progresses large joints such as knees and elbows become involved to produce a small and large joint inflammatory polyarthropathy (1). In contrast the most common form of PsA exhibits an asymmetric oligoarticular pattern (3) and the disease may also be characterised by DIP joint involvement which is not seen in RA (2,3). Spondyloarthropathy, dactylitis and enthesitis occur in other subgroups of PsA emphasising links with the seronegative spondyloarthropathies (4). Arthritis mutilans, a rare subtype of PsA, presents a distinctive clinical picture of extreme bony erosion and the development of "pencil-in-cup" joint deformities (2,3,4). One subgroup of PsA patients do develop a rheumatoid-like picture of symmetrical small joint involvement and confusingly some of these patients are seropositive for rheumatoid factor (5) blurring the distinctions between the two disorders. Extra-articular features are important in diagnosis as PsA by definition can only occur in the presence of psoriatic skin disease (2) while nodules and vasculitis are confined to RA, especially when this is seropositive.
Radiologic appearances also differ between these two forms of inflammatory arthritis. Assymmetric joint involvement, DIP joint changes and sometimes sacroileitis characterise PsA while RA joint involvement tends to be symmetrical, peripheral and more proximal (7). Although erosive joint damage occurs in both diseases, it is interesting that the erosions of PsA are often surrounded by bony hyperostosis as occurs also at sites of enthesitis (8). In contrast the bone eroded in RA is osteopenic (9) and does not seem to undergo proliferative change in response to the disease process. Thus these two diseases are both similar and dissimilar reflecting the genetic, environmental and immunological factors involved in their pathogenesis.

1.2 Genetics

The mode of inheritance of both RA and PsA appears to be polygenic (10,11) but strong associations with HLA antigens have been observed in both diseases. However these are with the Class II HLA-DR antigens in RA while PsA is linked primarily with Class I HLA antigens. The "shared epitope hypothesis" was proposed by Gregersen, Silver and Winchester in 1987 (12) as a unifying model to explain HLA-DR associations with RA. These investigators focused interest on the structure of the MHC antigen-binding groove. Detailed studies of Class II MHC molecules associated with RA revealed a shared conformational structure or "epitope" within this groove involving the third hypervariable region (3HVR) of the DRβ1 chain at amino acid positions 70 to 74 (QKRAA or QRRAAA sequence). The DR4 subtypes with this common hypervariable region which include DRB1*0401 (Dw4), DRB1*0404 (Dw14) and DRB1*0405 (Dw15) have been found to be associated with disease but the closely related DRB1*0402 (Dw10) with a different amino acid sequence in the crucial area has not (13,14). Fig 1. shows the recently determined crystal structure of the HLA-DR1 molecule (15) which also contains the 3HVR sequence and has been associated with the development of RA in some populations (16).
A New Zealand study has confirmed these findings and linked the rarity of rheumatoid arthritis in New Zealand Polynesians with the low prevalence of HLA-DRB1*04 (DR4) subtypes containing the shared epitope in this population (14).

Data from Weyand et al (17) has suggested that the 3HVR sequence may predispose not only to the development of disease, but to its progression and severity. This group has provided evidence for severe disease, associated with rheumatoid vasculitis, developing in individuals homozygous for the DRB1*0401 sequence implying a gene dosage effect. A hierarchy of DRB1 alleles has been proposed associated with a scale of clinical severity in RA (18) suggesting a strong correlation between genotype and clinical phenotype. Prediction of disease severity in RA using genetic, biochemical and immunological markers has important therapeutic implications. Patients deemed to be “at risk” for developing aggressive disease can be targeted for aggressive treatment using...
potentially toxic agents such as methotrexate whereas other patients lacking poor prognostic markers may be managed effectively with more conservative regimens (19).

In PsA, the strongest HLA associations are with the Class I antigens, Cw6, B13, B17,B7 and B27 (11,20). In the small subgroup of PsA patients with rheumatoid-like disease an association with HLA-DR4 has been observed (11) but not in the group as a whole. There is an association with the Class II molecule, DR7, but this is related to its linkage disequilibrium with Cw6 (20). The Class I HLA association implies that different immunological mechanisms may function in the initiation of this disease compared with RA (21). Class I HLA molecules usually present endogenous antigens such as viral proteins to CD8+ T cells (which may be cytotoxic or have suppressor function) and the shared epitope hypothesis as it applies in RA cannot be so readily invoked. However, some investigators have proposed a similar mechanism may underly the Class I association with Cw6. An increased frequency of the Cw*0601 allele has been described in psoriasis by one group (22) but as yet no specific epitope has been related to the development of PsA.

While the association of PsA with Class I HLA genes has been interpreted as suggesting a precipitating viral antigen in this condition (as opposed to bacterial in RA), even this paradigm of classical immunology has lately been shaken by evidence that Class I MHC molecules can present bacterial antigens to T cells (23) in certain circumstances. In addition PsA bears many resemblances, both clinically and radiologically, to forms of reactive arthritis such as Reiter's syndrome (24), known to be precipitated in some cases by bacterial antigens derived from organisms such as Salmonella and Shigella species. Moreover, the HLA alleles linked to the development of Reiter's syndrome are predominantly Class I (such as HLA-B27). Thus it is apparent that HLA associations of the different forms of inflammatory arthritis cannot accurately predict the nature of precipitating microbial antigens.
1.3 Immunohistology

Despite differences in clinical phenotype and genetic susceptibility, RA and PsA are extremely similar diseases in many respects. Chronic synovial inflammation typifies both forms of arthritis; the synovial membrane being infiltrated by large numbers of lymphocytes which are predominantly CD4+ and frequently these display the "memory" phenotype CD45Ro (25,26). Although the production of rheumatoid factor is not classically seen in PsA, aggregates of B lymphocytes in lymphoid nodules within the synovium are seen in this disease as in RA (26). Comparative studies of the synovial histology of PsA and RA have been remarkable for the similarities between these conditions (27) although Veale et al (26) recently demonstrated less thickening of the synovial lining layer and a lack of expression of the E-selectin adhesion molecule in PsA specimens studied. Despite presumed differences in the immunological mechanisms precipitating disease, convergence of disease mechanisms seems to occur to a final common pathway of chronic inflammation.

The collagen-induced arthritis model of inflammatory arthritis (28), where immunisation of rats with native Type II collagen and Freund's adjuvant gives rise to inflammatory synovitis with pannus formation and joint erosions, may mimic the second stage of the autoimmune process which is likely to be common to RA and PsA. In this model, an autoimmune reaction against self-collagen within the joint appears to be triggered by the highly immunogenic type II collagen molecule and results in activation of humoral and cellular immunity. Autoantibodies to type II collagen have been detected in patients with RA and PsA at higher levels than controls and T cell responses to components of the collagen molecule have also been observed in patients with both conditions (29). Trentham et al propose that the immunogenicity of self-antigens (such as type II collagen) is heightened due to enzymatic and cytokine-mediated damage causing a vicious cycle of inflammation perpetuating the autoimmune reaction which in turn causes more inflammation within the joint.
1.4 Cytokines

The development of synovitis in RA and PsA appears to be linked to the production of proinflammatory cytokines. These include IL-1β, TNFα and IL-6 (30). Production of IL-1 is likely to be upregulated in the rheumatoid joint as suggested by observations that monocytes from rheumatoid synovial fluids produce more IL-1 after in vitro stimulation than paired samples of peripheral mononuclear cells (31). Activation of IL-1 gene expression in RA synovial cells has also been demonstrated in vivo using in situ hybridisation techniques (32). Many actions of IL-1β are relevant to the maintenance of chronic joint inflammation and these are summarised in Fig. 2 (33).

![Figure 2](image-url) Cellular interactions and cytokines mediating joint destruction in rheumatoid arthritis. Adapted from Ref 31.
IL-1 has been shown to stimulate the release of prostaglandins and collagenases from rheumatoid synovial cells in culture and this can be inhibited by its naturally occurring competitive inhibitor, the IL-1 receptor antagonist (34). Moreover the injection of IL-1 into the joints of animals results in reduced proteoglycan synthesis (35), enhanced proteoglycan degradation and increased release of stromelysin (a potent cartilage degrading enzyme) into the synovial fluid (36).

Evidence is also available for an important role for IL-1 and other inflammatory cytokines in the pathogenesis of PsA. Elevated levels of IL1-β have been demonstrated in PsA synovial fluids (37) and IL-6 activity has also been shown to be high, comparable with levels in RA synovial fluids (38). As the expression of IL-6 by mononuclear phagocytes, endothelial cells and fibroblasts can be induced by IL-1 (39) the activities of these 2 cytokines are closely linked. Increased production of IL-1β by fibroblasts from psoriatic skin and synovium has also been demonstrated with correspondingly increased proliferative activity (40) suggesting a link between fibroblast production of IL-1β in this disease and the subsynovial fibrosis observed in endstage PsA (41).

Clinical evidence for direct links between IL-1β levels and disease activity in RA comes from a study of 51 patients where mean plasma levels of IL-1β were found to correlate with Ritchie score, pain score and ESR (42). As an extension of this work, a further study of IL-1β levels in premenopausal women was undertaken and is presented in Chapter 1 of this thesis (43). This examines the fluctuation of symptoms and signs of RA through the menstrual cycle and the relationship between clinical indices of disease activity and plasma IL-1β levels.

The interplay of hormonal influences and cytokine activity on joint inflammation in rheumatoid arthritis has been examined in a number of specific clinical situations and animal models. During pregnancy, amelioration of disease activity in rheumatoid arthritis is observed in up to 75% of patients and was first described by Hench in 1938 (44). In addition there is some evidence that female sex steroids may protect against the development of RA when used for contraception in premenopausal women (45). However other evidence implies that factors associated with female
sex exacerbate the cartilage degradation associated with inflammatory synovitis and that this may be associated with changes in proinflammatory cytokines. In an animal model, increased sensitivity to IL-1-induced inhibition of proteoglycan synthesis was observed in female rats compared with male rats (46). Furthermore, in human male RA patients, the addition of 17-β-estradiol to peripheral blood mononuclear cells in vitro has been shown to enhance secretion of IL-1 and IL-6 (47). In contrast, our findings (43) suggest that symptom fluctuations through the menstrual cycle in RA are not related to changes in IL-1β levels but may reflect more complex interactions between gonadotropins, cytokines and inflammation which await clarification.

1.5 Antigens

Streptococcal cell wall arthritis is an animal model of inflammatory arthritis induced in genetically susceptible Sprague-Dawley rats by the intra-peritoneal injection of heat-killed sonicated Group A streptococci (48). As these animals develop an erosive inflammatory form of chronic arthritis characterised by the formation of pannus and the destruction of cartilage, this has been regarded as a model of RA, but histological appearances would be equally consistent with PsA (49). Adjuvant arthritis is another important animal model of inflammatory arthritis whereby joint swelling develops in susceptible rats about two weeks after an injection of mycobacteria in mineral oil (50). Again the features of inflammatory synovitis mirror appearances in both RA and PsA but the development of extra-articular features such as sacroileitis and dermatitis suggest a closer approximation to PsA (51).

The hypothesis developed from these models proposes that inflammatory arthritis may result from a form of molecular mimicry between a foreign microbial antigen and a self-antigen in a genetically susceptible host (52). A recent refinement of this concept relates to the phenomenon of “epitope spreading” whereby the initial immune response to a foreign epitope is followed by intra- and intermolecular spreading superseded by an autoimmune reaction directed against a growing spectrum or “second wave” of self-antigens. This phenomenon has been demonstrated to occur in
a number of animal models of autoimmune disease including experimental allergic encephalomyelitis (EAE)(53), a model of multiple sclerosis, and the non-obese diabetic (NOD) mouse (54), a model of insulin-dependent diabetes mellitus. It has been noted that the late responses to “second-wave” determinants in these models may involve antigen presentation by different MHC molecules than did the response to the initial disease-inciting epitope confusing the interpretation of MHC-linked disease susceptibility markers. The observation that RA and PsA have different MHC associations remains consistent with the molecular mimicry hypothesis. Both diseases may have been triggered initially by different microbial antigens but then have evolved into phenotypically similar autoimmune conditions characterised by reactivity to self-antigens within the joint.

Streptococci have been implicated more directly in the pathogenesis of PsA by the known clinical association between pharyngitis due to a Group A streptococcal infection and the development of guttate psoriasis (Fig. 3) after two to three weeks (55). Studies have revealed enhanced humoral immune responses to streptococci amongst PsA patients compared with controls (56) and limited data suggests augmented cellular responses may also occur to streptococci in these patients (57). Work described in this thesis (Chapter 2) has been aimed at studying cellular responses to streptococcal antigen in psoriatic and rheumatoid arthritis using T cells from peripheral blood of patients and controls as well as synovial fluid T cells from patients with knee effusions. Further studies of γδ T cells from these patients show that they can be induced to proliferate in response to streptococcal antigens raising questions regarding the role of these rather unusual lymphocytes in inflammatory arthritis.

1.6 NK cells

Studies of NK (Natural Killer) cell activity in RA and PsA are presented in Chapter 3 of this thesis. NK cells are recognizable by light microscopy as Large Granular Lymphocytes and are capable of spontaneous cytotoxic activity against target cells without prior sensitisation (62). Effector
cytolytic mechanisms include the release of preformed granules containing molecules such as perforins which mediate target death by osmotic lysis and the secretion of cytokines such as TNFα which are capable of initiating apoptosis in target cells (62,63). NK cells function as part of the

Figure 3. Guttate psoriasis. From Baillieres Clinical Rheumatology, 1994;8(2):301
Innate immune system mediating early nonspecific responses to viral infection and in immune surveillance by eliminating neoplastic cells (62).

The relevance of the NK system to rheumatic disease derives from hypotheses that PsA and/or RA could be triggered by viral infection (64, 65). Initial upregulation of NK activity within the joint in response to such a virus could lead to the release of potent lytic enzymes from NK granules triggering the release of a cascade of proinflammatory cytokines into the joint and setting the stage for chronic erosive synovitis. Also relevant to their putative role in RA is the proposal by Hendrich and Schmidt (66) that these cells could be activated directly by rheumatoid factor (via crosslinkage of CD16, the FcyRIII receptor). These authors observed increased production of interferon-γ and TNF-α by NK-enriched cell lines from RA patients when incubated with purified rheumatoid factor. However, most studies of NK cells derived from inflammatory synovial fluids have revealed reduced cytolytic function compared with paired samples from peripheral blood (67, 68) leading to the general conclusion that NK cells are not activated at the site of the disease process and therefore unlikely to be of relevance to its pathogenesis.

Important recent advances in the understanding of NK physiology suggest that conclusions based on studies of NK cytolysis of tumor cell lines may be flawed. These cell lines are unusual in that they express very low levels of MHC Class I molecules and it is this which underlies their susceptibility to NK-mediated lysis (69). Studies by Karre et al (70) of MHC Class I negative mutant cell lines revealed their exquisite sensitivity to NK-mediated lysis which was abrogated by the reconstitution of MHC Class I expression by transfection of the β2 microglobulin gene (71). A new concept relating to the recognition of targets by NK cells has emerged suggesting that NK cells may survey normal tissues for MHC Class I molecules and in the absence of normal Class I expression, the target is lysed. Further studies have proposed that it is the presence of self peptide in the groove of Class I MHC molecules which prevents NK-mediated lysis of targets and that when this is replaced by viral peptide, susceptibility to NK lysis is increased (72).
In the context of PsA and RA, targets for NK cell attack within the joint have not been defined but could include autologous cells if these had been infected with virus. Certainly the tumor target line, K562, which expresses very low levels of Class I MHC (73) is an inadequate model for target cells in this context but has been used consistently in studies of NK activity in RA (67,68). Synovial tissue is likely to express much higher levels of Class I molecules than K562 cells and these levels may even be upregulated in response to cytokines such as IFN-γ during joint inflammation (74).

1.7 Adhesion molecules and cellular activation

More accurate information regarding the state of activation of NK cells from inflammatory synovial fluid may be obtained by flow cytometry when the expression of cell surface molecules is examined. NK cells do not express T or B cell surface receptors or the complete CD3 complex but they do express the ζ chain of the CD3/TCR complex (75) which is likely to mediate signal transduction into the cell. They also express the Fc receptor for IgG (CD16 or FcγRIII) which mediates their effector function in antibody-dependent cellular cytotoxicity (ADCC) (76). Cell adhesion molecules are expressed by NK cells isolated from the peripheral blood of healthy volunteers and include the β2 integrins CD11a-c/CD18, and the immunoglobulin superfamily molecule, ICAM-1(CD54) (77).

A new class of cell adhesion molecule utilising the β7 chain has recently been described (78,79) which includes a molecule recognized by the Mab, Human Mucosal Lymphocyte adhesion molecule-1 (HML-1) (79). This is expressed by intra-epithelial lymphocytes of the gut (80) and is structurally related to the α4β7 integrin molecule which is the murine homing receptor to the mucosal tissue of the gut. HML-1 is an activation antigen on T cells and epithelial cells where expression may be induced by mitogens and transforming growth factor β (81,82). In chapter 6 of this thesis we have examined the expression of this molecule on NK and T cells freshly isolated
from psoriatic and rheumatoid synovial fluids and found it to be elevated compared with paired peripheral blood samples (83). Since submission of this data for publication, others have reported similar findings (84) including high expression of HML-1 on T cells from rheumatoid synovial fluids and significantly lower HML-1 expression on synovial fluid T cells from patients with osteoarthritis. Interestingly this group found the majority of T cells expressing this antigen to be CD8+ and therefore of the cytotoxic/suppressor phenotype.

The implication of these findings is that both NK and T cells within inflammatory joint fluid are in an activated state. Their expression of HML-1 is of particular interest in view of the parallel expression of this antigen by a high percentage of CD8+ T cells in the gut mucosa (81). Other lymphoid tissues such as thymus, tonsils and spleen rarely or never express HML-1 (80). This raises the fascinating question of whether an antigen encountered by the cells of the gut mucosa might be important in initiating or perpetuating synovial inflammation in RA and/or PsA via such activated T cells. A candidate antigen might be the Eschericia coli heat shock protein, dnaJ, which is known to possess a B cell epitope cross reactive with the HLA DRB1*0401 molecule (85). Further studies of lymphocyte trafficking between the gut mucosa and synovium of patients with inflammatory arthritis are needed to better define the role of these adhesion molecules and antigens.

1.8 Conclusions

While RA and PsA are distinct diseases clinically, radiologically and genetically, they share the common feature of chronic inflammatory synovitis. Release of proinflammatory cytokines into synovial fluid is a characteristic of both disorders and the histological appearances of inflammation and hypertrophy within the synovial membrane may be identical. Indeed a review of the literature reveals a paucity of data obtained at a cellular level which distinguishes these two conditions (27,64). The experimental data discussed in this thesis largely relates to the final stage of the autoimmune process resulting in joint inflammation and convergence of pathology in PsA and RA. However events initiating the autoimmune reaction remain poorly defined in both disorders.
I.9 REFERENCES


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2.1 Menstrual Cyclicity of Symptoms and Relation to Plasma IL-1β Levels in Rheumatoid Arthritis

2.1.1 Measurement of plasma IL-1β

Blood samples for plasma IL-1β were taken into tubes containing EDTA (5 x 10^{-3}M) and aprotinin (bovine lung 15 - 30μg/ml; Sigma Chemical Co., St Louis, Missouri) at 0.67μg/ml. Plasma was separated from cells and platelets by immediate two-stage centrifugation at 400g and 10,000g and then stored in 500μL aliquots at -50°C until tested for IL-1β content. Collection and processing of samples for measurement of IL-1β levels was performed according to the method described originally by Cannon et al (1) and used in RA by Eastgate et al (2). IL-1β was measured by ELISA assay (Cistron Biotechnology, New Jersey) in chloroform-extracted plasma (Fig 1). This assay is sensitive to 20pg/ml and is suitable for use with RA plasma (2).

2.1.2 Measurement of β estradiol and progesterone

Serum samples for β estradiol and progesterone were also stored in 500μL aliquots at -50°C. Estradiol was measured by radioimmunoassay following ether extraction (Immunodiagnostics Ltd, Washington, England). Progesterone estimation was by a direct radioimmunoassay (3) with danazol as a displacing agent.

2.1.3 Patients and Clinical Methods See Chapter 3.

2.1.4 Statistical Methods See Chapter 3.
Monoclonal antibody to IL1β

RA patient plasma containing IL1β

Polyclonal rabbit anti-IL1β antibody

Anti-rabbit antibody labelled with enzyme

Addition of substrate → colour change α to IL1β

Figure 1. ELISA assay for measurement of IL-1β levels in chloroform-extracted plasma

2.2 γδ+ T Cells From Patients With Psoriatic and Rheumatoid Arthritis Respond to Streptococcal Antigen

2.2.1 Preparation of Streptococcal Antigens

A Lancefield Group B β haemolytic streptococcus (strept. agalactiae serotype 1A) was isolated from the throat of a patient with acute guttate psoriasis. This is referred to as “Strep 1”. A Lancefield group A β haemolytic streptococcus was isolated from a patient with acute rheumatic fever and is referred to as “Strep 2”. A pure culture of each streptococcal strain was grown for 48 hrs in Luria Bertani medium, irradiated with 5 - 6kG, and cultured to ensure no viable organisms remained. Bacteria were pelleted from culture medium by centrifugation (8000g at 4°C), washed twice in PBS, resuspended in distilled water, sonicated (5 x 12 sec bursts), lyophilised and stored at 4°C before use. A streptococcal cell wall preparation (SCW) derived from the crude Strep 1 material
was prepared (courtesy of Professor P Sullivan, Dept of Chemistry, University of Otago, NZ) using the method of Cromartie et al (4). Briefly the lyophilised material was reconstituted with PBS and mechanically disrupted for 3 mins using a Braun homogeniser. Following centrifugation at 10,000g for 30 mins the total pellet was washed x 3 with PBS and x 3 with H2O. Disruption was assessed as being at greater than 95% of organisms by phase contrast microscopy. Material was then treated with 0.025% RNase (incubation for 4 hours at 37°C), washed and treated with 0.025% trypsin (incubation for 4 hours at 37°C) and washed with PBS. Finally material was resorificated using 25 x 12 sec bursts and lyophilised for storage at 4°C.

2.2.2 Lymphocyte Proliferation assays

Mononuclear cells were isolated from blood and synovial fluid using Ficoll-Hypaque density centrifugation. Cells were washed twice and resuspended in medium containing 5% heat inactivated autologous serum, 1% glutamine (0.2 mg/ml) and RPMI 1640. Cells were cultured at a concentration of 2 x 10^5 cells/well in 96 well flat bottom plates with appropriate antigen at 37°C in a 5% CO₂ in air incubator for 7 days. After 6 days, cultures were pulsed for 18 hrs with 3H-thymidine (3HTdr) at 0.25 μCi per well and then cellular uptake of 3HTdr was assessed by liquid scintillation counting. Experiments were performed in triplicate and results recorded as mean +/- SD of the difference between the maximum response and background counts per minute (Δcpm). Data are also expressed as Stimulation Index (SI) calculated as cpm of lymphocyte responses to antigen divided by cpm of unstimulated cells.

2.2.3 γδ+ T cell proliferation assay

Cells were cultured at a concentration of 2.5 - 3.5 x 10^6/ml in 24 well flat bottom plates (total volume of 1.5 ml/well) with streptococcal antigens (2 - 10μg/ml) at 37°C in a 5% CO₂ in air incubator. On day 6 cultures were pulsed for 18 hrs with 1.88 μCi of 3HTdr. On day 7 cells were pooled, harvested by density gradient centrifugation and divided into 2 aliquots. One aliquot was
stained for γδ+ cells and the other was stained with IgG1-FITC as a background control. Cells were sorted using a Becton Dickinson FACS 440 flow cytometer to obtain a γδ+ enriched fraction (Figure 2). Percentages of γδ+ cells in this enriched fraction ranged from 8.8 to 79.1% and are presented in Chapter 4, Table 6. The gate for sorting was set to maximise enrichment of the γδ+ fraction. Cell numbers within the sorted γδ+ fraction were recorded: (median: 9.0 x 10^3, range: 1.7 x 10^3 - 2.9 x 10^4). The uptake of 3HTdr in each fraction and in the unsorted control was measured using liquid scintillation and results were expressed as Δcpm/10^5 cells.

**Figure 2.** Proliferation assay: response of γδ+ fraction to streptococcal antigen
2.2.4 Monoclonal Antibodies

Double labelling was performed using fluorescein isothiocyanate (FITC) conjugated anti-human TCR γδ1 (Becton Dickinson 11F2) which recognises a pan-γδ epitope. The following phycoerythrin (PE) conjugated monoclonal antibodies (mab) were used: anti-CD3 (Dako UCHT1), anti-CD8 (Dako DK25), anti-CD14 (Becton Dickinson MoP9). Isotype matched negative control antibodies used were FITC-conjugated mouse IgG1 (Dako DAK-GO1) and RPE-conjugated mouse IgG1 (Dako DAK-GO1). The following unconjugated mab’s were used to define 2 discrete subsets of γδ+ T cells: BB3 and A13, which recognise Vδ2 and Vδ1 respectively (5), courtesy of Professor L Moretta, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). A second FITC-conjugated polyclonal sheep anti-mouse IgG antibody (Fab fraction) was used for immunofluorescence (Silenus code DDAF).

2.2.5 Immunofluorescence labelling technique

Mononuclear cells were separated from synovial fluid and blood by Ficoll-Hypaque density centrifugation. The cells were washed twice and resuspended in 100μl of staining medium (PBS with 0.1% sodium azide and 5% heat-inactivated autologous serum). Cells were incubated with unconjugated monoclonal antibodies at 4°C for 20 min, washed twice and then incubated for 20 min with FITC-conjugated anti-mouse polyclonal antibody (2nd antibody). Cells were washed twice again and then incubated for a further 20 min with PE-conjugated mabs. When double labelling was performed using FITC- and PE- conjugated antibodies, cells were incubated with both antibodies simultaneously. Each analysis included a sample of unstained cells and a sample of cells incubated with either isotype-matched conjugated negative control antibodies or FITC-conjugated polyclonal antibody. Samples were analyzed within 4 hours of staining.

Cytofluorometric analysis, counting 2 - 3 x10^3 cells per second, was performed using a Becton Dickinson FACS 440 flow cytometer. Nonlymphoid cells were identified using “leucogate” (Becton-Dickinson anti-CD14-FITC/anti-CD45-PE) and in some samples anti-CD14-PE alone.
Results are expressed throughout as median (range) unless specifically stated otherwise.

2.2.6 Patients and Clinical Methods  See Chapter 4

2.2.7 Statistical methods  See Chapter 4

2.3 Natural Killer Cells in Psoriatic and Rheumatoid Arthritis: Their Cytotoxic Activity and Response to Cytokines and Prostaglandins

2.3.1 NK cell cytotoxicity assays
For NK assays, fresh mononuclear cells from PB and SF were separated by density centrifugation on Ficoll-Hypaque, washed and resuspended in medium containing RPMI 1640, 1% glutamine (0.2 mg/ml) and 10% fetal calf serum (FCS). K562 cells (derived from a human erythroleukemic cell line) and Molt-4 cells (from a lymphoblastic cell line) were used as NK-sensitive targets in a standard 4-hour $^{51}$Cr release assay. Mononuclear ‘‘effector’’ cells derived from PsA SF and PB were plated out in 96 well ‘‘v-bottom’’ tissue culture plates using 1 : 2 serial dilutions from an initial concentration of 1.25 - 5 x 10^6/ml depending on cells available. Target cells (2 x 10^6) were incubated with 100μCi of sodium $^{51}$chromate for 45 mins, washed twice and diluted to 5 x 10^4/ml before adding to effectors. Cells were cultured in a 5% CO_2 in air incubator for 4 hrs and $^{51}$Cr release into the supernatant determined after transfer of samples to a liquid scintillation counter (LKB 1270).

Experiments were performed in triplicate and cytotoxic activity of effector cells was calculated using the formula:

\[
\text{% specific lysis of targets} = \frac{\text{Experimental-spontaneous }^{51}\text{Cr release}}{\text{Maximum - spontaneous }^{51}\text{Cr release}} \times 100.
\]
Where sufficient cells were available, SFL were cultured for 20 hrs (3 x 10^6 /ml in 1.5 ml wells) alone or with IL-2 (100ng/ml), IL-6 (50ng/ml), PGE2 (5 x 10^-7M) or indomethacin (5 x 10^-7M) to assess their effect on NK cytotoxic activity. Cells were harvested the following day, counted and viability assessed by Trypan Blue exclusion. The NK 51Cr release assay was then performed using fresh targets.

2.3.2 Flow Cytometry
Phycoerythrin (PE) conjugated anti-NKH-1 (Coulter N901) was used to identify the NK subset of lymphocytes in SF and PB. Fluorescein isothiocyanate (FITC) conjugated anti-CD14 (Becton Dickinson MoP9) mab defined the population of monocytes. PE-conjugated anti-CD3 mab (DAKO UCHT1) labelled T lymphocytes. For experiments to determine NK and monocyte populations in PB and SF, double labelling was performed using a standard direct immunofluorescence technique as above (2.2.5). Briefly, cells were prepared as above, washed twice and resuspended in staining medium (PBS with 0.01% sodium azide and 5% heat-inactivated autologous serum). Cells were incubated with both antibodies simultaneously at 4°C for 20 mins and then washed twice before cytofluorometric analysis using a Becton Dickinson FACS 440 flow cytometer.

2.3.3 Patients and Clinical Methods See Chapter 5

2.3.4 Statistical methods See Chapter 5

2.4 Enhanced Expression of the β7 integrin molecule, HML-1, on NK and T cells from Synovial Fluid of patients with Psoriatic and Rheumatoid Arthritis
2.4.1 **Flow Cytometry**

For experiments examining expression of HML-1 on NK and T lymphocytes, a three-stage technique was followed as the anti-HML-1 mab (Immunotech S.A.) was unconjugated. Incubation of cells with anti-HML-1 mab was followed by 2 washing steps, then further incubation with polyclonal FITC-conjugated anti-mouse antibody. Lastly, cells were incubated with either anti-NKH-1PE mab or anti-CD3PE mab. Each analysis included a sample of unstained cells and a sample of cells incubated with isotype-matched negative control antibodies.

For experiments examining the influence of cytokines and prostaglandins on HML-1 expression by NK cells, PBL and SFL were cultured in 24 well plates at 3 x 10^6 cells/1.5ml for 7 days in 10% FCS medium. Cells alone were cultured as a control and compared with cells cultured with IL-2 at 20ng/ml, IL-6 at 50ng/ml or PGE_2 at 5 x 10^{-7}M. Cells were harvested and stained for flow cytometry as described above.

2.4.2 **Patients and Clinical Methods**

See Chapter 6

2.4.3 **Statistical methods**

See Chapter 6
2.5 REFERENCES


CHAPTER THREE

MENSTRUAL CYCLICITY OF SYMPTOMS AND RELATION TO PLASMA INTERLEUKIN-1β LEVELS IN RHEUMATOID ARTHRITIS

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3.1 ABSTRACT

Symptoms fluctuate with the menstrual cycle in some pre-menopausal female RA patients. Plasma IL-1β levels have recently been shown to correlate with disease activity in RA but variations in IL-1β levels through the menstrual cycle in RA patients have not previously been studied. A group of 12 seropositive rheumatoid patients was studied over 3 or 4 consecutive menstrual cycles, assessing clinical and laboratory parameters of disease activity and IL-1β levels (from extracted plasma). Measurements were made at 3 time-points per cycle: mid-follicular, mid-luteal and late-luteal. There was significant variability in mean values for the Camp articular index (p=0.03), pain score (p=0.04) and Index of Disease Activity (Mallya) (p=0.01) through the cycle, with the highest values occurring during the late-luteal phase. However there was no significant cyclical variation in hemoglobin, platelets, ESR or IL-1β.

Mean IL-1β levels in the RA patients were significantly higher than in controls when measured at all three phases of the cycle (p<0.05). However, no intra-cycle variability in IL-1β levels was found for patients or controls and levels remained remarkably constant for each individual studied. A positive correlation was found in the late-luteal phase between plasma IL-1β levels and Ritchie articular index (p=0.04), pain score (p=0.04) and Index of Disease Activity (p=0.04) in the RA patient group, but there was no such correlation during the mid-follicular or mid-luteal phases. In some individuals, variability of clinical disease activity through the menstrual cycle (termed cyclicity) was more marked than in others. This was measured by the change in Index of Disease activity from mid-follicular to the late-luteal phases and correlated strongly with mean IL-1β levels (P=0.004) and with ESR (p=0.02).

Conclusions: Menstrual cyclicity of articular index and pain score was demonstrated in these patients with RA but there was no cyclical variation in ESR or IL-1β levels. The articular index correlated with IL-1β levels only during the late-luteal phase when symptoms were most severe. Those patients with the greatest degree of clinical cyclicity were those with the most active disease.
3.2 INTRODUCTION

Disease activity in rheumatoid arthritis (RA) appears to be subject to hormonal influences. For example, symptoms often improve during pregnancy and worsen following parturition (1,2). There is also some evidence to suggest that female sex steroids protect against the development of disease both when used for contraception during the reproductive years (3,4) and for hormone replacement therapy in perimenopausal women (5). However, studies investigating the fluctuation of symptoms throughout the menstrual cycle have produced conflicting results and only one group has measured disease activity by objective criteria (6). The effect of the menstrual cycle on disease activity in RA is therefore still unresolved.

The cytokine interleukin-1 β (IL-1β) has been implicated as a pathogenic mediator in RA (7,8) and plasma levels have been shown to correlate significantly with changes in disease activity in a group of RA patients (8). IL-1β is released from activated mononuclear cells as well as fibroblasts and is likely to mediate joint inflammation and the formation of bony erosions in RA by stimulating production of collagenase and other enzymes (9). A previous study of normal women has suggested that IL-1β levels may be elevated in the post-ovulatory phase of the menstrual cycle (10) but there is no information concerning circulating IL-1β levels in premenopausal female RA patients.

This study aimed firstly to determine whether disease activity in RA fluctuates with the menstrual cycle in some women. Secondly correlations were sought between levels of IL-1β throughout the menstrual cycle and clinical and laboratory measures of joint inflammation.

3.3 PATIENTS AND CLINICAL METHODS

Patients with rheumatoid arthritis according to 1987 ARA criteria (11) attending a Rheumatology outpatient clinic were invited to participate if they conformed to the following criteria:

1) Female between age 20 and 45 years
2) not taking an oral contraceptive (currently or within the previous six months)
3) No gold, penicillamine, immunosuppressive drugs (including methotrexate, azathioprine or cyclophosphamide), oral corticosteroids or intra-articular steroid during the six months prior to the study.
4) History of a regular menstrual cycle

Criteria 1, 2 and 4 were used to select healthy controls. Women were excluded from both the patient and control groups if they had any chronic medical conditions requiring therapy. Twelve patients completed the study, 4 over 4 consecutive menstrual cycles and 8 over 3 consecutive cycles. Of the 8 healthy control individuals who completed the study, 7 completed 3 menstrual cycles and 1 completed 2 consecutive cycles. The mean age of the patients was 36 years (range 26-43 years) and of controls was 34 years (range 26-38 years). Of the 190 occasions on which cases and controls were seen, 177 fell within the limits of one of the 3 phases of the menstrual cycle that we defined.

Study design

The study was prospective, measurements being made over 3 - 4 consecutive menstrual cycles for each woman. Clinical and laboratory assessments were made at three timepoints per cycle i.e. mid-follicular (MF), mid-luteal (ML) and late-luteal (LL) phases, estimated on the basis of the previous cycle. The MF day was taken as midway between the start of the cycle and the day of ovulation (cycle length minus 14 days). The MF phase was MF day -2 to MF day +3. The ML phase was from days -9 to -4 from the end of the cycle and the LL phase was from days -2 to +3 of the next cycle. For a 28 day cycle, the mid-phase MF, ML and LL days corresponded to days 7, 21 and 28 respectively (see Appendix 1).

Clinical Assessments

At each visit a clinical assessment of disease activity was made by an investigator who was unaware of the patients' cycle dates. The articular index was assessed using both Camp (12) and Ritchie (13) scores. The Ritchie score grades tenderness over each joint from 0 - 3 and the Camp score includes a measure for pain at rest and the presence of swelling. Grip strength was measured
using a bag inflated to 20 mm Hg attached to a sphygmomanometer and the mean of 3 scores of maximum pressure generated was taken as the grip strength for each hand. A visual analogue pain score was completed by the patient on each visit and an estimate given of the duration of morning stiffness on the same day. Body weight was recorded and all measurements were made between 9 and 11 am to minimise possible diurnal fluctuations. An index of disease activity (IDA) was calculated at every assessment. This was derived according to the method of Mallya and Mace (14), incorporating articular index, grip strength, pain score, morning stiffness, hemoglobin concentration and erythrocyte sedimentation rate (ESR).

**Laboratory Studies**

At each visit blood was taken for routine hematology and biochemistry tests including hemoglobin, hematocrit, leucocyte count, platelets, ESR, urea and creatinine. One measurement of rheumatoid factor was made for each patient during the study. Samples for measurement of IL-1β, β estradiol and progesterone from each woman were processed and measured at the same time to minimise the effects of inter-assay variation (ref. Chapter 2, Materials and Methods, sections 2.1.1 and 2.1.2). Laboratory measurements as described above were also made for each member of the control group at three time-points within the cycle. Body weight was measured in these women at every assessment.

**Statistical Methods**

Previous studies (15,16) have treated the menstrual cycle as the unit of analysis and considered the total sample size to be the total number of menstrual cycles in all women. This may be misleading because different cycles in the same individual may show similar patterns and increase the significance of correlations found. An alternative is to consider the woman as the unit of analysis and calculate mean values at each phase of the cycle for all the cycles measured in that individual. The approach taken here, which takes into account the structure of the data, was to model the variation within and between women (multi-level modelling) (17). The analysis of the differences within and between groups used the programme P3V from the BMDP package (18). Some additional analyses were performed using the programme HLM, kindly made available by
Professor S Raudenbush of Michigan State University (19). The correlation of means for each variable at each time-point between individual women and “within cycle” analysis was performed by calculating rank correlations of patient means using the Spearman correlation coefficient.

3.4 RESULTS

Menstrual cyclicity of disease activity

One of the aims of this study was to determine whether clinical activity of RA was affected by the menstrual cycle. Table 1 shows mean values for clinical and laboratory variables in the RA patient group during each phase of the cycle. Units for Camp score, Ritchie score and pain score are arbitrary, grip strength was in mm Hg, morning stiffness in minutes and weight in kg. There was significant cyclical variability for the Camp score (p=0.03) (Fig. 1), pain score (p=0.04) and IDA (p=0.01). Similar trends were seen for Ritchie score (p=0.07) and grip strength (p=0.06), negative correlation. On each occasion, the lowest articular index, pain score and IDA and the highest grip strength occurred at the mid-follicular timepoint while the highest articular index, pain score, IDA and the lowest grip strength occurred during the late-luteal phase.

<table>
<thead>
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<th>LL</th>
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<td>12.6</td>
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<td>Ritchie</td>
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<td>5.6</td>
<td>7.7</td>
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</tr>
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<td>Pain</td>
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<td>3.1</td>
<td>3.8</td>
<td>0.04*</td>
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<tr>
<td>Grip strength</td>
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<td>119.1</td>
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<tr>
<td>Morning stiffness</td>
<td>20.3</td>
<td>36.6</td>
<td>57.3</td>
<td>0.01*</td>
</tr>
<tr>
<td>IDA</td>
<td>1.68</td>
<td>1.82</td>
<td>1.96</td>
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<td>Weight</td>
<td>62.96</td>
<td>63.00</td>
<td>63.25</td>
<td>0.12</td>
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Table 1. Mean values of clinical variables by phase of cycle: RA patients
Interestingly, laboratory measures of disease activity for the patient group including the ESR (mm/hr), platelet count (x10^9/L) and plasma IL-1β (pg/ml) did not change significantly throughout the cycle whereas the expected fluctuations in β oestradiol (pmol/L) and progesterone (nmol/L) were observed (Table 2). Hemoglobin (g/L) appeared to fall in the late-luteal phase but this was not significant (p=0.09). Body weight tended to increase during the late-luteal phase. The difference between the mid-follicular and the late-luteal mean weight was significant (p=0.02).

Table 3 shows clinical and laboratory data for controls revealing a significant cyclical fluctuation in weight (p=0.02) and as expected, cyclical fluctuations of progesterone and β oestradiol, both peaking in the mid-luteal phase and falling to their lowest point in the late-luteal phase (p<0.001).
### Table 2. Mean values of laboratory variables by phase of cycle: RA patients

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<tr>
<td>β oestradiol</td>
<td>346.5</td>
<td>527.7</td>
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<tr>
<td>Progesterone</td>
<td>2.00</td>
<td>38.43</td>
</tr>
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</table>

* p<0.05

### Table 3. Mean values by phase of cycle: Controls.

<table>
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<tr>
<th>Variables</th>
<th>Means by Phase of Cycle</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
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<td>ML</td>
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<tr>
<td>Weight</td>
<td>62.35</td>
<td>62.45</td>
</tr>
<tr>
<td>ESR</td>
<td>3.40</td>
<td>3.56</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>13.4</td>
<td>13.5</td>
</tr>
<tr>
<td>Platelets</td>
<td>294.1</td>
<td>274.1</td>
</tr>
<tr>
<td>Interleukin I β</td>
<td>57.0</td>
<td>57.0</td>
</tr>
<tr>
<td>β oestradiol</td>
<td>330.0</td>
<td>457.5</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.97</td>
<td>60.2</td>
</tr>
</tbody>
</table>

* p<0.05
Figure 2. shows data from one patient where cyclicity of clinical joint inflammation was clearly observed with rising articular indices, pain score and IDA in the late-luteal phase, falling again to their lowest points during the mid-follicular phase. Fluctuation of laboratory parameters including ESR, Hb, platelet count and IL-1β did not show predictable cyclicity.

To test whether late-luteal fluid retention contributed to cyclical changes in clinical variables, the multi-level analyses for Camp score, Ritchie score, pain score, IDA, grip strength and hemoglobin were repeated with weight included as a covariant. Adjusting for weight made very little difference to the cyclical fluctuation of variables apart from hemoglobin where the difference between the phases of the menstrual cycle were reduced suggesting that at least some of the late-luteal fall in hemoglobin was attributable to fluid retention.

A comparison between levels of IL-1β at each of the three phases for RA patients and controls is
presented in Figure 3. Mean levels were significantly higher in patients compared with controls when measured at all 3 phases (p<0.05). However no significant differences were found between phases for patients or controls and in fact levels remained remarkably constant for each individual studied.

![Graph showing mean plasma IL-1β levels in 3 phases of menstrual cycle for RA Patients and Controls.](image)

**Figure 3.** Mean plasma IL-1β in 3 phases of menstrual cycle for patients and controls.

**Correlation between plasma IL-1β and disease activity**

Mean levels of Camp score, Ritchie score, pain score, duration of morning stiffness, grip strength, index of disease activity, ESR, hemoglobin and platelets were compared with IL-1β levels for each phase of the menstrual cycle. A positive correlation was found in the late-luteal phase between IL-1β and Ritchie index (p=0.04), pain score (p=0.04) and index of disease activity (p=0.04).
Duration of morning stiffness and Camp articular index showed similar trends but were not statistically significant (p=0.06 and p=0.16 respectively). The same comparisons were made during the mid-luteal and mid-follicular phases but no correlations were found between any measure of disease activity and IL-1β activity at these phases of the cycle. Figure 4, depicts the correlations between Ritchie score and IL-1β during the late luteal phase compared with the mid-follicular phase for the RA patient group.

![Graph](image)

**Figure 4.** RA patient group: Ritchie score correlates with IL-1β levels during the late-luteal phase (left) but not during the mid-follicular phase (right).

**Correlation between plasma IL-1β and the degree of cyclicity of disease activity**

The difference between mean IDA at the mid-follicular phase and mean IDA at the late-luteal phase for any individual is a measure of cyclicity of disease activity. This change in IDA correlated significantly (p=0.004, R=0.77) with the mean plasma IL-1β concentration in the RA patient group (Fig. 5) and also with ESR (p=0.02). There was a negative correlation with hemoglobin (p=0.008). Thus those individuals with the greatest degree of cyclicity of disease activity also had
the greatest disease activity, as assessed by laboratory measures including plasma IL-1β.

Hormonal profiles

Hormonal profiles for patients and controls revealed a consistent pattern. In all cycles there was a marked mid-luteal progesterone surge showing that ovulation had occurred. There was also a marked mid-luteal rise in β estradiol with a lower late-luteal value. There was no difference in hormonal pattern between patients and controls. When these cyclical fluctuations of hormones were examined with the cyclical fluctuations of disease activity, it was apparent that the maximum index of disease activity (during the late-luteal phase) occurred when both progesterone and beta estradiol levels were falling (see Figure 7). However, there was no correlation between levels of sex hormones and disease activity as assessed by any of the parameters measured at any phase of the cycle. It may be that the rate of fall of β estradiol could influence the clinical level of disease activity during the late-luteal phase but this could not be adequately assessed using the data available. To answer this question, daily assessments of disease activity and hormone levels

![Figure 5. Change in IDA (LL-MF) correlates with mean IL-1β levels (p=0.004).](image-url)
would be required during the last week of the cycle and was beyond the scope of this study.

![Graph showing IDA, β estradiol, and progesterone levels vs cycle phase for individual RA patients](image)

**Figure 6.** Mean levels of IDA, β estradiol and progesterone vs phase of menstrual cycle for 4 individual RA patients (designated A, B, C and D).

### 3.5 DISCUSSION

This study was designed to answer two questions about the pattern of disease activity in pre-menopausal women with RA. The first was whether there was any variation of disease activity related to the menstrual cycle. Three previous studies have addressed this question. One reported a series of 14 women assessed over an average of 4.9 menstrual cycles and suggested an increase in disease activity during the pre-ovulatory phase compared with the post-ovulatory phase when the patients' assessments of pain score and morning stiffness were lowest (15). In contrast, results from the present study indicated that the time of maximum symptoms and the highest articular score was the late-luteal phase during the last 3 days of one cycle and the first 3 days of the next. In the earlier study, this period was divided between the post-ovulatory and pre-
ovulatory groups, making comparison between the two difficult.

Our findings are in agreement with other studies. Rudge and Kowanko (6), analysing 2 menstrual cycles in 7 female RA patients found the lowest grip strength and maximum finger joint swelling at 28 days corresponding to the mid-point of our late-luteal phase. Goldstein (16) reported a study of menstrual cyclicity in RA where increased disease activity occurred during the post-ovulatory phase. This was interpreted as being a manifestation of the "pre-menstrual syndrome" and therefore unrelated to increased rheumatoid inflammation.

In the present study, clinical assessment was made by an investigator who was unaware of the phase of the patient's menstrual cycle. Results showed statistically significant variations through the menstrual cycle for Camp score, pain score, morning stiffness and index of disease activity (Mallya). Ritchie index and grip strength showed the same trend (p=0.07 and 0.06 respectively). Clinical activity of disease was lowest during the mid-follicular phase, greatest during the late-luteal phase and intermediate at the mid-luteal phase. It should be noted that two of the other studies cited above (15,16) used the menstrual cycle as the unit of analysis, which as already described may lead to different results.

It has been argued that pre-menstrual weight gain might be the basis for apparent cyclicity of clinical disease activity and previous authors have attempted to correct for this variable (6). We found evidence for late-luteal weight gain in cases and controls, but it was possible to control for this during analysis of results. When this was done, the significance of the cyclical fluctuation observed in disease activity was not diminished implying that pre-menstrual weight gain was not the basis for cyclicity. Although we found a statistically significant fluctuation of clinical activity through the menstrual cycle in the patient group as a whole, those patients with the most active disease demonstrated the greatest cyclicity. Conversely, the patients within the group who could be classified as having inactive disease remained inactive during all the phases of the cycle.
There are many reports on the influence of sex hormones on the immune system in general and specifically on the course of many rheumatic diseases such as SLE and RA. Hench (1) in the 1930's observed improvement in many RA patients during pregnancy and more recently this has been shown to occur in up to 75% of patients (2,20). Surveillance of combined oral contraceptive usage in the general population has revealed that RA develops less frequently in women taking these drugs than non-users (3), suggesting a protective effect of the estrogen/progesterone preparation. A clinical trial investigating the effect of low dose estrogen therapy in RA found some improvement in certain indices of disease activity during hormone therapy over a twelve week period but the effect was not pronounced and the numbers small.

Plasma interleukin 1 beta levels have been shown to be related to clinical and immunological indices of disease activity in RA (8,22,23). We have found that immunoreactive levels of IL-1β remain constant through the menstrual cycle despite evidence of fluctuation of disease activity using clinical parameters in many patients. Interestingly, for this patient group, IL-1β levels only correlated with clinical disease activity during the late-luteal phase of the cycle when symptoms were most severe. One explanation for this could be that falling levels of estrogen may augment biological effects of IL-1β, while high or increasing estrogen levels as are found during the mid-follicular and mid-luteal phases may reduce the biological activity of IL-1β. This latter effect could be mediated by an estrogen-induced IL-1 inhibitor or by estrogen modulation of IL-1 receptors on target cells.

Disparity between cyclicity in clinical and and laboratory/immunological features might be interpreted as a fluctuation in the patients' perception of symptoms independent of disease activity. Appreciation of pain influences all the clinical variables measured here apart from morning stiffness and joint swelling. IL-1β has recently been shown to be a very powerful hyperalgesic agent in animals with as little as 0.05pg injected systemically producing an augmented withdrawal response.
to foot pad pressure (24). We have also previously shown (8) a significant correlation between pain score and plasma IL-1β levels in RA patients and this was confirmed in the present study during the late-luteal phase (p=0.04).

Little is known regarding the interaction between IL-1β and sex hormones. An early report (10) investigating 5 normal women found IL-1β levels to be consistently high during the luteal phase compared to the follicular phase of the menstrual cycle but in that study IL-1β was measured indirectly by its ability to reduce serum Fe and Zn concentration and produce fever in experimental animals. Our results using an ELISA measuring immunoreactive IL-1β directly in extracted plasma did not show any such fluctuation in the control or RA group and in fact levels within each individual remained remarkably consistent.

In conclusion, our results reveal definite menstrual cyclicity of several conventional clinical measures of disease activity in RA and have shown that the degree of cyclicity correlates with overall disease activity. Laboratory variables including plasma IL-1β levels did not show such cyclicity. Changes in pain perception could be postulated to explain this apparent disparity between clinical and laboratory data. However, plasma IL-1β did correlate with disease activity during the late-luteal phase when estrogen and progesterone levels were falling. This raises the possibility that the biological activities of inflammatory cytokines in vivo may be modulated by sex hormones.

Acknowledgements

We are indebted to the following physicians who allowed us to study their RA patients: Professor G Nuki, Dr TM Chalmers and Dr J N McCormick.
Phases of the Menstrual cycle

<table>
<thead>
<tr>
<th>Phase</th>
<th>Definition</th>
<th>28 day cycle</th>
<th>26 day cycle</th>
<th>29 day cycle</th>
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<tr>
<td>DO (Day of ovulation)</td>
<td>Previous cycle length - 14</td>
<td>14</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>MF (midfollicular)</td>
<td>DO/2</td>
<td>7</td>
<td>6</td>
<td>7.5</td>
</tr>
<tr>
<td>Range</td>
<td>MF-2 to MF+3</td>
<td>5 - 10</td>
<td>4 - 9</td>
<td>5 - 10</td>
</tr>
<tr>
<td>ML (midluteal)</td>
<td>cycle length - 7</td>
<td>21</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Range</td>
<td>ML-2 to ML+3</td>
<td>19 - 24</td>
<td>17 - 22</td>
<td>20 - 25</td>
</tr>
<tr>
<td>LL (luteal)</td>
<td>cycle length</td>
<td>28</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>Range</td>
<td>LL-2 to LL+3</td>
<td>26 - 3</td>
<td>24 - 3</td>
<td>27 - 3</td>
</tr>
</tbody>
</table>

A six day window is allowed for each of the three phases of the menstrual cycle.

Every woman had at least one assessment in each of the three phases made for all variables tested.

Of the 190 occasions on which cases and controls were seen, 177 of them were captured in one of the three windows as follows:

<table>
<thead>
<tr>
<th>Phase</th>
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</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
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<tr>
<td>ML</td>
<td>58</td>
</tr>
<tr>
<td>LL</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>177</td>
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</table>
3.7 REFERENCES


19) Raudenbush S, Bryk AS: A hierarchical model for studying school effects. Soc of Education 1986;59,1-17


CHAPTER FOUR

γδ+ T CELLS FROM PATIENTS WITH PSORIATIC AND RHEUMATOID ARTHRITIS
RESPOND TO STREPTOCOCCAL ANTIGEN

F M GRINLINTON
M A SKINNER
N M BIRCHALL
P L J TAN

DEPARTMENT OF MOLECULAR MEDICINE
AUCKLAND SCHOOL OF MEDICINE
AND DEPARTMENT OF RHEUMATOLOGY
AUCKLAND HOSPITAL
4.1 ABSTRACT

Objective: To investigate cellular immune responses to streptococcal antigens in patients with psoriatic arthritis (PsA). To specifically examine responses of the γδ+ T cell subset.

Methods: Proliferation of PsA synovial fluid lymphocytes (SFL) and peripheral blood lymphocytes (PBL) cultured with streptococcal antigen was measured using a 3HThymidine (3HTdr) uptake assay system. γδ+ T cells from PsA PBL and SFL were phenotyped by flow cytometry. Following culture with streptococcal antigen, γδ+ enriched SFL were sorted by automated flow cytometry and 3HTdr uptake measured.

Results: PsA patient and control groups did not differ significantly in their PBL responses to 2 strains of streptococci, one of which was isolated from a patient with guttate psoriasis (Strep 1) and the other from a rheumatic fever patient (Strep 2). There was also no difference in their responses to a cell wall preparation derived from the former strain. SFL from 8 of 9 PsA patients responded to both streptococcal strains as did SFL from 3 RA patients. γδ+ SFL from 7 PsA and 3 RA patients responded only to the psoriasis-associated strain.

Conclusions: PsA PBL and SFL responded to stimulation by streptococcal antigen but this reactivity was not disease-specific. We have demonstrated that γδ+ T cells from PsA SF proliferated when cultured with a psoriasis-associated strain of streptococcus (Strep 1). However RA γδ+ SFL responded similarly suggesting that γδ+ T cell reactivity to streptococcal antigen may be a feature of inflammatory arthritis.
4.2 INTRODUCTION

Psoriatic arthritis (PsA) is generally reported to occur in 5-10% of patients with psoriasis (1) but a recent study suggests the prevalence may be as high as 21% (2). While the etiology of psoriasis remains poorly understood, there is evidence to suggest that it is an immunologically mediated disorder (3). The psoriatic plaque is characterised by hyperproliferation of keratinocytes in close spatial proximity to T cells, monocyte/macrophages and dermal dendritic cells, all of which contribute to the release of chemotactic and proinflammatory cytokines. These findings bear similarities to the inflammatory changes within the synovium in PsA. Furthermore increased keratinocyte HLA-DR expression has been shown to correlate with the presence of arthritis in a group of psoriatic patients (4).

While the factors causing skin and joint lesions remain unclear, clinical and radiological similarities between PsA and Reiter's syndrome suggest that both forms of arthritis share common mechanisms of pathogenesis (5). PsA could be regarded as a form of "reactive" arthritis triggered by antigenic components of bacteria resident on the psoriatic plaque. In support of this proposal is the clinical observation that acute guttate psoriasis often follows a β haemolytic streptococcal throat infection after an interval of 10-14 days (6). An exacerbation of psoriasis has also been noted to occur at the time of concurrent streptococcal infection. Enhanced humoral immunity to streptococcal exotoxins has been reported in PsA patients (7) but cellular immune responses to streptococci have only been studied in a small number of patients (8).

Our aim was to investigate proliferative responses of peripheral blood lymphocytes (PBL) and synovial fluid lymphocytes (SFL) from PsA patients to streptococcal antigens. We observed that a subset of CD3+ synovial lymphocytes bearing the γδ T cell receptor (TCR) responded to a whole-cell streptococcal sonicate derived from a patient with guttate psoriasis. Proliferation of γδ+ T cells from PsA and RA patients to streptococcal preparations have been further examined.
4.3 PATIENTS AND CLINICAL METHODS

21 patients with PsA as defined by the criteria of Moll and Wright (1) and 15 age-matched healthy controls were entered into the study. 15 PsA patients provided PBL samples and in 9 patients with chronic knee effusions, SFL samples were obtained. Patients were selected from a hospital outpatient rheumatology clinic. Informed consent was obtained from all patients and this study was approved by the Auckland Area Hospitals Research Ethics Committee.

For each patient a brief history was taken and clinical examination performed by one of the investigators (SY or FG) to assess activity of arthritis. Duration of skin and joint disease was recorded and an assessment made of disease distribution and activity. Activity of skin disease was graded using a PASI score (Psoriasis Activity and Severity Index) (9). Activity of arthritis was graded using the Ritchie score (10) and visual analogue pain score. For comparative whole SFL and γδ+ SFL proliferative assays, SF was obtained from 3 seropositive RA patients. Demographic characteristics of PsA patients and controls are presented in Table 1.

Laboratory Studies

An ESR was performed on all patients on entry into the study as an objective indicator of inflammatory activity (Westergren method).

Immunological studies

See Materials and Methods, Chapter 2, Sections 2.2.1 to 2.2.5

Statistical Analyses

For analysis of PBL proliferation assays, the SAS programming language, General Linear Models Procedure (mixed models) was used (11). For comparison of clinical parameters with PBL proliferation in PsA patients, Spearman’s correlation coefficients (12) were determined as data were nonparametric. The Wilcoxon signed ranks test (13) was used to analyze flow cytometry staining profiles of paired peripheral blood (PB) and synovial fluid (SF) data.
<table>
<thead>
<tr>
<th></th>
<th>PsA patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>M/F</td>
<td>7/14</td>
<td>5/10</td>
</tr>
<tr>
<td>Age (yrs)*</td>
<td>37.1 (22-67)</td>
<td>36.1 (21-60)</td>
</tr>
<tr>
<td>Disease Duration (yrs)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>arthritis</td>
<td>7.5 (0.1-27)</td>
<td></td>
</tr>
<tr>
<td>psoriasis</td>
<td>12.0 (0.1-30)</td>
<td></td>
</tr>
<tr>
<td>Disease Activity*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritchie Score</td>
<td>4.5 (0-16)</td>
<td></td>
</tr>
<tr>
<td>Pain Score</td>
<td>3.9 (0-9.2)</td>
<td></td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>15 (3-74)</td>
<td></td>
</tr>
<tr>
<td>PASI Score</td>
<td>1.2 (0-18)</td>
<td></td>
</tr>
<tr>
<td>Drug Treatment</td>
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</tr>
<tr>
<td>None</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>NSAID alone</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>NSAID + 2nd line agent **</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Disease Distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assymetric oligoarticular</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Assymetric polyarticular</td>
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</tr>
<tr>
<td>Symmetric polyarticular</td>
<td>1</td>
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</tr>
</tbody>
</table>

* Expressed as median (range)

** Salazopyrin 500-2000mg/day in 3 patients, Azathioprine 100mg/day and prednisone 5mg/day in 1 patient

Table 1. Characteristics of PsA and control populations
4.4 RESULTS

PBL and SFL responses to streptococcal antigens

PBL obtained from 15 patients with PsA and 15 controls were cultured with Strep 1, Strep 2 and SCW streptococcal preparations (Ref. Materials and Methods, Section 2.2.2). The proliferative responses to these 3 particulate antigens were not significantly different between PsA patients and controls (p>0.05) (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Strep 1</th>
<th>Strep 2</th>
<th>SCW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control PBL</strong></td>
<td>(15)</td>
<td>(15)</td>
<td>(12)</td>
</tr>
<tr>
<td>SI (median)</td>
<td>8.5</td>
<td>11.6</td>
<td>1.8</td>
</tr>
<tr>
<td>range</td>
<td>0.5-40.2</td>
<td>4.3-132.0</td>
<td>0.3-10.3</td>
</tr>
<tr>
<td><strong>PsA PBL</strong></td>
<td>(15)</td>
<td>(15)</td>
<td>(15)</td>
</tr>
<tr>
<td>SI (median)</td>
<td>10.8</td>
<td>19.7</td>
<td>3.8</td>
</tr>
<tr>
<td>range</td>
<td>1.4-159.0</td>
<td>3.5-391.0</td>
<td>0.6-13.8</td>
</tr>
</tbody>
</table>

Table 2. Proliferative responses of PsA and control PBL to streptococcal antigens. Stimulation index (SI) for cultures pulsed with antigen is given as a median and a range.

In both groups proliferation to the SCW antigen was significantly less than the response to the whole-cell sonicates (p<0.05). There was no correlation between clinical indices of disease activity and proliferative responses to any of the antigens in the patient group. To determine whether SFL were more responsive to streptococcal antigen than PBL, 6 paired PsA PBL/SFL samples were obtained (Table 3). In 3 of 6 pairs, PBL responses exceeded those of SFL implying no special sensitization of SFL to streptococcal antigen in this group of patients.
From a total of 9 PsA patients were examined (Table 4). In 8 samples, PsA SFL responded to both Strep 1 and Strep 2, whereas in one sample, SFL responded to Strep 2 alone. Because SFL samples were not available from healthy individuals, SFL from 3 RA patients were studied for comparison.
As observed for psA sFL, the 3 RA SFL samples also responded to both whole-cell streptococcal sonicates. Proliferation to SCW antigen was minimal in the 3 PsA SFL tested. To demonstrate that the appropriate antigen dose was used, Figure 1 shows the dose-response curve of SFL from one PsA patient (MH). Each point on the graph represents the mean of triplicate cultures (SD < 20%). This pattern of SFL responses to both whole streptococcal sonicates (maximal at 2 - 10μg/ml) but not to the SCW preparation, was representative of SFL responses in PsA patients.

<table>
<thead>
<tr>
<th></th>
<th>Strep 1</th>
<th>Strep 2</th>
<th>SCW</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsA SFL</td>
<td>(n)</td>
<td>(9)</td>
<td>(9)</td>
</tr>
<tr>
<td>SI(median)</td>
<td>11.6</td>
<td>6.2</td>
<td>0.7</td>
</tr>
<tr>
<td>range</td>
<td>1.6-24.7</td>
<td>3.5-33.6</td>
<td>0.7-4.0</td>
</tr>
<tr>
<td>RA SFL</td>
<td>(n)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>SI(median)</td>
<td>14.7</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>7.7-30.5</td>
<td>2.6-11.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Proliferative responses of PsA and RA SFL to streptococcal antigens

As observed for PsA SFL, the 3 RA SFL samples also responded to both whole-cell streptococcal sonicates. Proliferation to SCW antigen was minimal in the 3 PsA SFL tested. To demonstrate that the appropriate antigen dose was used, Figure 1 shows the dose-response curve of SFL from one PsA patient (MH). Each point on the graph represents the mean of triplicate cultures (SD < 20%). This pattern of SFL responses to both whole streptococcal sonicates (maximal at 2 - 10μg/ml) but not to the SCW preparation, was representative of SFL responses in PsA patients.

Flow cytometry of SFL after culture reveals proliferation of γδ+ cells to Strep 1

Although no differences were observed between the responses of whole SFL to Strep 1 and to Strep 2, we sought to determine whether subsets of lymphocytes exhibited a preferential response to streptococcal antigen. Following culture for 7 days with Strep 1 or Strep 2, SFL from PsA patient AB were phenotyped using flow cytometry (Fig. 2). FITC-labelled γδ+ mab staining is shown overlaid on cells stained with IgG-1FITC. The gate was set at channel No. 120. The proportion of γδ+ cells increased from 2.4% of CD3+ cells before culture to 6.5% after culture with Strep 1 (2μg/ml). In contrast no γδ+ T cells were detected after culture with Strep 2 (2μg/ml).
Figure 1. PsA patient MH, SFL: Dose response curves to streptococcal antigens.

Figure 2. FACS analysis of SFL from PsA patient AB. a) Before culture: 2.4% γδ+ cells  
b) After culture with Strep 1: 6.5% γδ+ cells  
c) After culture with Strep 2: 0% γδ+ cells.
In PsA patient MH, SFL were sorted using the flow cytometer into a population enriched for γδ+ T cells and these cells were then cultured in IL-2 (100ng/ml) for 48 hrs. A scanning electron micrograph of a cell obtained from this culture revealed structural characteristics of the TCS1 subset of γδ+ T cells including uropod formation (14)(Figure 3).

**Figure 3.** Scanning electron micrograph of a γδ+ T cell isolated from SF of PsA patient MH. Original magnification 4433x

**Phenotypic characterisation of SFL and PBL γδ+ T cells**

SFL and PBL obtained concurrently from 7 PsA patients with chronic knee effusions were analyzed using double labelling with mab and flow cytometry. As shown in Table 5, γδ+ T cells comprised a similar proportion of CD3+ cells in SFL (1.3 - 7.1%) and PBL (1.0 - 7.6%). A variable proportion of SFL γδ+ cells were CD8+ (7.0 - 62.5%). Subpopulations of γδ+ T cells expressing δ1 and δ2 chains were identified using the monoclonal antibodies BB3 and A13.
<table>
<thead>
<tr>
<th>PsA patients</th>
<th>sample</th>
<th>γδ/CD3</th>
<th>γδ/CD8</th>
<th>BB3/CD3</th>
<th>A13/CD3</th>
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</thead>
<tbody>
<tr>
<td>LR SFL</td>
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<td>0.5</td>
<td>2.4</td>
<td>2.2</td>
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<tr>
<td>LR PBL</td>
<td>4.8</td>
<td>0</td>
<td>4.6</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>JS SFL</td>
<td>6.2</td>
<td>3.6</td>
<td>1.1</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>JS PBL</td>
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<td>0.8</td>
<td>0.5</td>
<td>1.1</td>
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<tr>
<td>AB SFL</td>
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<td>1.8</td>
<td>7.0</td>
<td>1.8</td>
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</tr>
<tr>
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<td>3.4</td>
<td>0.4</td>
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</tr>
<tr>
<td>DJ SFL</td>
<td>4.1</td>
<td>1.9</td>
<td>2.5</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>DJ PBL</td>
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<td>1.3</td>
<td>2.3</td>
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<td></td>
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<tr>
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<tr>
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<td>0.8</td>
<td>3.5</td>
<td>1.6</td>
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</tr>
<tr>
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<td>1.9</td>
<td>6.6</td>
<td>3.0</td>
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<td>1.7</td>
<td>8.5</td>
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<tr>
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<tr>
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<td>0.4</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

* Double staining expressed as %CD3+ cells. ND: not done.

**Table 5** Flow cytometry of SFL and PBL from PsA patients
respectively (Chapter 2, Ref 5). There was no significant predominance of either subtype in either PBL or SFL but small numbers precluded accurate statistical analysis.

**Proliferation of γδ+ T cells after culture with streptococcal antigen**

Following culture for 7 days with streptococcal antigen, SFL from 7 PsA patients were phentyped. The median percentage population of γδ+ cells after culture with no antigen was 2.7% (0.6 - 14.0%) compared with 3.0% (1.4 - 15.0%) after exposure to Strep 1 and 1.1% (0 - 10.5%) after culture with Strep 2. Moreover in 3 of 7 SFL no γδ+ staining was detected after culture with Strep 2 suggesting a different response of the γδ+ subset to the 2 streptococcal strains.

As the γδ+ T cell subset constituted a small fraction of total SFL, percentage changes in this population were below the limits of resolution by flow cytometry. Therefore to confirm the differential effects of Strep 1 and Strep 2 on PsA γδ+ SFL, we assessed antigen-induced stimulation of this cell population using a modification of the 3HThd uptake assay (Ref. Materials and Methods, Section 2.2.3). In brief, SFL from PsA patients were cultured with streptococcal antigen, pulsed with 3HThd and then processed by the fluorescence activated cell sorter to obtain an enriched γδ+ fraction.

Figure 4 compares the response of the γδ+ fraction to Strep 1 and Strep 2. There was marked proliferation of γδ+ SFL cultured with Strep 1 in samples from all patients (Δcpm/10^5 cells: 2579 - 65992) (also shown in Table 6). Responses to Strep 2 in 4 patients were minimal (Δcpm/10^5 cells: 441 - 7820) and in 3 patients there was insufficient staining with γδ mab to obtain an enriched γδ+ fraction precluding further analysis. The pattern of γδ- proliferation (not shown) mirrored that of unsorted cells with no significant difference in response to Strep 1 and Strep 2. Stimulation indices to Strep 1 for γδ- T cells ranged from 1.1 - 15.1, median 2.8 (Δcpm/10^5 cells: 2321 - 22360, 3953) and to Strep 2 from 2.1 - 15.8, median 2.2 (Δcpm/10^5 cells: 3635 - 5390, 4521).
A comparison with γδ+ SFL from patients with rheumatoid arthritis is shown in Table 6. These cells also proliferated in response to Strep 1 (Δcpm/10⁵ cells: 1360 - 5270) but not to Strep 2 (Δcpm not detectable).

To determine whether SCW antigens would stimulate γδ+ T cell proliferation, SFL from 2 PsA patients were cultured with SCW. γδ+ SFL from patient MH did not proliferate in response to this antigen. SFL from patient LR responded only minimally (Δcpm/10⁵ cells: 672) despite a dramatic response (Δcpm/10⁵ cells: 65,019) to Strep 1 (the whole-organism streptococcal preparation from which SCW was derived). This suggests that the component of Strep 1 responsible for its antigenicity to γδ+ T cells does not reside in the cell wall.
<table>
<thead>
<tr>
<th>PsA patients</th>
<th>Control (%γδ+)</th>
<th>Strep 1 (%γδ+)</th>
<th>Strep 2 (%γδ+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH</td>
<td>915 (17.8%)</td>
<td>8136 (30.5%)</td>
<td>1665 (17.4%)</td>
</tr>
<tr>
<td>StC</td>
<td>3762 (76.9%)</td>
<td>25640 (85.3%)</td>
<td>7820 (72.9%)</td>
</tr>
<tr>
<td>SaC</td>
<td>2380 (50.0%)</td>
<td>27252 (81.0%)</td>
<td>3077 (60.0%)</td>
</tr>
<tr>
<td>JS</td>
<td>510 (30.3%)</td>
<td>2706 (23.1%)</td>
<td>441 (8.8%)</td>
</tr>
<tr>
<td>AB</td>
<td>800 (21.4%)</td>
<td>17240 (64.5%)</td>
<td>0 (0%)**</td>
</tr>
<tr>
<td>TW</td>
<td>474 (42.0%)</td>
<td>2579 (53.8%)</td>
<td>0 (7.7%)**</td>
</tr>
<tr>
<td>LR</td>
<td>903 (28.6%)</td>
<td>65922 (41.4%)</td>
<td>0 (0%)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>694 (62.2%)</td>
<td>3116 (77.5%)</td>
<td>0 (0%)**</td>
</tr>
<tr>
<td>MB</td>
<td>0 (0%)**</td>
<td>1360 (76.1%)</td>
<td>0 (0%)**</td>
</tr>
<tr>
<td>MH</td>
<td>169 (46.4%)</td>
<td>5270 (38.7%)</td>
<td>0 (0%)**</td>
</tr>
</tbody>
</table>

*Percentage enrichment of γδ+ fraction
**Insufficient +ve γδ staining to sort.

Table 6. Proliferation of SFL γδ+ cells to streptococcal antigens in PsA and RA patients as determined by ³HThd uptake (cpm/10⁵ cells) of the γδ+ fraction after 7 days culture.

**DISCUSSION**

Streptococcal antigens may be important factors in the pathogenesis of PsA. Gross et al (15) studied PBL from patients with psoriasis alone and reported specific cell mediated immune responses to Group A streptococcal cell wall and membrane antigens in leucocyte migration inhibition assays. Additional studies have examined the humoral response to streptococcal
Antigens in PsA. Antibodies to the streptococcal exotoxin anti-DNA aseB are found at higher levels in PsA patients than in controls (7) leading Vasey et al to propose that PsA represents a form of reactive arthritis triggered by the presence or persistence of bacterial antigen. We have studied proliferative T lymphocyte responses to streptococcal antigen in PsA. Using SFL and PBL from PsA patients we have detected proliferative responses within the whole lymphocyte population to the streptococcal antigens tested which included a preparation derived from an organism originally isolated from a patient with guttate psoriasis. However, these responses were also detectable in PBL from controls and in SFL from RA patients.

Ford (16) proposed that the specificity of SFL proliferative responses in reactive arthritis may identify micro-organisms responsible for disease pathogenesis. Others have cautioned the interpretation of SFL responses as antigen-specific mononuclear cells may be recruited from the periphery into the joint after any recent infection (17). Sheldon (8) noted stimulation of SFL from patients with different forms of inflammatory arthritis, including two with PsA, to multiple strains of streptococcus suggesting a nonspecific response. Responses to streptococcal antigen may contribute to promoting joint inflammation in inflammatory arthropathies as epitopes shared between streptococcal M protein and joint tissue have been described (18). We did not establish differences between unfractionated PsA lymphocyte responses to streptococci isolated from a psoriatic patient (Strep 1) and responses to streptococci isolated from a rheumatic fever patient (Strep 2). However whole SFL represents a heterogeneous population and our analysis of the γδ+ T cell subset did reveal a difference between the two strains of streptococci.

It has been proposed that peptidoglycan, a component of the bacterial cell wall, may constitute the "arthritogenic factor" in PsA (19). Patients with PsA have elevated levels of antibodies to a synthetic peptide with the same sequence as the immunodominant moiety of peptidoglycan (20). These antipeptidoglycan antibodies are also elevated in patients with Reiter's syndrome and ankylosing spondylitis (19). Interestingly our results show that cellular proliferative responses to
the SCW antigen were consistently low in all groups suggesting that the stimulatory antigen present in the whole-organism preparation was eliminated during preparation of the SCW material. Thus, the peptidoglycan component of the micro-organism which remains intact in the SCW preparation, seems unlikely to be responsible for the T cell responses observed.

The main observation of our study was that a rare subpopulation of T cells with γδ receptors were responsive to Strep 1 but not Strep 2. As the percentages of γδ+ T cells were low, changes in their frequency before and after streptococcal stimulation were too small to be reliably detected by flow cytometry. We were able to confirm the responsiveness of γδ+ SFL to Strep 1 but not Strep 2 by measuring their thymidine uptake following isolation by cell sorting.

What is the relevance of γδ+ T cells in PsA? These cells represent 1-15% of normal PBL (21). We have shown them to be present in PsA SF at 3.2-7.1% and in PsA PBL at 1.2-7.6% of CD3+ cells. These frequencies are similar to those reported for PBL and SFL from patients with RA and other inflammatory arthropathies (22). Despite their low frequency, γδ+ cells are thought to participate in inflammation (23,24). The chronically inflamed joint in PsA is often clinically and histologically indistinguishable from that of RA suggesting convergence of disease mechanisms to a final common pathway of chronic inflammation. γδ+ T cells have been shown to proliferate in response to heat shock proteins and it has been proposed that they may play a role in immune surveillance by eliminating stressed autologous cells (25). Heat shock proteins are highly homologous between species and responses to the mycobacterial 65kD protein have been observed in SFL from patients with Reiter’s syndrome and RA (26). Whether the γδ+ T cells present in PsA SF contribute to inflammation via hsp stimulation remains speculative.

Responsiveness of γδ+ T cells to streptococci has not been reported previously. Data in this report indicates that further characterisation of streptococcal sonicates may provide clues to the nature of antigen/s which activate γδ+ T cells in inflammatory arthritis.
ACKNOWLEDGMENTS

We are grateful to Mr Stephen Edgar, Senior Technical Officer in Electron Microscopy, Dept of Pathology, Auckland School of Medicine for preparing electron micrographs, Ms Elizabeth Robinson, Statistician, who has assisted with statistical analyses and Mrs Sue Yeoman who has provided invaluable help with collection of samples and clinical data. We also acknowledge the technical assistance of Ms Serey Kong and thank the following physicians who referred the patients enrolled in this study: Dr D Caughey, Dr R Grigor, Dr N Lynch, Dr H Hart, Dr M Butler, Dr P Gow.
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CHAPTER FIVE

NATURAL KILLER CELL FUNCTION AND RESPONSE TO CYTOKINES AND PROSTAGLANDINS IN PSORIATIC ARTHRITIS

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M A SKINNER
GW KRISANSEN
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P L J TAN

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AND DEPARTMENT OF RHEUMATOLOGY
AUCKLAND HOSPITAL
5.1 ABSTRACT

Objective: To examine the cytotoxic activity of natural killer (NK) cells from peripheral blood (PB) and synovial fluid (SF) of patients with psoriatic arthritis (PsA). The influence of selected inflammatory mediators on the cytolytic function of NK cells was also studied.

Methods: Paired samples of PB and SF lymphocytes (PBL and SFL) were obtained from 8 PsA patients for comparison of NK activity between PBL and SFL using a Cr\textsuperscript{51} release assay on tumor target cell lines. NK activity was examined in fresh samples and after incubation for 7 days with interleukin 2 (IL2), interleukin 6 (IL6), prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and indomethacin. In 5 patients, flow cytometry was used to determine the percentage of SF cells bearing the NK cell marker, NKH-1. The association between the proportion of NK cells present on flow cytometry and NK functional activity of synovial fluid was examined. The proportion of monocytes in SF samples was also determined by flow cytometry using a monoclonal antibody to the surface marker, CD14.

Results: NK cytolytic activity of PB samples was significantly greater than paired SF (p=0.015). SF NK activity was enhanced by overnight culture with IL-2 (p<0.05). A trend towards reduction of NK activity by PGE\textsubscript{2} was noted (p=0.06) whereas IL-6 and indomethacin had no significant effect. NK activity did not correlate with the percentage of NK cells in PB or SF. However, all SF samples contained a greater proportion of monocytes than PB samples.

Conclusions: NK activity of PsA SF was lower than that of paired samples of PB. NK activity could be enhanced by culture with IL-2 and reduced by PGE\textsubscript{2}. No correlation was found between the population of NK cells and NK activity. It is proposed that cytolytic activity of fresh NK cells may be influenced by monocyte-derived PGE\textsubscript{2}. 
5.2 INTRODUCTION

Cells with cytolytic activity are present within SF obtained from inflamed joints of patients with PsA (1). These include NK cells, a subset of lymphocytes which are capable of spontaneous cytotoxic activity against neoplastic cells and cells infected with viruses (2). NK activity is not MHC-restricted and does not require prior sensitisation. When activated these cells are capable of releasing pre-formed granules containing perforins and serine esterases which permit osmotic lysis of target cells (3). In addition they secrete soluble toxins including tumor necrosis factor (TNF) which mediate target cell death by apoptosis (4). It has been proposed that these cells may play a role in the immunoregulatory disorder promoting chronic synovitis in rheumatoid arthritis (RA) and PsA both via direct cytotoxic effects on cells of cartilage and bone (5) and by indirect effects such as the suppression of immunoglobulin production (6).

NK activity may be detected in SF and PB from patients with RA but at lower levels than in PB from healthy persons (7). Some reports have shown NK activity of SF from RA patients to be lower than that of matched RA PB samples (8) but other studies have provided evidence to the contrary (9). Studies in PsA have mostly shown that NK function is reduced within the synovial fluid compartment being equal to or lower than that of matched PBL (1,10). The reason for reduced NK activity in SF from patients with inflammatory arthritis has not been elucidated. However, the proliferation of NK cells and their cytolytic activity are known to be modified by cytokines including IL-2 and IL-6 (11) as well as prostaglandins (12). These mediators are frequently released at sites of inflammation and could potentially influence NK activity in vivo in patients with active synovitis.

We have compared NK activity of SF from PsA patients with that of paired PBL and have examined the effects of exogenous cytokines and prostaglandins on the function of SF NK cells. In addition we have determined the percentage of NK cells present in PsA SF samples as measured by detection of the NK surface marker NKH-I which is expressed by 95% of these cells (13). The proportion of SF cells bearing the NK phenotype has been compared with NK cytotoxic activity. We have also examined the proportion of monocytes in matched PB and SF
samples from PsA patients as these cells are potentially an important source of prostaglandins.

5.3 PATIENTS AND CLINICAL METHODS

11 PsA patients (according to the definition of Moll and Wright) (14) with knee joint effusions were studied. All were selected from a rheumatology outpatient clinic population. Informed consent was obtained from each patient and this study was approved by the Auckland Area Hospitals Research Ethics Committee. For each patient a brief history was taken and clinical examination performed according to a protocol previously described (15). The following parameters of disease activity were recorded: visual analogue pain score, Ritchie score (16), PASI score (Psoriasis Activity and Severity Index) (17) and ESR. Demographic characteristics with details of clinical activity, disease subtype and medication are presented in Table 1. Where SF was obtained from patients on more than one occasion, the mean disease activity scores are presented. For 2 of the patients listed (GT and DT), data appears in Chapter 6.

Slow-acting anti-rheumatic drugs (SAARDs) were used at the following dosages: sulphasalazine 500mg-2g/day in 3 patients, methotrexate at 7.5-10 mg/week in 2 patients and prednisone 5-10mg/day in 2 patients. Patient TW was taking no medication at the time of his first assessment (for NK studies) but on his second assessment (see Chapter 6) he was taking the non-steroidal anti-inflammatory drug (NSAID) diclofenac at 100mg/day and sulphasalazine at 2g/day.

Comparative NK cytolytic activity in matched SF and PB samples was examined in 8 patients (Table 2). In 5 of these patients and one other (MH), further SF samples were available for studies of NK activity after incubation with cytokines and prostaglandins (Table 3 and Figure 1). In 5 patients with recurrent knee effusions further samples were obtained for flow cytometry to determine NK and monocyte populations (Table 4 and Figure 2).

Immunological Studies

See Materials and Methods, Chapter 2, Sections 2.3.1 - 2.3.2
Statistical Analyses

The Statistical Analysis System (SAS) programming language (18) was used for all analyses of data. Multivariate techniques including regression, analysis of variance and a repeated measures analysis were used to investigate NK activity. The Dunnet's multiple comparison test was used to investigate the effects of cytokines and prostaglandins on NK activity.

<table>
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<td>naproxe</td>
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</table>

*see text
** sulphasalazine

Table 1 Characteristics of PsA patients
5.4 RESULTS

NK activity of SFL and PBL in PsA patients

NK activity of paired PBL and SFL samples from 8 PsA patients was measured by the lysis of K562 and Molt-4 tumour cells (Table 2). NK activity was detectable in all 8 PB samples and in 7 SF samples.

<table>
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<tr>
<th>PsA pt.</th>
<th>% specific lysis of target cells by effector cells (SFL or PBL)*</th>
<th></th>
</tr>
</thead>
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<td></td>
<td>Target</td>
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</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>RB</td>
<td>K562</td>
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<td>K562</td>
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<td>Molt</td>
<td>10.0(1.4)</td>
</tr>
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<td>K562</td>
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</tr>
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<td></td>
<td>Molt</td>
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</tr>
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<tr>
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</tr>
<tr>
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<td>K562</td>
<td>10.3(1.2)</td>
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</table>

*mean of triplicates(SD)
**mean of duplicates(SD)

Table 2. NK activity of SFL and PBL in patients with PsA
A repeated measures analysis revealed NK activity of PB to be significantly greater than SF (p=0.014). No correlation was found between NK activity of SF or PB samples and clinical parameters including pain score, Ritchie score, PASI score, ESR or the presence of erosive disease. Pain score and ESR were highly correlated (r=0.92).

**NK activity of SFL cultured with IL-2, IL-6, PGE2 and Indomethacin.**

Where sufficient synovial fluid was available, NK activity of SFL from PsA patients was also examined after preincubation with cytokines and prostaglandin E2 (Ref. Materials and Methods, Chapter 2, 2.3.1). Using a repeated measures analysis, significant differences were found between NK activity of SFL cultured alone and SFL cultured with cytokines and prostaglandins (p=0.01) (Table 3). Analysis of the influence of IL-2 (100 ng/ml) on NK activity in 4 PsA patients revealed that this cytokine significantly increased NK activity (p=0.01). In 3 of 4 patients, increases in specific target lysis of 15.0%, 20.2% and 31.4% were recorded (K562 at effector:target ratio of 25:1) while in one patient no change was found.

IL-6 (50 ng/ml) did not influence lysis of K562 targets but the addition of PGE2 (5x10^-7M) reduced NK activity in 4 patients at an E:T ratio of 25:1 (p=0.06). The effect of indomethacin (5x10^-7M) was only studied in 3 patients (insufficient for statistical analysis). In 2 of these, no effect was seen, but in one patient (RB) there was an apparent increase in NK activity (see below). Figure 1 shows experiments from PsA patient RB demonstrating the increased NK activity of PBL compared with SFL against K562 targets (A) and enhanced NK activity of SFL after incubation with IL-2 (B). Incubation with IL-6 had no effect. In this patient, NK activity was not significantly altered by PGE2 but was increased after incubation with indomethacin (C). This suggests that prostaglandins present in freshly aspirated synovial fluid may have already suppressed NK activity prior to the addition of exogenous PGE2.
<table>
<thead>
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<th>Patient</th>
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<th>IL-2</th>
<th>IL-6</th>
<th>PGE₂</th>
<th>indo</th>
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*mean of duplicates (SD)
**one well only

| Table 3. | Cytolytic activity of SF NK cells on K562 targets (% specific lysis) |

NK function of SFL and PBL was not associated with number of NKH-1 cells.

In 5 PsA patients NK activity of paired SFL and PBL was compared with the percentage of cells bearing the NKH-1 surface marker using flow cytometry. The size of the NK population was not found to be associated with the degree of NK activity in SFL or PBL (Table 4). As activated monocytes are capable of producing PGE₂ (19), the population of these cells was also determined using an anti-CD14 mab. In all SFL samples a larger proportion of monocytes was detected than in matched PBL (Table 4), but this was not found to be a significant predictor of relative NK activity. Figure 2 shows the FACS display from patient LR demonstrating staining of NK cells (with NKH-1 mab) and monocytes (with CD14 mab) in SFL and PBL.
Figure 1. PsA patient RB. (A) NK activity of SFL vs PBL on K562 targets. (B) Effect of IL-2 and IL-6 on SFL NK activity. (C) Effect of PGE$_2$ and indomethacin on SFL NK activity.
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<tr>
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<th>Sample</th>
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<th>% monocytes (CD-14)</th>
<th>% lysis K562 at E:T of 25:1</th>
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</table>

Table 4. Flow cytometry of paired PsA PBL and SFL to demonstrate NK and monocyte populations with concurrent NK activity.

**Figure 2.** PsA patient LR. Flow cytometry of SFL (A) and PBL (B) demonstrating NK and monocyte populations.
5.5 DISCUSSION

NK cells are active physiologically in the elimination of neoplastic and virally infected cells (2) and are present in synovial effusions of patients with inflammatory arthritis (2). In the present study we have observed NK activity of SFL from PsA patients to be low compared with matched PBL samples although in a number of individuals activity in PBL and SFL was virtually identical. This finding is consistent with other published reports in PsA (1,10) and in seropositive RA (6,7). In RA, Tovar et al (6) observed a facilitatory effect of low NK activity on immunoglobulin and autoantibody synthesis suggesting that these cells could influence immune-dysregulation within the joint via an effect on rheumatoid factor production. It is not clear whether a similar mechanism could operate in PsA as this disease is not characterised by autoantibody production. We sought to identify factors which might explain low SF NK activity in PsA.

Firstly we studied the effects of exogenous inflammatory mediators including cytokines and prostaglandins on NK activity. Koren et al (20) observed that production of PGE1 by stimulated monocyte in vitro down-regulated PBL NK activity. We confirmed a weakly suppressive effect of PGE2 on SF NK function in the five patients studied which almost reached statistical significance \((p=0.06)\). In the one patient where the addition of prostaglandin had no apparent influence (RB, Fig 1,C), the addition of indomethacin to SF resulted in enhancement of NK activity suggesting that this may have been previously suppressed by endogenous prostaglandins. Interestingly, this patient had not been taking NSAIDs at the time of SF aspiration while the other 4 patients were all taking these medications orally for suppression of joint pain and inflammation. This may also account for the relative lack of response of their SF NK cells to exogenous indomethacin.

The addition of IL-2 to cultures of SFL from these patients significantly stimulated NK activity; a finding consistent with reports that IL-2 is a potent inducer of NK proliferation and activation (21,22). In contrast to the observations of Smyth et al (11), we were unable to demonstrate an effect of IL-6 on SF NK activity. High levels of IL-6 have been found in SF from patients with inflammatory arthritides (23) as opposed to IL-2 levels which are generally low (24). The differing
responsiveness of PsA SF NK cells to these cytokines in vitro could be influenced by their previous exposure in vivo resulting in a dramatic response to IL-2 compared with an apparent lack of response to IL-6.

We also examined the proposal that low levels of NK effectors in PsA SF could account for the observed low NK cytolytic activity against K562 targets. Using flow cytometry and K562 lysis assays we were unable to find any correlation between the percentage population of NK cells in the samples studied and NK functional activity. We were interested to note that in all patients, the population of monocytes as defined by the CD14 surface marker was much greater in SFL than in matched PBL samples and propose that production of mediators such as prostaglandins by these cells in vivo may serve to suppress SF NK function as measured in vitro. Thus it seems likely that multiple stimulatory and inhibitory factors influence the function of NK cells within the inflamed joint and the measured ability of these cells to lyse K562 targets in vitro reflects the integration of all factors operating at the time of joint aspiration.

Although no correlation was found between disease activity (as determined by measures of joint and skin inflammation) and SFL NK activity (as determined by lysis of K562 targets), the question of whether NK cells could be influencing joint destruction in PsA remains unanswered. Recent studies by Karre et al (25,26) followed by Chadwick et al (27) have shed light on the mechanisms involved in the recognition and attack of targets by NK cells. Although NK-mediated cytolysis has classically been regarded as “non-MHC restricted”, it appears that Class I MHC molecules do play a crucial role. Thus NK cells are “switched off” by Class I molecules on target cells which bear self-peptides in their antigen-presenting grooves and “switched on” by those bearing foreign (often viral) peptides (27). This provides a strategy to prevent autoreactivity mediated by the highly reactive products of NK cell activation. It is interesting that the K562 cell line expresses very low levels of Class I MHC molecules and cannot deliver the appropriate “NK switch off” signal. These cells are therefore exquisitely sensitive to NK-mediated lysis and have been used in most assays to measure NK activity in SF and PB of patients with inflammatory arthritis. However this model is unphysiological as any cells within the joint which might be targets for endogenous NK-mediated
lysis would express much higher levels of Class I MHC molecules plus peptide of self or foreign origin. Thus conclusions regarding the importance of NK cells in rheumatic disease based on K562 cytolysis assays may be flawed and new ways to assess activity of these cells need to be explored. In Chapter 6 a different approach is taken by measuring the surface expression of activation markers on NK cells from PsA PBL and SFL.

ACKNOWLEDGMENTS

We are grateful to Ms Lisa Watson and Ms Serey Kong for their technical assistance in this project and Mrs Sue Yeoman, Arthritis Foundation nurse metrologist, who helped in the collection of samples and clinical data. We thank the following physicians who referred patients enrolled in this study: Dr D Caughey, Dr R Grigor, Dr N Lynch, Dr H Hart, Dr M Butler and Dr P Gow.

5.6 REFERENCES


9) Reinitz E, Neighbour PA, Grayzel AI: Natural killer cell activity of mononuclear cells from rheumatoid patients measured by a conjugate-binding assay. Arthritis Rheum 1982;25:1440-1444


CHAPTER SIX

EXPRESSION OF β7 INTEGRIN BY NK AND T CELLS IN PSORIATIC AND RHEUMATOID ARTHRITIS

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6.1 ABSTRACT

Objective: To examine the surface expression of the β7 integrin molecule, human mucosal lymphocyte adhesion molecule (HML-1), on NK and T cells from peripheral blood (PB) and synovial fluid (SF) of patients with psoriatic and rheumatoid arthritis (PsA and RA). To examine the influence of IL-2, IL-6 and prostaglandin E2 on HML-1 expression by NK cells.

Methods: Paired samples of PB and SF lymphocytes (PBL and SFL) were obtained from 6 patients with PsA and 6 RA patients. The phenotype of NK cells and T cells was determined by flow cytometry using monoclonal antibodies (mab) to the natural killer associated antigen (NKH-1), the CD3 complex and the β7 integrin, HML-1. In 3 patients, PB and SF samples were incubated for 7 days with IL-2, IL-6 and PGE2 and changes in HML-1 expression by NK cells were observed.

Results: The expression of HML-1 by NK cells was highly correlated with T cell expression of this adhesion molecule (r = 0.82). In PsA and RA samples, HML-1 expression was significantly greater on SF than on PB (p<0.05). Expansion of the NK population of PBL and SFL occurred after culture with IL-2 but the effect of IL-2 on HML-1 expression was variable. Culture with IL-6 and PGE2 did not influence HML-1 expression.

Conclusion: HML-1 is known to be an activation marker on T cells. Our data suggests it is also an NK cell activation marker. Moreover it is strongly expressed by SFL of patients with active inflammatory arthritis suggesting that NK cells within the joint are in an activated state.
6.2 INTRODUCTION

Lymphocyte activation on antigenic challenge is accompanied by increased adherence of these cells to neighbouring cells or background matrix ensuring they remain at sites of inflammation (1). This is mediated by the expression of adhesion molecules including those of the integrin family which are heterodimers of $\alpha$ and $\beta$ chains and play a critical role in mediating strong adhesive interactions (2). A new class of integrin utilising the $\beta 7$ chain has recently been described (3,4,5) which includes the molecule recognised by the human mucosal lymphocyte 1 (HML-1) monoclonal antibody. This antigen has been found on >95% of human intra-epithelial lymphocytes (IEL) which reside on the external face of the basement membrane adjacent to the basolateral surface of intestinal epithelial cells (4). These cells are predominantly CD4-CD8+ memory T lymphocytes and have been found to have a restricted T cell receptor repertoire suggesting they may play a role in the recognition of antigen presented by cells of Peyer’s patches or the intestinal epithelium (6).

It has been proposed that $\beta 7$ integrins play a role in “homing” of recirculating lymphocytes to the intestinal mucosa where they may be activated by, or mediate tolerance to, gut-derived antigens (4,5,6).

Constitutive expression of $\beta 7$ integrins by other human tissues is very low and less than 2% of resting PBL express HML-1(4). However this is increased on activation by mitogens such as phytohaemaggulitin (PHA) and phorbol 12-myristate 13-acetate (PMA) suggesting that the appearance of this molecule on the surface of T lymphocytes may denote their activation (7). NK cell expression of HML-1 has not been examined but these cells are known to express other adhesion molecules including the $\beta 2$ integrins LFA-1 (CD11-a) and LFA3 (CD58) which may be important in binding to target cells and potentially triggering the cytolytic process (8).

The expression of HML-1 by cells of the synovial membrane has not been investigated. However this gut-associated adhesion molecule may be relevant in inflammatory arthritis as synovial membrane has been compared structurally to mucosal tissue and some evidence has suggested a
role for gut-derived antigens in disease pathogenesis (9). We have therefore used flow cytometry to look for the presence of the HML-1 antigen on the surface of T cells and NK cells from SFL and PBL of patients with PsA and RA. We have also studied the effects of cytokines and prostaglandin on HML-1 expression by these cells.

6.3 PATIENTS AND CLINICAL METHODS

6 PsA patients (according to the definition by Moll and Wright) (10) and 6 RA patients (1987 ARA criteria) (11) with recurrent knee joint effusions were studied (Ref. Patients and Clinical Methods section, Chapter 6). Demographic characteristics are recorded in Table 1, Chapter 5.

Paired samples of PB and fresh SF were obtained from patients for flow cytometry to determine HML-1 expression of NK and T lymphocytes and comparisons were made with PBL taken from 5 age-matched normal controls. In 3 patients, further samples were obtained for investigation of the effects of cytokines and prostaglandin on HML-1 expression by NK cells.

Immunological Studies

See Materials and Methods, Chapter 2, Sections 2.4.1

Statistical Analyses

Multivariate analysis of variance was used to investigate the levels of HML-1 expressed on NK and T lymphocytes. Pearson's correlation coefficients are quoted where appropriate.

6.4 RESULTS

Expression of β7 integrin (HML-1 antigen) by NK cells from PB

PBL samples were obtained from 6 PsA patients and 5 controls. NK cells were defined by flow cytometry using the anti-NKH-1 mab which stains 95% of these cells (12). HML-1 expression on NK cells from PsA PBL (mean 4.2, range 0-15.1) did not differ significantly from HML-1 expression on NK cells derived from controls (mean 5.2, range 1.2-10.5) (Table 1).
The expression of HML-1 by NK cells from PsA patients' PBL was then compared with expression by paired samples of SFL in 6 patients (Table 2). PB and SF samples were also

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Table 1. Flow cytometry of PBL from PsA patients and controls to show expression of HML-1 by NK cells.

Expression of HML-1 by NK and T cells from SF

The expression of HML-1 by NK cells from PsA patients' PBL was then compared with expression by paired samples of SFL in 6 patients (Table 2). PB and SF samples were also
obtained from a "control" group of 6 RA patients to determine whether findings were disease-specific. For both PsA and RA patients, expression of HML-1 was greater on NK cells derived from SF than those from paired PBL (p=0.03 and 0.05 respectively using a univariate analysis).

<table>
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<th>% CD3 cells expressing HML-1</th>
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Table 2. Flow cytometry of SF and PBL to show expression of HML-1 on NK and T cells.
Multivariate analysis revealed a strong correlation between the percentage of NK cells from PsA patients expressing HML-1 and the percentage of CD3+ cells expressing HML-1 ($r = 0.82$).

Figure 1 shows results from PsA patient LR who had active chronic disease treated with NSAIDs alone. HML-1 was expressed on 29.2% of NK cells (HML-1/NKH-1 stained 8.3% total cells) and 13.8% of CD3+ cells (HML-1/CD3 : 5.4% total cells) derived from SF compared with 2.3% of NK cells (HML-1/NKH-1 : 0.4% total cells) and 0.5% of T cells from PB (HML-1/CD3 : 0.3% total cells).

![FACS display of PBL and SF to show staining of NK and T cells with HML-1](image)

**Figure 1.** Data from PsA patient LR. FACS display of PBL and SF to show staining of NK and T cells with HML-1 (expressed as total cells).

(A) PBL: HML-1/NKH-1=0.4%

(B) SF: HML/NKH-1=8.3%

(C) PBL: HML-1/CD3=0.3%

(D) SF: HML-1/CD3=5.4%
Figure 2. shows results from a patient with newly diagnosed PsA (DT, female, age 33) who presented acutely with a knee effusion and dactyliitis of one toe. She was taking NSAIDs alone and had not been treated with SAARDs or intra-articular steroid injections previously. FACS analysis of SFL from the knee effusion with a paired PBL sample is shown below (processed using a Coulter XL cell analyser). HML-1 was expressed on 55.7% of NK cells from the SFL sample (11.84% of total cells), whereas HML-1 staining of NK cells from PBL was much lower (2.8% NK cells, 0.57% total cells).

To determine whether HML-1 was a marker of disease activity in PsA and/or RA, the relationship between cellular expression of this integrin and clinical parameters of disease activity was investigated. The median ESR of the RA group was higher than that of the PsA group but no association between clinical indices and cellular HML-1 expression was found.
Effect of culture with IL-2, IL-6 and PGE2 on NK expression of HML-1.

The effect of culture with cytokines and prostaglandin on HML-1 expression by NK cells, was first examined in PBL from controls. In 3 of 5 control PBL samples, incubation with IL-2 modestly increased expression of HML-1 by NK cells (increments of 0.1%, 4.4% and 10.3%) while in 2 samples, no increase was observed. No significant effect was noted from incubation with IL-6 or PGE2. In 3 PsA patients (GF, DT and RB), paired samples of PBL and SFL were cultured for 7 days with medium alone, IL-2 (20ng/ml), IL-6 (50ng/ml), or PGE2 (5x10^-7M) and then stained for co-expression of NKH-1 and HML-1 antigens. Data are presented in Table 3.

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<th>PsA patient</th>
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<th>PGE2 (5x10^-7 M)</th>
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*expressed as % NK cells  
**Not Done

Table 3. Data from PsA patients, GF, DT and RB. HML-1 staining of NK cells from PBL and SFL before and after incubation for 7 days with cytokines and PGE2.
Expansion of the NK population of PBL and SFL occurred after IL-2 stimulation in all patients compared with samples cultured in medium alone. In patient GF, HML-1/NKH-1 staining of PBL and SFL was also increased after incubation with IL-2 and is depicted in Figure 3. However, in the other 2 patients studied (DT and RB) expression of HML-1 by NK cells from fresh SFL was very high and was not increased after culture with IL-2. Culture with IL-6 or PGE2 did not affect HML-1 expression by NK PBL or SFL in any of the 3 patients studied.

![Data from PsA patient GF. FACS display of PBL and SFL to show the influence of IL-2 on NK expression of HML-1 (expressed as % total cells).](image)

(A) PBL: HML/NK, control=1.4%  
(B) PBL: HML/NK,+IL-2 (20ng/ml)=9.6%  
(C) SFL:HML/NK, control=3.1%  
(D) SFL: HML/NKH,+IL-2=8.6%
6.5 DISCUSSION

Adhesion molecules including integrins are expressed by circulating lymphocytes and play a crucial role in the migration of cells to tissue sites of inflammation (1,2). Studies in RA have revealed expression of β2 integrin molecules including LFA-1 (CD11a/CD18) to be increased on SFL (13) whereas β1 integrins are down-regulated on RA synovial membrane (14). The HML-1 antigen is a recently described member of the β7 integrin family which is expressed by activated T lymphocytes (3,4,5) and has been identified on RA PBL and SFL (13,15). Data concerning the expression of β7 integrins by NK lymphocytes is sparse but Gismondi et al demonstrated increased expression of α4β7 integrin on activated vs fresh NK cells from normal human PBL (16). We present the first study to investigate the expression of HML-1 by NK cells in the context of chronic inflammatory disease.

We have demonstrated that HML-1 is expressed by NK and T lymphocytes in patients with PsA and RA. Interestingly, HML-1 expression by NK cells was strongly correlated with expression by T cells implying that this molecule may be an activation marker for NK cells also. NK cell activation is known to be accompanied by the upregulation of other adhesion molecules. Experiments by Schmidt et al (17) showed that LFA-1 was increased following NK stimulation through the CD2 molecule and this resulted in enhanced cytolytic function, possibly via improved adhesion to targets. Our finding of HML-1 expression by NK cells in PsA and RA and its possible upregulation with cellular activation, suggests that these cells may share homing characteristics with T cells and that this molecule may help direct activated cells to mucosal or in this case synovial tissues.

Our results show that in both PsA and RA, the expression of HML-1 on NK and T cells derived from SF was significantly greater than expression on cells from paired PB samples. In one patient (DT) with an acute synovial effusion and recent onset disease, HML-1 expression by SF NK cells
was twenty-fold greater than expression by NK cells from a paired sample of PBL (Table 2, Figure 2). This may indicate the presence of a large population of activated NK cells in SF which could well be contributing to the maintenance of chronic inflammation within the joint via elaboration of such pro-inflammatory cytokines as TNFα (18). Whether NK activation might have implications regarding the pathogenesis of PsA or RA remains a tantalising but unanswered question. A viral antigen could certainly trigger NK activation and the known association of PsA with Class I MHC antigens (19) would be consistent with antigen presentation via the endogenous pathway. In a patient with acute onset disease, evidence of large scale NK cell activation in SF would be predicted and indeed this appeared to be the case in our patient DT if HML-1 expression is taken as a sign of NK activation. Further study of patients in the acute phase might provide more information about a precipitating viral antigen in this disease.

Samples from RA patients also showed upregulation of HML-1 on NK and T cells from SF compared with PB. A viral antigen is more difficult to implicate in disease pathogenesis here as RA is associated with Class II MHC antigens which commonly present extracellular antigens such as those derived from bacteria to CD4+ T cells. However once the disease reaches the chronic stage, the spectrum of antigens stimulating an autoimmune response within the joint is likely to have shifted, confusing the interpretation of Class I and Class II MHC disease associations. The same reasoning would explain the evidence of T cell activation (HML-1 upregulation) in the PsA SF samples and this is consistent with other evidence from studies of synovial tissue which has revealed T cell activation in PsA despite the Class I associations of this disease (20).

If the high expression of HML-1 by SFL from PsA and RA patients does indicate NK cell activation, this is at odds with data presented in Chapter 5 of this thesis which implied low cytotoxic activity of NK cells using the traditional Cr51 release assay against tumor target cell lines. As has already been discussed, conclusions based on cytotoxicity data may be flawed due to the use of unphysiological target cells (Ref. Chapter 5, Discussion, Para 5). However the display
an activation phenotype by NK cells may not correlate with increased cytotoxic capability within
the joint where inflammatory mediators such as prostaglandins may have a negative effect on NK
cytolytic function (21).

IL-2 is known to induce expression of HML-1 on T lymphocytes (7) and we therefore sought to
determine whether this cytokine could induce expression of HML-1 on NK cells from PsA
patients. Effects were variable in the 3 patients studied. Apparent induction of HML-1 expression
by IL-2 was noted in 2 of the 3 PB samples but only in 1 of the 3 samples of SF. A striking
feature of results from patients DT and RB, both of whom had active oligoarticular PsA and neither
of whom were receiving SAARDs for disease suppression, was the high level of HML-1
expression on NK cells derived from fresh samples of SF. The lack of further induction of HML-
1 by exogenous IL-2 in these patients suggests that their SF NK cells were already in an activated
state when aspirated from the joint and therefore unable to respond further after culture in vitro.
In contrast, SF NK cells from patient GF did not initially exhibit high levels of HML-1 expression
but these increased after culture with IL-2 (Figure 3) suggesting they were not initially fully
activated. However, one of the difficulties in interpreting data from PsA patients is the clinical
heterogeneity of this disease and patient GF exhibited the rare form of arthritis mutilans which
differs radiologically and clinically from the oligoarticular form (22) and may have a different
immunopathology.

In summary, we have presented data from studies of PBL and SFL from patients with PsA and
RA. We have observed significantly increased expression of the β7 integrin, HML-1, on NK cells
and T cells from SF compared with paired PB samples. Moreover, levels of HML-1 on NK cells
were strongly correlated with levels on T cells where this antigen is a known activation marker.
We propose that HML-1 is also an activation marker on NK cells and that these cells are present in
an activated state in the synovial fluid of many patients with active inflammatory arthritis where
they may participate in the maintenance of chronic synovitis.
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CHAPTER SEVEN

DISCUSSION

7.1

Research presented in this thesis has investigated aspects of the immunopathology of two forms of inflammatory polyarthritis; rheumatoid and psoriatic arthritis. Although these 2 conditions are distinct on clinical and radiological grounds, the histopathology and immunopathology of affected joints is frequently indistinguishable (1) suggesting convergence of disease processes to a final common pathway of chronic inflammation.

Chapter 2 deals specifically with RA and focuses on the cyclical variability of symptoms and signs through the menstrual cycle in young women. In addition serum levels of the cytokine, IL-1β were monitored and correlated with clinical disease activity. This work confirmed that in some patients joint pain and tenderness did fluctuate cyclically with apparent deterioration in the late-luteal phase, but laboratory measures of disease activity such as the ESR and CRP did not change. Levels of IL-1β were considerably higher in the RA group than in matched controls confirming previous work (2) but did not fluctuate through the cycle and in fact remained remarkably constant when followed longitudinally in individual patients.

These observations highlight the importance of investigating a disease process within its physiological context which in this case was the menstrual cycle. Definitions of disease activity in RA have traditionally involved assessments of pain and joint tenderness which are generally highly correlated with laboratory measures of joint inflammation (3). Levels of the inflammatory cytokines, IL-1β, IL-6 and TNFα have also been found to reflect the degree of synovitis in RA and other inflammatory arthropathies (2,4,5). However findings from our study imply that rheumatoid joint pain is also influenced by factors other than inflammatory mediators and that fluctuating levels of female sex hormones throughout the menstrual cycle may have a profound effect. One of the
important clinical consequences of this is that patients whose symptoms are only severe in 2 weeks out of every 4 may not comply with regular second-line disease suppressing medication (6) and this was confirmed by a number of patients in our study.

Another question raised by this study is whether endocrine manipulation using gonadotropic hormones might be used therapeutically in RA. Two recent studies have investigated the effects of hormone replacement therapy (HRT) on disease activity in post-menopausal patients with RA (7,8). Hall et al (7) found that in those patients who complied with therapy, there were significant improvements in articular index and pain score compared with placebo after a six month period while MacDonald et al (8) reported similar findings. Interestingly, in both these studies there was no significant change in ESR implying a degree of disassociation between clinical and laboratory measures of disease activity just as was observed in our study within the menstrual cycle. The mechanisms whereby female sex hormones modulate joint inflammation remain obscure but Jorgenesen et al (9) have suggested that neuropeptides might provide an important link between the immune response and the neuro-endocrine axis. Clearly further studies in this area are required to tease out the complex and dynamic relationships between cytokines, hormones and inflammation which exist in the patient with RA.

The focus of research presented in Chapters 3 to 6 was narrowed to more closely investigate the cellular immunology of inflammatory arthritis. Lymphocyte responses to streptococcal antigens were examined initially in view of the known clinical association between guttate psoriasis and group A streptococci (10). Although proliferative lymphocyte responses by SFL from PsA and RA patients to streptococcal antigens were demonstrated, these were nonspecific as control PBL also responded. However when fractionation of lymphocyte populations was performed it was found that the γδ group of T cells within SF responded to a psoriasis-associated streptococcal strain only and not to a rheumatic fever-associated strain which was also tested. This result might have implicated streptococci in the pathogenesis of PsA if it had been disease specific but
somewhat disappointingly, findings were similar in SF from RA patients. What conclusions can be drawn from this work?

Firstly it raises questions regarding the implication of proliferative responses of SFL from patients with inflammatory arthritis to bacterial antigens. Ford et al (11) proposed that demonstrating such responses in patients with reactive arthritis could indicate the microbiological cause for their disease. However Keat et al (12) have disputed this, citing the work of Pelton and colleagues in RA who showed that cells activated by the influenza virus were recruited nonspecifically into already inflamed joints (13). Our findings would be consistent with this, suggesting that some SFL in patients with PsA and RA could have originated from PBL which had previously been exposed to streptococcal antigen in the environment and on further exposure proliferated vigorously. The similar findings in control PBL would suggest that streptococci are a common source of immunogenic environmental antigens.

Secondly, the data concerning γδ T cells invites speculation about how these cells may contribute to chronic joint inflammation in PsA and RA. Their physiological function remains elusive as does the nature of the antigens which stimulate them. Studies in the late 1980s revealed that γδ cells proliferated in response to mycobacterial antigens including the heat shock protein, hsp-65 (14). This led to the suggestion that these cells were involved in “first-line” defense against invading micro-organisms and might also destroy stressed autologous cells if these displayed similar heat shock proteins (15). However more recently γδ cell stimulation by small nonpeptidic ligands has been described including synthetic alkyl phosphates (16) and phosphorylated thymidine containing compounds (17). This would imply that γδ T cell physiology is even more fundamentally different to that of αβ T cells than had been expected and raises further questions about the nature of these cells and the significance of their activation.

Some evidence suggests that γδ T cells may have an immunomodulatory function in RA.
Holoshitz et al (18) demonstrated γδ T cells reactive to the acetone-precipitable fraction of mycobacteria tuberculosis (AP-MT) among synovial cells of rheumatoid arthritis patients and suggested that these cells might be involved in autoimmunity. Expanded populations of T lymphocytes bearing γδ receptors have also been detected in patients with Felty’s syndrome and a recent study has shown that these cells, while anergic to most T cell mitogens, can influence B cell production of immunoglobulins (19). Their role has also been investigated in other autoimmune disease including multiple sclerosis (MS) where they have been found to be overrepresented in parenchymal lesions of patients with early disease (20). Our finding of γδ T cell stimulation by streptococci is in accord with others in this field who have reported in vitro stimulation with Gram positive and Gram-negative bacteria (21, 22) and in vivo expansion during infection with malarial parasites (23) and in leprosy (24). The impression is that these cells are important in defense against a wide variety of pathogenic bacteria and parasites. Whether their presence in PsA and RA joint fluid implicates such micro-organisms in the pathogenesis of these disorders remains unclear.

Our observations of reactivity of RA SFL to bacterial antigens should be viewed in the light of a growing body of evidence for multiple synovial antigens in this disease (25). Numerous studies have examined RA SFL to look for oligoclonal populations of T lymphocytes which might indicate responsiveness to a unique antigen (26,27,28). Such T cell oligoclonality has been detected in the cerebrospinal fluid of MS patients and therapies are currently being designed in that disease to eliminate of these pathogenic and presumably antigen-specific T cells (29). However in RA, results have not been conclusive, with different groups reporting different TCR Vβ genes as being locally expanded (25) and many investigators now believe that T cells within RA joints are heterogeneous with respect to antigen recognition. Findings in PsA have been similar but a report from Lu et al did demonstrate oligoclonality of T cell clones derived from SF of a patient with early onset disease (30). Five of the 11 clones examined from that patient demonstrated identical rearrangements of their TCR. While no conclusions can be drawn from an isolated case report, this does suggest that answers regarding the nature of the inciting antigen in inflammatory arthritis...
may only be found from study of patients in the very earliest phases of their disease, before reactivity to multiple self antigens blurs the immunological picture.

In Chapters 5 and 6, studies of NK cell activity and expression of the β7 integrin molecule, HML-1, are presented. Cytotoxic activity of NK cells was initially assessed in SF and PB from patients with PsA with a standard Cr51 release assay using the susceptible tumour cell lines K562 and Molt-4 as NK targets. NK activity of PsA SF was found to be low compared with PB samples, a finding consistent with reports in RA and other inflammatory arthropathies (31,32). NK activation following culture with IL-2 was demonstrated and an inhibitory effect of PGE2 on NK function was also observed. A large monocyte population was noted in SF from both PsA and RA patients and we have hypothesised that production of prostaglandins by these cells may affect the cytotoxic activity of NK cells in SF.

Mention has already been made of the use of non-physiological target cells in the assessment of NK cell cytotoxicity. The K562 cell line is exquisitely sensitive to NK-mediated lysis by virtue of its very low expression of Class I MHC molecules (33). Usually, these molecules present self antigen to NK cells delivering a "switch off" signal which prevents NK-mediated target lysis (34). In the absence of that signal, K562 targets are quickly lysed by NK cells in an easily reproducible manner. The use of autologous target cells to assess NK activity would more closely mimic the physiological situation. However these cells are not usually lysed by NK cells as they will deliver the "switch off" signal as described above unless presenting foreign eg. viral peptide. As an example of this, Chadwick et al have described the conversion of NK-resistant normal lymphoblasts to a state of NK-sensitivity when these cells were incubated with viral peptides (35). Therefore, if one proposes that a viral antigen could trigger PsA or RA, increased NK cytolytic activity in joint fluid could only be demonstrated at the time when viral antigens were actually being presented by macrophages or other APCs within synovial tissue. Current evidence in RA suggests that by the time disease is established, multiple synovial antigens are exciting T cell responses and
in all probability any triggering microbial antigen has disappeared (25). Thus the likelihood of detecting NK cells in SF which are active against autologous target synovial cells is very low.

Data presented in Chapter 6 has examined the question of whether NK cells are important in the pathogenesis of inflammatory arthritis from a different angle. Expression of the β7 integrin molecule, HML-1, on the surface of NK cells was studied in PBL and SFL samples using flow cytometry. This integrin is a known activation marker on T cells (36) and our findings of high HML-1 expression on both T and NK cells from PsA and RA SF suggest that it may also indicate NK cell activation. Interestingly, the highest HML-1 levels on NK cells from SF were seen in a patient with active, recent onset PsA. HML-1 expression by PBL in this patient was negligible implicating the inflamed, effused joint as the site of NK activation. In 1 of 3 PsA patients, further HML-1 expression by SFL could be induced by incubation with IL-2 while in 2 patients high levels in fresh samples were not augmented.

These findings suggest that both NK and T cells are present in an activated state in the joints of patients with PsA and RA. The degree of SF cellular activation (HML-1 expression) in individual patients did not correlate with clinical or traditional laboratory measures of disease activity. This is not surprising as many patients had only one or a few inflamed joints with comparatively normal ESRs and low Ritchie scores. Thus these clinical measures did not reflect the degree of inflammation in the individual joints from which synovial fluid was aspirated but rather indicated the overall activity of disease. Further studies of synovial membrane would be interesting using simple closed biopsy procedures as are currently being developed in some centres (37,38). These would be of special relevance to HML-1 expression as this molecule is involved in homing to gut mucosal tissue (39) and might serve a similar function in directing lymphocytes to inflamed synovial membrane (40).

Both PsA and RA are now regarded as autoimmune forms of arthritis developing on a background
of genetic susceptibility after a triggering environmental event. Decades of research have focused on trying to define triggering antigens but these remain elusive despite the powerful tools of molecular biology available today. Other approaches are now being developed which may render identification of these antigens irrelevant. Studies of oral tolerance to autoantigens have confirmed the gut associated lymphoid tissue (GALT) as a potential source of activated T cells in animal models of organ-specific autoimmune disease (41,42). The phenomenon of "bystander suppression" has been studied by Weiner and colleagues using the adjuvant arthritis model and they have demonstrated marked improvement in disease activity following oral feeding of collagen to affected animals (43,44). Interestingly, the mechanism of disease suppression here appears to neatly bypass the need for identification of putative arthritogenic antigens and depends instead on lymphocytes activated by collagen (via cells of the GALT), which migrate to the joints and on meeting further collagen, produce suppressive cytokines such as TGFβ. These cytokines then suppress immunological activity of neighboring lymphocytes (bystanders) without the need to identify the particular cells causing disease (43). Immunotherapy using oral tolerance to Type II collagen has already been used in rheumatoid arthritis and preliminary results are encouraging (45). Similar approaches may be useful in management of PsA as evidence of an autoimmune response directed against joint collagen has been identified in both conditions (46).

To better define initiating immunopathological events in PsA and RA it will be necessary to study patients in the early phases of disease and this provides a challenge both for the clinician, in diagnosis and recruitment of such individuals, and for the medical scientist, in obtaining relevant biological samples when synovial effusions may be fleeting and synovial tissue difficult to obtain. Even then, if disease is triggered by a common pathogen it may be difficult to identify. Atkinson (47) has proposed that subtle defects in the immune system of some individuals may render them susceptible to infection with certain organisms leading to an exaggerated or prolonged immune response and ultimately an autoimmune reaction. Thus the key to understanding initiating events in inflammatory arthritis may lie with elucidation of these subtle immune defects as coded for by
relevant immune response genes in affected patients and their unaffected relatives. As a sequel to the work presented in this thesis, further studies by our group are now underway to better define the genetic markers associated with disease progression in RA using a longitudinal study of patients with early disease. It is to be hoped that ultimately the immune processes underlying these disabling forms of arthritis will be clarified and curative treatment may become a possibility.

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