Synovial Immune Mechanisms in Rheumatoid Arthritis: Prospects for Immunotherapy

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University of Cape Town

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LIAM RATCLIFFE

This work is dedicated to the memory of my late father, Brian Ratcliffe (1928 - 1991)

and to my mother, Mary Ratcliffe,

and for my wife, Penny.

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ABSTRACT

The identification of relevant pathogenetic processes is crucial for the developement of rational therapy in rheumatoid arthritis. Several aspects of the function of CD4+ T-cells in the rheumatoid synovial compartment remain largely unknown, including the nature of the T-cell stimulus, the role and regulation of CD4+ T-cell mediated antigen-specific cytotoxicity, and the potential modulatory effects of eicosanoids, present in significant concentrations in the rheumatoid synovial compartment, on T-cell effector functions. The aim of this study was to address these aspects of T-cells in the immunopathogenesis of rheumatoid arthritis and identify novel targets for the therapeutic modulation of CD4+ T-cell effector functions.

Antigen-induced proliferation and antigen-specific cytotoxic function of peripheral blood and synovial fluid mononuclear cells were investigated in a total of 20 patients with seropositive rheumatoid arthritis using purified protein derivative of *Myocbacterium tuberculosis* (PPD) as the antigen. Proliferation was determined in a standard *in vitro* tritiated thymidine incorporation assay and cytotoxicity measured in a ^{51}chromium release assay, confirmed as a specific indicator of CD4+ T-cell cytotoxic function. *In vitro* findings were correlated with clinical data. A significant positive linear relationship between proliferation and cytotoxicity was demonstrated in cells from both compartments. Patients were classified according to their synovial fluid mononuclear cell proliferative and cytotoxic responses: low or absent proliferation and cytotoxicity was associated with significantly longer disease duration, while moderate to high levels of both parameters was associated with non-steroidal anti-inflammatory drug monotherapy. A novel finding of intact proliferation but impaired antigen-specific cytotoxicity was observed in the synovial fluid mononuclear cells of 5 out of 6 patients receiving disease modifying therapy. This observation was confined to cells from the synovial compartment in these patients, with moderate to high levels of proliferation and cytotoxicity demonstrated in autologous peripheral blood mononuclear cells.

The potential mechanisms for these findings were explored by investigating the *in vitro* effects of several disease modifying agents and prostaglandin E2 on proliferation and cytotoxicity of peripheral blood mononuclear cells from normal controls. In addition, prostaglandin E2 production by CD14+ synoviocytes from the patients was quantified in cell supernatants using a commercial radioimmunoassay and correlated with proliferative and cytotoxic reponses demonstrated previously. The data obtained indicate that impaired synovial CD4+ T-cell mediated cytotoxicty in the setting of intact proliferative responses may be due to the suppressive effects of prostaglandin E2. Thus, in addition to providing functional evidence of the cytotoxic potential of the CD4+ T-cell infiltrate in rheumatoid synovium, these findings

highlight potential regulatory mechanisms of this function, with clinical and therapeutic correlations.

The mechanism of prostaglandin E2 inhibition of CD4+ T-cell cytotoxicity and the effects of this agent and other eicosanoids on CD4+ T-cell effector functions and phenotypic expression were investigated further. Studies were performed *in vitro* using bulk peripheral blood mononuclear cells from normal individuals, and panels of CD4+ T-cell clones from the synovium of three patients with inflammatory synovitis and the circulation of a healthy control. CD4+ T-cell clones were isolated by limiting dilution cloning of T-cell lines generated from synovial explants of the patients or the peripheral blood mononuclear cells of the healthy control. Cloning was performed using both specific and non-specific stimuli. Prostaglandin E2 inhibition of antigen-specific cytotoxicity was demonstrated in polyclonal peripheral blood mononuclear cell effectors and in the panel of CD4+ T-cell clones. Misoprostol (a prostaglandin E1 analogue) and forskolin (a cyclic AMP agonist) also displayed inhibitory effects on the clones. Inhibition of both binding and post-binding events by PGE2 was indicated by data from lectin-dependent and antigen-specific cytotoxicity assays. Conversely, moderate but significant augmentation of cytotoxicity by leukotriene B4 and C4 was observed in the clones. Modulation of CD4+ T-cell cytotoxicity by the eicosanoids tested was mainly due to effects on calcium-dependent pathways of cytotoxicity. Reciprocal effects of prostaglandin E2 and leukotrienes on interferon-γ production by the clones was also demonstrated. This study provides a novel illustration of the regulatory effects of eicosanoids on CD4+ T-cell cytotoxicity and confirms and extends previous reports of the regulatory effects of these agents on cytokine production. In addition, the findings indicate the potential to modulate CD4+ T-cell function at a chronic inflammatory site by therapeutic manipulation of local eicosanoid synthesis.

The observation of dissociated proliferation and cytotoxicity (proliferation in the absence of cytotoxicity) in synovial fluid mononuclear cells of a subset of rheumatoid arthritis patients, and in normal peripheral blood mononuclear cells assayed in the presence of prostaglandin E2, suggested that these functions of CD4+ T-cells may be independently regulated. This hypothesis was tested by measuring a range of morphologic, phenotypic and functional parameters of a panel of 6 CD4+ T-cell clones as a function of time following a single initiating stimulus. Morphologic, phenotypic and cell cycle analyses were performed by flow cytometry, and cytokine concentrations of cell supernatants quantified using commercial kits. Dissociation of functions was noted, with maintenance of cytolytic potential independently of other parameters. These findings provide evidence, at a clonal level, of the separate regulation of cytotoxicity in CD4+ T cells and demonstrate that CD4+ T-cells retain cytolytic potential irrespective of their size, activation status, cell cycle progression or cytokine production. Based

on these *in vitro* observations, it is possible that the rheumatoid synovial CD4+ T-cell infiltrate may retain cytolytic potential *in vivo*, despite their small size and paucity of cytokine production. In addition, the different kinetics of effector pathways in CD4+ T-cells suggests the possibility of independent therapeutic modulation of effector functions.

Previous reports have suggested that mycobacterial antigen reactivity of synovial fluid mononuclear cells in rheumatoid arthritis may have aetiological significance. In this study, the specificity of mycobacterial antigen reactivity in rheumatoid arthritis was investigated in patients and controls drawn from a population where tuberculosis has been endemic for several decades. The data demonstrate that in this population, mononuclear cell reactivity to purified protein derivative of *Mycobacterium tuberculosis* is a non-specific phenomenon, observed with similar frequency in rheumatoid arthritis patients and the control non-rheumatoid inflammatory synovitis patient group, and with similar kinetics of proliferation in both patient groups. In addition, PPD-specific cytotoxicity was also demonstrated in synovial fluid mononuclear cells and CD4+ T-cell clones from the control patient group. Interestingly, the frequency of peripheral blood mononuclear cell reactivity to a recombinant 65kDa heat shock protein of *Mycobacterium bovis* (HSP65) in PPD-responsive rheumatoid arthritis patients was significantly lower than that found in PPD-responsive healthy controls, and specific localisation of HSP65 reactive T-cells to the site of acute synovial inflammation was found in a patient with gout. Given current theories on the potential immunoregulatory role of HSP65 reactive T-cells, these observations may have pathogenetic significance for rheumatoid arthritis.

Explants of synovial membrane from patients with rheumatoid arthritis and other arthritides were cultured under conditions that favour the growth of *in vivo* activated T-cells. Prolonged out-growth of T-cells from synovial explants passaged repeatedly in the presence of recombinant interleukin-2 (100iu/ml) was confined to rheumatoid synovium. Phenotypic and functional characterisation of the synovial explant-derived T-cell lines was performed. Synovial membrane was subject to SDS-polyacrylamide gel electrophoresis and proliferative responses of synovial T-cell lines to autologous synovial constituents investigated using a T-cell immunoblot technique. Clustering of responses to a 114-142 kDa component of autologous synovium was found in 5 out of 6 patients with rheumatoid arthritis, but not in control patients. Components of extracellular matrix proteins, including collagen and fibronectin, were demonstrated in the stimulatory fraction of synovium by western blotting, and cross reactivity between this fraction and type I collagen found in a T-cell line from a single patient.

This study therefore contributes to the understanding of the function and regulation of CD4+ T-cells at the site of pathology in rheumatoid arthritis and indicates novel targets for the therapeutic modulation of CD4+ T-cell effector functions at chronic inflammatory sites.

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1. INTRODUCTION

Identifying the aetiological agent(s) and the pathogenetic mechanisms involved are critical for the development of rational therapy for rheumatoid arthritis (RA). Recent progress in the comprehension of fundamental mechanisms in immunology may have considerable implications for RA and herald the advent of a new generation of anti-rheumatic agents. This review encompasses a spectrum of clinical and scientific publications that provide the background and indicate recent developments in the understanding of the immunological processes involved in the pathogenesis of RA, and their potential therapeutic relevance.

2. CLINICAL FEATURES

2.1. Epidemiology

The global prevalence of rheumatoid arthritis is estimated to be in the order of 1% of the adult population, affecting females predominantly[1]. Geographical variations in the prevalence and severity of disease have been reported[2]; with particular relevance to southern Africa, there has been a perception that RA is rare in black Africans[3]. Earlier reports, on which this perception is based, may have failed to take into account the lack of accessible clinics or hospitals for many disabled patients. More recent epidemiological surveys have revealed that the prevalence of RA in urban black African populations is similar to that reported from the northern hemisphere[4]; however, considerable variation in prevalence rates have been reported in studies of rural African populations (0.1% to 3.0%)[5,6,7]. Higher prevalence rates associated with urbanisation may reflect increased population densities and suggest a transmissible aetiological agent. Historical record and studies of skeletal remains of native North Americans also indicate the possibility of an infectious agent as the cause of RA[8].

2.2 Morbidity and mortality

RA can affect any diarthrodial joint, but typically presents as a symmetrical polyarticular inflammatory arthritis involving joints of the hands, wrists, knees and feet[9]. Extra-articular manifestations occur with variable frequencies and may include abnormalities of virtually every major organ system[9]. There is now considerable evidence that the severity of rheumatoid arthritis has been underestimated, largely due to the application of diagnostic criteria in the past that failed to distinguish between different forms of the disease in population studies[10]. In clinical settings, the application of the revised criteria in studies performed shows that rheumatoid arthritis is associated with significant mortality and rare spontaneous remissions[11]. Accelerated mortality rates in patients with RA have been noted in several studies and are similar to mortality rates in patients with triple vessel coronary artery disease or Hodgkin's

lymphoma[12]. Maximal joint destruction occurs early, within the first two years of disease onset[13]. Hospital-based studies from southern Africa have shown similar disease severity among black RA patients when compared to patients of other race groups in other settings[14,15]. The disease has substantial economic impact; in addition to the high costs of medical care, fifty per cent of those working at the time of diagnosis will be unemployed within 10 years[16].

Several markers that predict a particularly poor outcome in patients with RA are known, and include: high titres of rheumatoid factor, subcutaneous nodules, early bone erosions, polyarticular onset of the disease, older age onset and level of formal education[17]. Recent studies have shown that the combination of HLA DR4 (DR1), positive rheumatoid factor and persistent elevation of CRP indicate a poor prognosis in patients who satisfy the revised ARA classification criteria[18,19].

3. PATHOLOGY

The pathogenic processes in RA are essentially, but not exclusively, centred in the synovial membrane and surrounding structures of diarthroidal joints. Normal diarthroidal joints are lined by a superficial layer of synvoiocytes 1 - 3 cells deep, in a bed of loose vascular connective tissue[20]. Synoviocytes have been classified into types A and B according to their appearance, phenotypic characteristics and suggested function and lineage. Type A synoviocytes have an appearance suggestive of phagocytic function and are thought to be bone-marrow derived cells of macrophage lineage[21]. Type B synoviocytes contain a rough endoplasmic reticulum and Golgi appartus, suggestive of a secretory function, and are thought to be specialised mesenchymal cells of fibroblastic origin[22].

Synovial membrane undergoes dramatic changes during the course of RA. Early disease is characterised by synovial hypertrophy with a predominant increase in type A synoviocytes, although local proliferation of type B synoviocytes may also occur[23,24]. These changes are accompanied by neovascularisation, a range of vascular abnormalities, and subintimal infiltration of lymphocytes which typically form perivascular aggregates[25,26,27,28]. At the junction of synovial membrane and cartilage a wedge-shaped tongue of synovial tissue extending over cartilage has been described and termed pannus[20]. Synoviocytes in this area, particularly those of fibroblastic origin, have a transformed appearance typified by expression of ras and myc oncoproteins[29,30]. In addition, clusters of immature polyhedral cells with large folded nuclei may be found at the site of synovial attachment[20]. As the disease progresses, cartilage and subchondral bone is progressively invaded and destroyed, with increasing villus hypertrophy of synovial membrane[31]. Other cells present in synovium include natural killer

(CD16/CD57+) and dendritic cells (CD14/CD44+); the latter cell type may appear similar to classical follicular dendritic cells found in lymphoid follicles and germinal centres and have enhanced antigen processing functions[27,32]. Polymorphonuclear neutrophils (PMN) are rarely found in synovial membrane in established disease but are the predominant cellular constituent of synovial fluid[31]. End stage rheumatoid arthritis is characterised by loss of cartilage and bone with progressive fibrous ankylosis, accompanied by reduction in vascularity and cellularity[31].

3.1 Cellular products

A wide range of enzymes, cytokines, growth factors and inflammatory mediators have been described in the rheumatoid synovial compartment (TABLE 1.I).

TABLE 1.I. Cellular products identified in rheumatoid synovial compartments*

CELLULAR SOURCE	CYTOKINES & GROWTH FACTORS	ENZYMES / OTHER
Monocyte/macrophage or type A synoviocyte	IL1, IL6, IL8, TNFα, CSF-1 / MCSF, IFNα, GM-CSF, EGF, acidic and basic FGF, Insulin-like growth factor, PDGF, TGFβ,	Eicosanoids, Collagenase, neutral proteinases, MMP -1 -2 -3, stromelysin, TIMP, collagen
Fibroblast or type B synoviocyte	IL1, IL6, IL8, GM-CSF, MCSF, TGFβ,	Collagenase, neutral proteinases, MMP -1 -2 -3, stromelysin, TIMP, collagen
Endothelial cells	IL1, IL8, GM-CSF, MCSF, FGF, PDGF, TGFβ	heparinase, plasmin, tissue plasminogen activator
Platelets	FGF, PDGF	
T-lymphocyte	IL2, IL6, TNFα, IFNγ, GM-CSF, TGFβ	perforin, granzymes
B-lymphocyte / plasma cell	IL6,	Immunoglobulin

* Table adapted from refs [27,33,34,35]

IL(n) - interleukin(n); TNFα - tumour necrosis factor α; CSF-1 - colony stimulating factor; MCSF - macrophage colony stimulating factor; IFNα - interferon α; GM-CSF - granulocyte macrophage colony stimulating factor; EGF - epidermal growth factor; FGF - fibroblast growth factor; PDGF - platelet-derived growth factor; TGFβ - transforming growth factor β; MMP (n) - matrix metalloproteinase (n); TIMP - tissue inhibitor of matrix metalloproteinase.

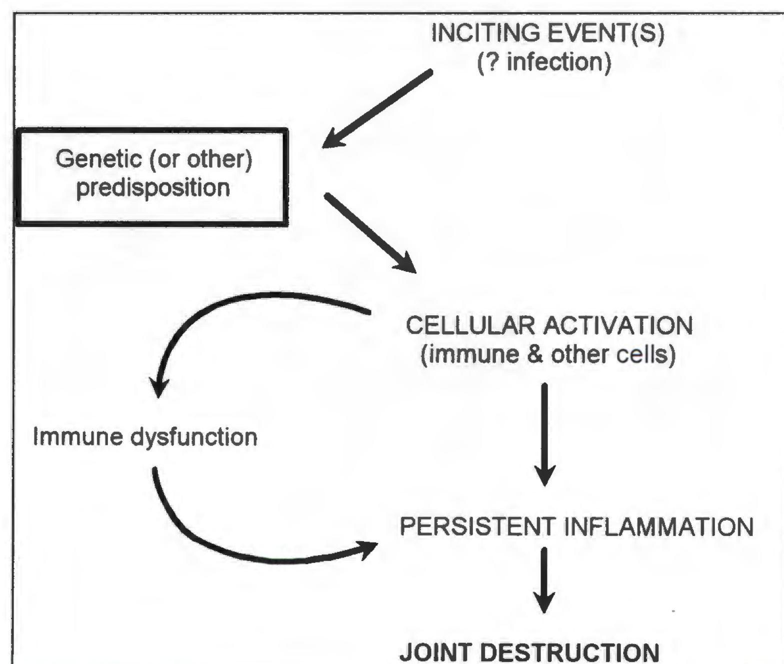
3.2 Lymphocytic infiltrate

The lymphocytic infiltrate in the subintimal layer of synovium includes CD4, CD8 and γδ T-cells and B-cells and plasma cells[28,36]. CD4 T-cells with memory phenotype (CD29+ and CD45RO) predominate and are typically described as clustered around monocyte/macrophages or dendritic cells[37,38,39,40]. Significant quantities of CD4 T-cells are also found in synovial fluid, although the proportion of CD4:CD8 T-cells may differ from that found in synovial membrane[41]. Cytolytic mediators, granzymes and perforins, within the cytoplasm of synovial CD4 and CD8 T-cells have been described in RA[42,43]. Evidence of CD4 T-cell activation include upregulated expression of HLA DR, soluble IL2 R, VLA -1, VLA-4 and CD69 and down-regulated expression of CD2 and CD3 [28,44,45,46,47,48,49]. The presence of CD4 T-cell derived cytokines or messenger RNA, although often difficult to detect and somewhat controversial, has also been cited as evidence of CD4 T-cell activation[50,51,52]. Oligoclonality of T-cell receptor expression in RA synovium is also controversial. Preferential usage of Vβ 14 and Vβ 2 have been reported, but may reflect *in vitro* manipulation of cells[53,54,55,52]. Histological appearances of RA synovial membrane similar to a secondary lymphoid organ or indistinguishable from tuberculosis have been described, and, in the case of the latter, led to the use of gold therapy in RA [56,57].

4. AETIOPATHOGENESIS

It is widely accepted that the cause of RA is multi-factorial, and that a variety of events and mechanisms combine to result in a common chronic inflammatory and destructive process [Figure 1.1]

FIGURE 1.1
Multi-factorial aetio-pathogenesis of rheumatoid arthritis.
(references indicated in text)



4.1. Infectious agents

There is evidence from epidemiological studies that suggests a transmissible agent as the cause of RA (see section 2.1). In addition, investigation of RA in monozygotic twins has indicated that genetic factors contribute in the order of 30% to the development of the disease, thereby implying an important role for environmental factors in its pathogenesis[58]. A wide variety of micro-organisms can indeed cause inflammatory arthritis [TABLE 1.II]; in some cases the arthritis may closely resemble RA.

TABLE 1.II. Infectious aetiological agents of arthritis*

MULTIPLY WITHIN JOINT	LOCALIZE TO JOINT	OUTSIDE JOINT	DIRECT TOXIC EFFECT
Pyogenic bacteria	Borrelia burdorferi	Rheumatic fever / streptococci	Arbovirus
Mycobacteria	Mycoplasma	Shigella	
Fungi	Rubella	Salmonella	
Viruses: small pox	Herpes	Yersinia	
	Hep. B (in immune complex)		
	Mycoplasma (in immune complex)		
	? Reactive arthritis		

* Table adapted from[59]

Several infectious agents have been implicated in the pathogenesis of RA [TABLE 1.III]. These associations have resulted from either the isolation of the organism, its antigens or DNA from joints (or other sites, including blood and stool) of RA patients, or the identification of elevated or persistent immune responses to the organism or its components. In some studies control samples from the joints of OA patients have also yielded evidence of foreign antigens[60]. Thus the presence of a foreign microorganism or its antigens at the site of pathology are not necessarily indicative of a causative association in the disease process. Shared clinical and laboratory features of infections by slow-growing microorganisms and RA have also been cited as evidence for a role of infectious agents in the aetiology of the disease[61].

TABLE 1.III. Organisms implicated in the aetiopathogenesis of RA

BACTERIA	VIRUSES
Clostridia[62]	Adenovirus[63]
Diptheroids[64]	Cytomegalovirus[60,65]
Mycoplasma[66]	Epstein-Barr Virus[67]
Mycobacteria[68]	Parovirus[69]
Streptococci[70]	Retroviruses[71]
	Rubella[72,73]

In order to define a causative association between a microorganism and a disease, fulfilment of Koch's postulates is required. These postulates include: (i) isolation of the microbe from an infected host, (ii) identification of the microbe by culture or direct methods and (iii) transfer of the microbe to a susceptible host, human or animal, results in similar symptoms of disease[74]. The difficulty in fulfilling these postulates to define a causative association between *Borrelia burdorferi* and Lyme arthritis may have implications for identifying an infectious cause of RA[75]. In particular, the causative agent of Lyme arthritis remained undetected for several decades, and *B. burgdoferi* is infectious only in the minority of cases.

4.1.1 Current models of infectious agents in the pathogenesis of RA

Several models have been proposed to address the potential pathogenic mechanisms involved in the association between infectious agents and RA. [Figure 1.2] Although indicated as separate models, a combined mechanism involving aspects of persistent local infection, molecular mimicry and superantigenic stimulation is feasible.

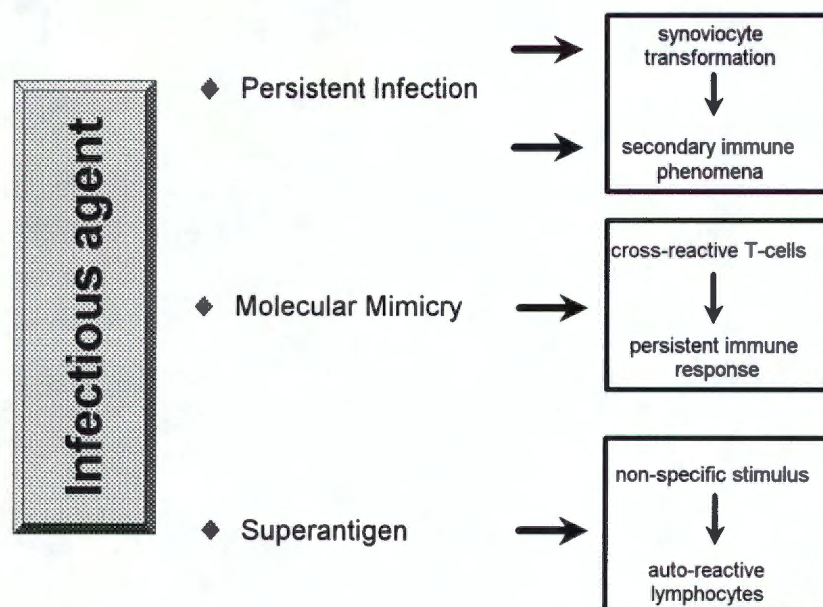


FIGURE 1.2
Current paradigms of the infectious aetiopathogenesis of rheumatoid arthritis.
(References indicated in text)

a) *Persistent infection:* the persistent presence in the synovial compartment of a foreign microbe, either actively replicating or in a latent phase of growth, may result in persistent local immune activation and chronic inflammation. Lyme arthritis and possibly reactive arthritis, where the antigens of microorganisms can be identified within the synovial compartment, are examples of this 'permanent hit' model[74,76]. At present there is no consistent evidence of microbes in RA joints, although limitations of isolation techniques may partly be responsible. It has been suggested that PCR technology will contribute considerably to the identification of infectious agents, particularly viruses, in RA[77]. Retroviral-like particles have been identified in synovial fluid samples of a small number of RA patients[71]. Their discovery has led to the suggestion that retroviral activation of fos and myc oncogenes in synoviocytes may be an important initiating event in RA, leading to the transformation of synoviocytes into an aggressive invading component of pannus, and giving rise to a secondary immune response[34].

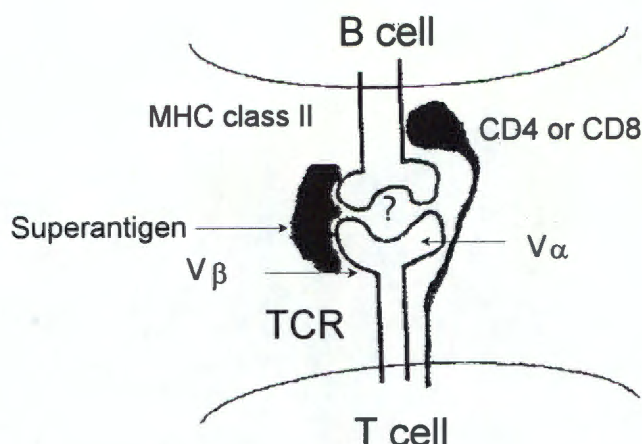
b) *Molecular mimicry:* this paradigm invokes triggering of an immune response by an initial infection, which may subsequently be cleared. However, the activated immune response may be maintained by the recognition of self components with similar (cross-reactive) antigenic structures to the initiating pathogen or its products[78]. Cross-reactivity between bacterial 65kDa heat shock proteins (HSP65) and human 60 kDa heat shock proteins or between EBV glycoprotein and HLA DR4 have been proposed as important pathogenic mechanisms that conform to this paradigm of RA[79,67]. The role of HSP65 in RA is controversial and is discussed in the review on mycobacteria and RA (pg. 11) and in chapter 5 (pg. 126). A short sequence of homology between HLA DR4 and EBV glycoprotein gp110 has been identified, but its potential role in the pathogenesis of RA, particularly activation of CD4 T-cells, requires further delineation[74].

c) *Superantigen stimulation:* Given the ability of superantigens to activate a large proportion of T-cells in a non-specific manner (figure 1.3), a potential role for these bacterial and viral products in the pathogenesis of RA has been suggested[80]. Stimulation of quiescent but potentially autoreactive T-cells, which clearly exist in normals, may give rise to persistent autoimmune T and B-cell responses[81,82,83]. Restricted TCR Vβ usage has been reported in synovial T-cells in RA, and suggested to be indicative of prior superantigenic stimulation[84,53]. However, the finding of restriced TCR Vβ usage in RA synovium is not universal[54]. In addition, the effect of prior superantigenic stimulus on subsequent antigen-specific responses by T-cells is unclear; anergy or unresponsiveness may be the result[85,86]. Conversely, evidence from an animal model of autoimmune disease indicates that a superantigen is capable of causing disease relapse, thereby implying intact *in vivo* function of post superantigen-stimulated T-cells[83].

FIGURE 1.3

Superantigenic stimulation of T cells. Binding of a specific region of the Vβ chain, distinct from the peptide binding groove, may activate a population of T cells with diverse antigenic specificities.

(figure reproduced from [80])



4.1.2. Reactive arthritis: implications for RA

Classical reactive arthritis is usually defined as inflammatory arthritis that occurs approximately four weeks following infection by a specific microorganism, with a typical pattern of involvement, systemic features and strong HLA class I association[87]. Wider definitions have included rheumatic fever and Lyme disease, and reactive arthritis shares several common features with other spondarthropathies including psoriatic arthritis and ankylosing spondylitis[88]. Indeed, it has been suggested that ankylosing spondylitis is a form of reactive arthritis caused by Klebsiella[89].

TABLE 1.IV. Differences between RA and reactive arthritides*

	RA	Reactive Arthritis
Microbiological organisms recognised	No	Yes
Peripheral joint destruction	Typical	Rare
Sacroiliac involvement	None	Frequent
Association with skin infections	None	Frequent
rheumatoid factor production	Frequent	None
Response to antibiotic treatment	None	Yes
HLA linkage	Class II, DR4	Class I, HLA B-27
Clinical course upon HIV infection	Remission	Severe

*Table reproduced from [74]

While there are several distinct differences between classical reactive arthritis and RA (TABLE 1.IV), similarities in the histological appearance of synovium have been noted[90]. In common with RA, the pathogenesis of reactive arthritis would appear to involve an environmental trigger in individuals with a genetic predisposition (HLA association), leading to cellular and humoral immune activation and the development of chronic inflammatory

arthritis[88]. Earlier theories invoked antibody cross-reactivity between microbes and HLA-B27 as a mechanism for the persistent immune activation, although this does not explain localisation to synovial joints[91]. Recent studies have indicated the presence of inciting organisms in the joints, usually in low numbers and perhaps latent forms[92]. The distinction between reactive and septic arthritis may thus be a function of the intra-articular pathogen load and rate of multiplication (figure 1.4).

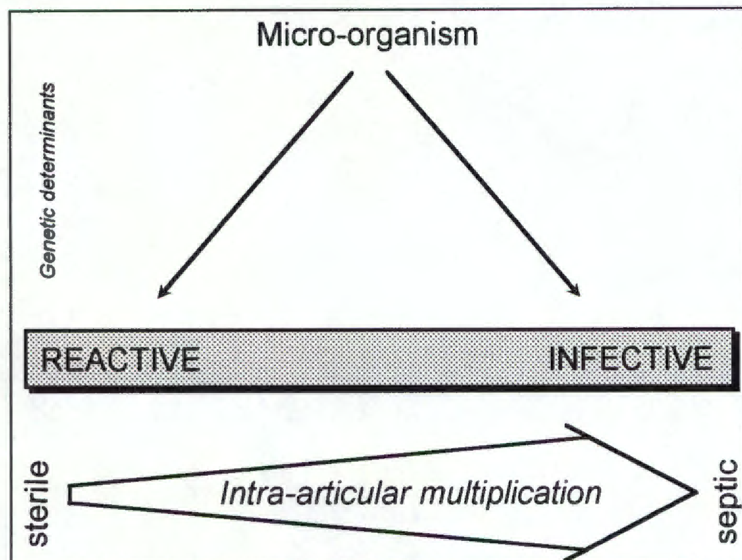


FIGURE 1.4
Reactive and septic arthritis may represent extremities of a continuum that reflects the rate of intra-articular multiplication of the initiating infectious agent

4.1.3 Mycobacteria

Mycobacteria and RA have had a long and complex association. Historically gold therapy was first used to treat RA based on similarities in histological appearance of RA and tuberculous tissue, and its perceived beneficial effects when used to treat RA patients with tuberculosis[57]. Mycobacteria are capable of causing inflammatory arthritis in both experimental animal models and in humans. Immunization of susceptible strains of laboratory rodents with dead *Mycobacterium tuberculosis* in oil (Freund's adjuvant) causes progressive polyarthritis of small joints of extremities and extra-articular phenomena, an experimental disease termed adjuvant arthritis[93,94]. Although there are some important differences, adjuvant arthritis has been widely accepted as a laboratory model of RA. Transfer of the disease to naive rats can be achieved using T-cell clones specific for aminoacids 180-188 of the HSP65 of *M. tuberculosis* that cross-react with cartilage proteoglycan[95]. Interestingly, prior immunisation with this peptide or the whole HSP65, can also confer protection against the development of adjuvant arthritis or other animal models of autoimmune diseases[96,97,98].

In humans, *Mycobacterium tuberculosis* infection can cause septic arthritis, with bacilli readily identifiable in affected joints, usually axial skeleton or single peripheral joints[99]. Rarely a reactive-type polyarticular inflammatory arthritis, without identifiable intra-articular bacilli

and similar to RA can occur in individuals with tuberculosis, and has been termed Poncet's disease[100,101,102]. BCG immunization or therapy for bladder cancer has also been associated with the development of inflammatory arthritis[103].

Several features shared by RA and the mycobacterioses have been cited as evidence for a causative association [TABLE 1.V]. Further evidence has included proliferative and cytotoxic responses of RA synovial T-cells to mycobacterial antigens, including HSP65, and the identification of mycobacterial antigens in synovial joints[104,105,106,107,68]. In addition, γδ T-cells specific for HSP65 have also been identified in RA synovial fluid[108]. Thus it was suggested that T-cells activated by mycobacterial HSP65 may cross-react with the human homologue, present in significant amounts in affected joints, thereby leading to a persistent autoimmune response[109]. However, the role of mycobacteria in the pathogenesis of RA is not clear; recent evidence has indicated that mycobacterial antigen-reactivity is not specific to RA, but is a feature of chronic inflammatory sites of diverse aetiology[110]. It has also been suggested that lower than normal responses to HSP65 in RA, found in a recent study, may be relevant to the immunopathogenesis of the disease[111].

TABLE 1.V. Shared features of rheumatoid arthritis and the mycobacterioses*

Lesions	Systemic illness Synovitis Skin nodules with eosinophilic necrosis Abnormal alveolar lavage
Immunogenetics	Partly genetic, some HLA links Possible restricted usage of T-cell receptor Vβ sequences Different incidences in males and females
Serology	↑rheumatoid factor ↑autoantibodies ↑agalactosyl IgG ↑antibody to mycobacterial HSP65 Serological changes in spouses
Epidemiology	Transmission of disease more to children than to spouse Cause is about 70% environmental (ca 30% genetic)
Skin-test responses	↓response to common mycobacterial antigens ↑response to tuberculin in DR4+ (RA and leprosy)
Endocrinology	Improvement in pregnancy and worsening post partum Defective cortisol response

Table reproduced from[111]

4.2. Genetic Predisposition

Classical genetic studies have indicated that a genetic component is relevant in the pathogenesis of RA. One third of the overall inherited risk has been ascribed to sex and an equal proportion to HLA haplotype[112].

4.2.1 HLA and RA

A strong association between RA and HLA-DR4/DR1 subtypes has been found in most populations studied[113,114,115,116]. In addition, DR4 homozygosity has been associated with juvenile RA, Felty's syndorme and severe adult RA[117,118,119]. The molecular basis for the association has been addressed in the shared epitope hypothesis[120]. DR4, DR1 and DR6 subtypes associated with RA share a common pentapeptide in the third hypervariable region of the HLA-DR-β-1 chain, a region that lines the peptide binding cleft of the HLA molecule, and may thus influence the nature of the peptide being presented to CD4 T-cells[121,122]. Additional functions of class II HLA in shaping the T-cell repetoire and as a ligand for superantigens may have relevance in the association of HLA-DR4 and RA[123,124,125]. However, DR subtypes with the shared epitopes are found in 45% of caucasian populations without RA and the short arm of chromosome 6 contains approximately 70 other genes, in addition to HLA genes, which may exhibit linkage disequilibrium with DR4 subtypes[126,127]. In particular, transporter proteins encoded by the TAP2 gene localised to this region may have relevance in RA[112]. Cytokine gene polymorphism may also contribute to the inherited risk for the development of RA[128].

4.3 Angiogenesis

Cartilage in normal diathroidal joints is avascular[129]. In early RA neovascularisation is a prominent feature in pannus tissue, and may contribute to the maintenance of the chronic inflammatory state by several mechanisms[29]. These mechanisms include the ability of endothelial cells to regulate the inflammatory cell influx and produce several soluble factors that have local effects on these cells[130,131,132,133,134]. Normally angiogenesis is tightly regulated by a dominance of inhibitory over stimulatory factors. The neovascularisation that occurs in RA pannus may represent a shift in favour of stimulatory factors that include heparin-binding growth factors, angiogenin, IL1, IFNα and TGFβ[135]. It has been suggested that a cartilage-derived matrix metalloproteinase inhibitor that is responsible for the inhibition of angiogenesis in normal cartilage may have therapeutic relevance in RA[136].

4.4 Cytokine-hypothalamic-piturity-adrenal axis (C-HPA axis)

The endocrine and immune systems have complex interactions that combine to regulate the response to stress or other stimuli[137]. In particular, stimulation of the HPA axis by pro

inflammatory cytokines may be important in the control of an acute inflammatory response (FIGURE 1.5). Evidence for endocrine involvement in the pathogenesis of RA includes the female preponderance of the disease and the influence of pregnancy and postpartum period on disease activity, although the precise role of sex hormones remains unclear[138]. Small animal models of autoimmune diseases are uniformly associated with abnormalities in HPA function[139]. Abnormalities in HPA function in RA have also been noted, and have been ascribed to hypothalamic dysfunction[140]. The defect results in subnormal cortisol secretion, causing an imbalance favouring the immune stimulatory effects of prolactin over the immuno-suppressive effects of corticosteroids[141]. Given the differential susceptibility of CD4 T-cell subsets to cortisol, normal secretion of this hormone favours a Th2 response that is capable of inhibiting rheumatoid synovitis by IL4 production[142]. Subnormal cortisol secretion in response to infection or other initiating stimuli may result in prolonged Th1 responses. A possible role for dehydroepiandrosterone (DHEA) in opposing glucocorticoid effects and promoting Th1 responses in RA has also been suggested[143,144,111].

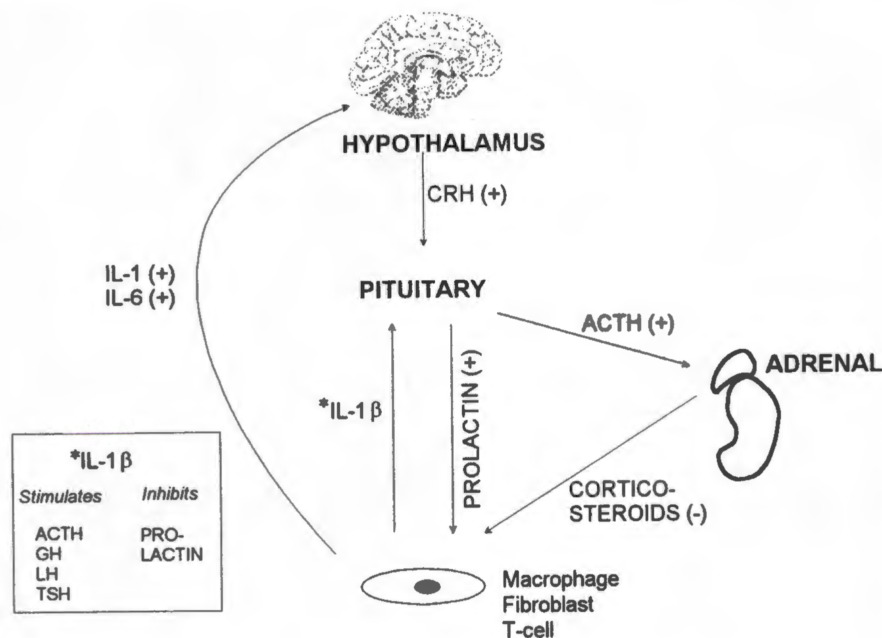


FIGURE 1.5
The cytokine-hypothalamic-piturity-adrenal axis
ACTH - adrenocorticotrophic hormone; GH - growth hormone; LH - leutinizing hormone; TSH - thyroid stimulating hormone
IL1 secretion indicated refers to macrophage and fibroblast secretion and not T-cell.
(references indicated in text)

4.5 Other factors that mediate joint damage

A state of relative hypoxia can occur within rheumatoid joints. Progressive accumulation of fluid causes marked intra-articular hypertension, reducing blood flow to hypertrophied synovium and leading to ischaemia and focal infarction[31]. It has been suggested that reperfusion injury may contribute significantly to joint damage in RA[145,146]. Free radicals released by activated macrophages, granulocytes or endothelial cells may mediate damage by several mechanisms[147].

5. IMMUNOPATHOGENESIS

Earlier models of the pathogenesis of RA focused on the role of rheumatoid factor production by B-cells and invoked immune complex-mediated damage as a prime pathogenetic mechanism in rheumatoid joints[148]. Current paradigms favour complex interactions, mediated by a wide range of soluble factors, between T-cells, monocyte/macrophages and synoviocytes[20,33,34,35,52]. The relative importance of each cell type and its products in initiating and perpetuating the disease process is the subject of considerable debate[20,33,52,149].

5.1. T-cells in RA

5.1.1 Evidence for a role in RA

There is a substantial body of evidence that supports a role for T-cells in the pathogenesis of RA. CD4+ T-cells are a significant component of the cellular infiltrate in rheumatoid synovium and their typical perivascular aggregation and expression of surface markers compatible with a state of activation (see earlier) have been cited as indications of their role in RA[52]. Further evidence includes the strong HLA class II association with RA, especially in view of current understanding of the role of class II molecules in peptide presentation to CD4 T-cells[112,122]. The presence of T-cell cytokines is somewhat controversial, although it would appear that IFN-γ and IL2 messenger RNA in RA synovium can be detected with the use of the polymerase chain reaction (PCR)[35].

Several parallels have been drawn between models of diseases with known antigen-driven T-cell participation and RA[35,52,149]. Rheumatoid synovium and tuberculous tissue may have similar immunohistological appearance[57]. In tuberculosis, tuberculoid leprosy and leishmaniasis, T-cells specific for the inciting antigen occur in low frequencies at the site of pathology, such that the detection of oligoclonality by current techniques would be difficult[150,151,152]. In addition, the majority of T-cells from tuberculous effusions are not activated, according to IL2-R and HLA-DR status, and are in the G0-G1 phase of the cell cycle [P.T. Lukey, personal communication]. The detection of oligoclonality or limited T-cell receptor usage of T-cells from RA joints has yielded conflicting results and in the light of the above would appear unnecessary as evidence of their role in the disease[35].

Therapeutic manipulation of CD4 T-cells modulates the disease. IL-2 therapy for malignancy has been associated with reactivation and flares of RA[153]. Thoracic duct drainage, total lymphoid irradiation and lymphocytopharesis cause significant improvement in disease activity[154,155,156]. In addition, Cyclosporin A and monoclonal antibodies directed at various antigens on CD4 T-cells may have similar beneficial therapeutic effects in RA[157,158]. It has been suggested that the resolution of pre-existing RA in patients developing AIDS is indicative

of the role of CD4 T-cells in the disease although effects of HIV on other immune functions may be relevant[34]. Evidence of T-cell involvement in several experimental animal models of arthritis has also been cited in support of a role for T-cells in RA[52,149]. Perhaps the most compelling indications come from adoptive transfer experiments in which even small numbers of CD4 T-cell clones are capable of transferring adjuvant arthritis to otherwise healthy animals[159,160].

5.1.2. The nature of the T-cell stimulus

Candidate antigens: While there is evidence to support the hypothesis that RA is an antigen-driven, T-cell mediated disease, the nature of the rheumatoid antigen remains elusive. Locally sequestered microbial antigens or persistent infection may implicate foreign antigens as the source of the T-cell stimulus (see infectious causes)[74,161]. Alternatively, self-reactive T-cells, activated non-specifically or by cross-reactive microbial antigens, may be relevant. While clonal deletion may exclude most potentially self-reactive T-cells in the thymus, it is evident that many autoreactive cells escape this mechanism. Potentially auto-reactive T-cells to several immunologically 'noisy' proteins have been identified in normal individuals, including responses to myelin basic protein, heat-shock proteins, acetylcholine receptor and thyroid peroxidase[162,163,164,165]. These findings appear to be confined to *in vitro* studies, and autoreactive T-cells may be suppressed *in vivo* in normals by several potential mechanisms, loosely termed peripheral tolerance[165,166]. Peripheral tolerance may be the result of anergy or apoptosis of autoreactive cells when stimulated by their specific antigen, or may be due to a network of complex regulatory pathways[167,168,169,170,171]. In any event, activation of T-cells that react to self antigens *in vivo* may require disruption of tolerance mechanisms. Several self-antigens have been proposed as the rheumatoid auto-antigen, including native type II collagen, cartilage proteoglycan and as yet unspecified chondrocyte antigens[172,173,174,175]. T-cell responses to type II collagen have been demonstrated in RA[176]. However, the lack of DR-restriction of collagen-reactive T-cells and the suggestion that pepsin contaminants may have been responsible for some of the earlier findings raise some doubts about collagen as the rheumatoid antigen[176,177]. The immunologically priveleged nature of cartilage has led to the suggestion that the rheumatoid antigen may be a component of cartilage[178]. Screening of chondrocyte expression libraries for evidence of T-cell reactivity is currently in progress.

Clues from antigen processing and presentation: cells of several lineages in rheumatoid synovium display marked upregulation of class II HLA expression, possibly as a result of IFN-γ or other cytokine stimulation[161]. In addition, the rheumatoid synovium is enriched with activated and mature dendritic cells that have enhanced antigen-presenting capabilities[32]. Taken together these findings are indicative of active antigen processing and presentation to CD4 T-cells in the rheumatoid joint[179]. In general, peptides presented by the class II pathway

are derived from exogenous protein sources, in contrast to cytoplasmic products that are presented in association with class I HLA[180]. The majority of peptides occupying the groove of the MHC molecules are derived from self constituents[181,182,183]. The molecular basis of HLA-DR4 association with RA has suggested that an important region of the peptide binding groove may govern the nature of the antigenic peptide being presented to pathogenic CD4 T-cells[120]. Recent advances in the understanding of peptide-HLA interactions may contribute significantly to the identification of the elusive rheumatoid antigen. However, unlike HLA class I bound peptides which are of 8-10 amino acids in length with fairly rigid allotype-specific binding motifs containing between one and three anchor positions, class II bound peptides are of variable length (10-34 amino acids) with a higher degree of allelic promiscuity[180,184,185,186]. The recently-solved DR1 structure has indicated that the class II groove is open ended; the variation in bound peptide length may be due to portions of the peptide extending beyond the edges of the groove[122]. At least three anchor positions have been defined for class II bound peptides, although as few as 2 and as many as 6 different amino acids may be found at each anchor position[187,188]. A fairly conserved and deep hydrophobic pocket binds aromatic or aliphatic residues near the amino terminal of the peptide, forming the first anchor position. Other anchor positions have been defined at residues 4 (hydrophobic) and 6 (small amino acid). In addition to variable residues at anchor positions, allelic promiscuity may result from the fact that not all anchors need to be used by a specific ligand[189]. Nevertheless, investigation of DR4-bound peptides in RA patients is currently being actively pursued and may have therapeutic relevance, particularly if relevant CD4 T-cell responses can be elicited[190]. Recent advances in the understanding of the interaction of HLA-DR2 and myelin basic protein-derived peptides in patients with multiple sclerosis may have significance for RA[191].

Non-specific stimulus: It is now well-established in known antigen-specific models of chronic inflammation that the vast majority of T-cells at the site of pathology are not specific for the inciting antigen[150,151,152]. Non-specific accumulation of "memory" CD4 T-cells (CD4+ CD45 RO CD29+) at chronic inflammatory sites, including RA synovium, may be due to the increased expression of adhesion molecules (ICAM-1, ELAM-1 and VCAM-1) on endothelial cells[192,193,194]. These adhesion molecules bind their specific ligands (LFA-1,asylyl Lewis-X, VLA-4) which are expressed in increased quantities on memory CD4 Tcells, initiating a process that results in transendothelial migration of the T-cells and binding to cellular and extracellular components at the inflammatory site[195,196]. Release of granzymes and other proteolytic enzymes by the T-cells may be important in transendothelial migration, resulting in cleavage of basement membrane collagen type IV[197]. Once present at the site of pathology, non-specifically recruited memory CD4 T-cells may (i) remain quiescent and not participate in the inflammatory process - the so-called bystanders at the fire, (ii) become activated by a variety of non-specific mechanisms including cytokines, inflammatory mediators, adhesion to

extra-cellular matrix, and homotypic adhesion, and (iii) exert an effect even in their resting state[33,35,198,199]. It is also possible that non-specifically recruited memory CD4 T-cells may encounter their specific or cross-reactive antigen in the joint, thereby initiating antigen-specific responses[161].

5.1.3. Pathogenetic role of CD4 T-cells in RA

The current paradigm invokes a central role for CD4 T-cells in the initiation and perpetuation of the disease process in RA[35,52,149,200]. Stimulation of the pathogenic T-cell initiates a cascade of events that results in activation of macrophages and synoviocytes, the main effector cell populations, and the subsequent release of a wide range of soluble factors including cytokines, growth factors, enzymes and arachidonic acid metabolites (Figure 1.6). Some of these factors combine to promote the formation of pannus and activate osteoclasts, together contributing to erosive destruction of cartilage and bone[20]. Several autocrine loops may amplify the responses, although there is evidence of potential negative feedback regulation, including the presence of an IL1 receptor antagonist and the anti-inflammatory effects of certain cytokines (see 5.3)[201,202]. It is not yet known whether the pathogenic CD4 T-cells are activated in the circulation and then home to the joints, or are activated in the joints themselves[35]. According to the T-cell paradigm of the immunopathogenesis of RA, the persistent autoimmune response is also centrally controlled by CD4 T-cells, and may include the effects of non-specifically activated CD4 memory T-cells localised to the chronic inflammatory site[35,149].

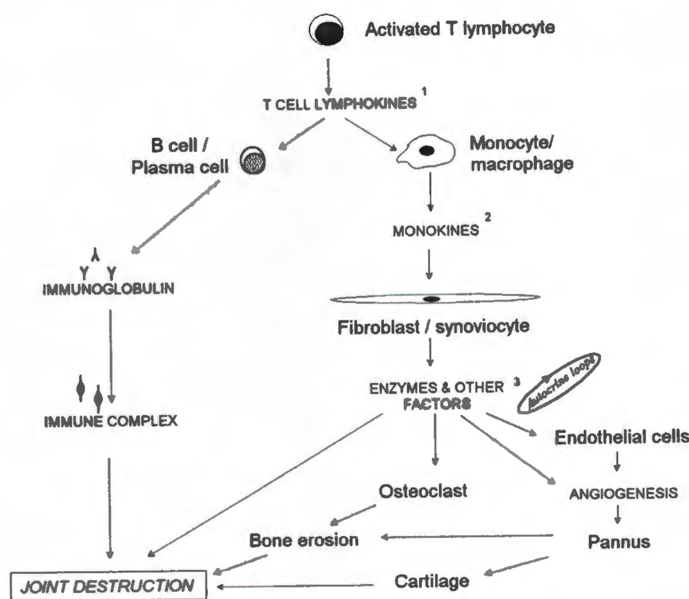


FIGURE 1.6
Immunopathogenesis of Rheumatoid Arthritis: T-cell Centric Paradigm
(References indicated in text)

1 **T-cell lymphokines**: IL-2, IFN-γ, IL4, IL5; 2 **Monokines** (and growth factors): IL1, tumour necrosis factor α, granulocyte macrophage colony stimulating factor (GMCSF), IL6, platelet derived growth factor, acidic and basic fibroblast growth factors, transforming growth factor (α and β); 3 **Enzymes and other factors**: collagenase, matrix metalloproteinase, metalloproteinase, stromelysin, tissue inhibitor of metalloproteinase, IL1, IL6, GMCSF, epidermal growth factor and eicosanoids.

CD4 T-cell subsets: Three subsets of murine and, more recently, human CD4 T-cell clones have been defined according to their cytokine production profile (TABLE 1.VI)[203,204]. Th1 and Th2 represent reciprocal subsets that are mutually antagonistic. Additional functional distinctions also exist between these two subsets: Th1 cells are primarily involved in interactions with members of the phagocytic cell system (monocytes and macrophages) and Th2 cells with B-cells and antibody production[205]. Important differences in signalling pathways between these two subsets have been identified by investigating their responses to prostaglandins (see section 5.5)[206,207]. Macrophage-derived interleukin-12 (IL 12) is probably the most potent stimulus of Th1 responses[208]. Th0 cells are a hetergenous population that may have predominant Th1 or Th2-like functions or a combination thereof[206]. At present, data of CD4 T-cell subset prevalence in RA is somewhat conflicting, although analysis of synovial T-cell clones has indicated a predominant Th1 infiltrate[209,210,211]. Additional evidence in support of a role for Th1 cells in the pathogenesis of RA includes the presence of intracellular perforins and granzymes in RA synovial CD4 T-cells (indicating cytolytic potential that is usually confined to Th1 cells) and the fact that the predominant Th2 product, IL4, actually inhibits rheumatoid synovitis[42,43,212,213,142].

TABLE 1.VI. CD4 T-cell Subsets: Cytokine profiles*

TH1	TH2	TH0
IL-2	IL-4	IL-2
IFN-γ	IL-5	IFN-γ
TNFα	IL-6	IL-4
TNFβ	IL-10	IL-5
GM-CSF	GM-CSF	TNFα
IL-3	IL-3	TNFβ
IL-10		GM-CSF
IL-6		IL-3
		IL-10
		IL-6

*Table adapted from [204,214,215,216]

CD4 T-cell mediated cytotoxicity: Although CD4 T-cells are traditionally termed helper T-cells (reflecting their cytokine production) and CD8 cells cytotoxic T-cells, it is now well-established that these functional distinctions are no longer valid. A subset of CD4 T-cells is capable of mediating antigen-specific, class II HLA-restricted cytotoxicity, and CD8 T-cells are capable of cytokine production and providing 'help' for B cell immunoglobulin synthesis[212,217,218,219]. CD4 T-cell cytotoxicity is almost exclusively confined to Th1 and Th0

cells and exhibits a positive linear relationship with the amount of IFN-γ production[208,220]. Cytotoxic CD4 T-cells are thought to be important in the immune response to intracellular pathogens where they mediate cytolysis of infected monocyte/macrophages resulting in release of the pathogen to alternate defence mechanisms[221]. Two distinct pathways (calcium dependent and calcium independent) may be involved in mediating T-cell cytolytic function [222,223,224,225]. The calcium dependent pathway is probably effected by perforins and granzymes (serine protease constituents of cytoplasmic granules)(FIGURE 1.7a)[226,227]. Following specific binding with the target cell, cytoplasmic granules are transported to the binding pole in the cytotoxic cell by the action of microtubules[228]. The granules are then exocytosed into the intercellular space and the released perforin polymerizes in the target cell membrane causing a series of holes 15nm in diameter, and leading to osmotic rupture of the target cell[229]. In addition to cytolysis, granule constituents of cytotoxic T cells may also mediate apoptosis[230,231,232]. A calcium independent pathway of T-cell cytotoxicity has been described and involves the binding of fas antigen, expressed on the surface of the target cell, and its ligand on the T-cell (FIGURE 1.7b)[223,224]. The fas ligand has been cloned and is a type II trans-membrane protein of the TNF receptor family[233]. Binding of the fas antigen and its ligand results in apoptosis, and subsequently lysis, of the target cell[234]. Cytolytic granules have been demonstrated within RA synovial CD4 T-cells and an isolated previous report has indicated cytolytic function of mycobacterial antigen-reactive CD4 T-cell clones from RA synovial fluid[42,43,107]. The current paradigm of CD4 T-cell mediated pathogenesis of RA does not address the potential role of CD4 T-cell mediated cytotoxicity.

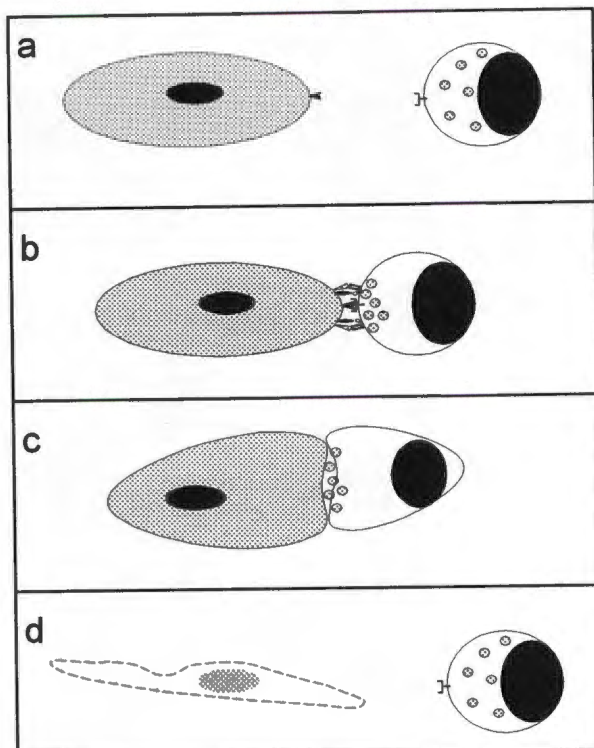


FIGURE 1.7

Mechanisms of T-cell mediated cytotoxicity: (a) Granule exocytosis model

a) Resting T-cell containing cytotoxic granules distributed randomly throughout the cytoplasm.

b)Specific target cell recognition: receptor/ligand binding and polarization of cytotoxic granules.

c) Delivery of the 'lethal hit'. Conjugate formation is associated with morphological changes. Cytotoxic granules are released into the intercellular space. Perforin, a constituent of the granules, polymerizes in the target cell membrane, creating multiple pores. Other components of the cytotoxic granules may mediate apoptosis.

d) Target cell cytolysis occurs due to increased osmotic gradient, but may also be secondary to apoptosis.

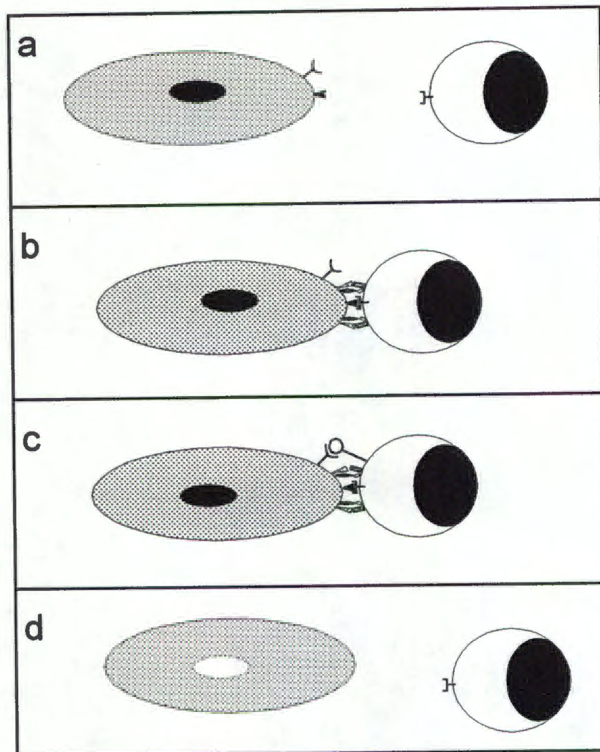


FIGURE 1.7

Mechanisms of T-cell mediated cytotoxicity: (b) Fas and fas ligand interaction model

a) Fas antigen is constituitively expressed on potential target cells

b) Specific target cell recognition: receptor/ligand binding of adhesion molecules

c) T-cell fas ligand expression follows specific triggering of activation pathways through the T-cell receptor

d) Fas/fas ligand binding results in target cell apoptosis prior to cytolysis

(References are indicated in the text)

5.2. Macrophage and Synoviocyte centric paradigms of RA

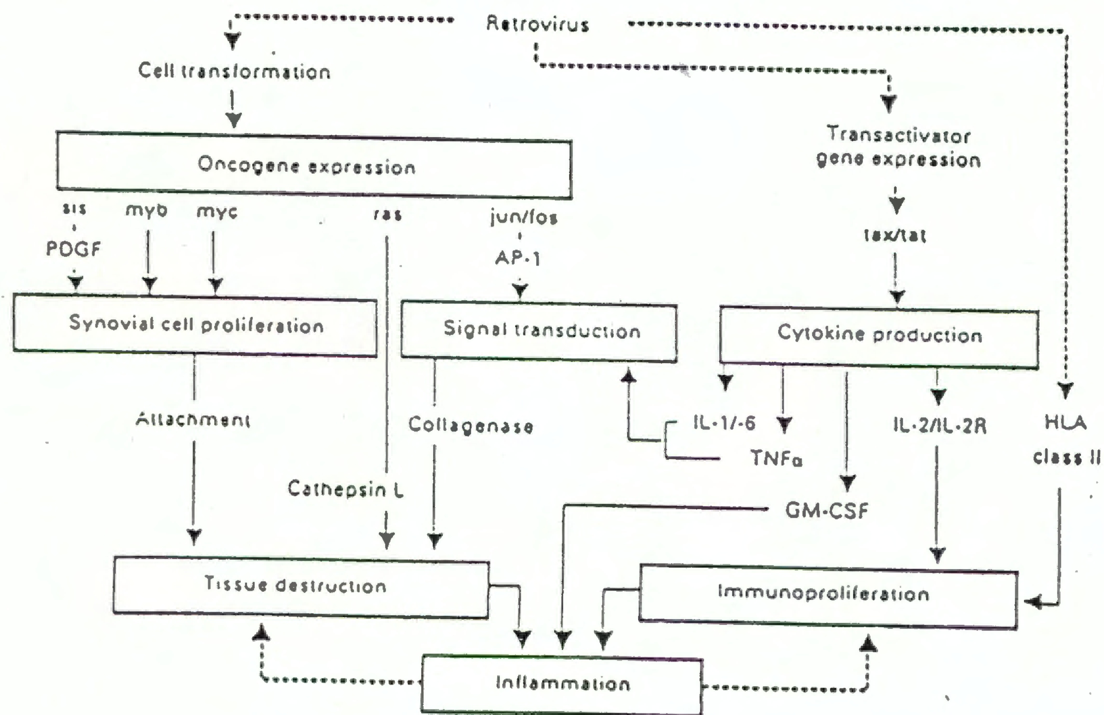
T-cell independent models of the pathogenesis of RA have been suggested[20,33,34]. These models focus on activated synovial macrophages and fibroblastic-origin synoviocytes as the centre of complex autocrine and paracrine pathways and suggest that T-cell responses are secondary to initial destructive processes. Evidence cited in support of these models include (i) prominent macrophage activation compared to T-cell activation status, (ii) abundant macrophage as opposed to scarce T-cell derived cytokines in rheumatoid joints, (iii) the ability of GMCSF and TNFα to upregulate class II HLA expression, independently of IFN-γ, (iv) MRL lpr/lpr mouse model of arthritis, where proliferation, attachment and invasion of transformed synoviocytes preceeds inflammatory cell infiltrate, and (v) the spontaneous development of erosive arthritis in TNFα transgenic mice that is associated with markedly increased TNFα production and cured by anti-TNFα monoclonal antibody therapy[33,235,236,237,238]. The effectiveness of anti-TNFα monoclonal antibody therapy in RA may also support this view[239]. It has been suggested that retroviral infection may be responsible for initiating the pathogenetic process by stimulation of synoviocyte oncogenes that are responsible for transcriptional control of collagenase and stromelysin (FIGURE 1.8)[34].

While it is generally accepted that monocyte/macrophages and synoviocytes contribute the main effector mechanisms of damage in the rheumatoid joint, in defence of a T-cell centric model of RA and in addition to evidence noted above, it has been argued that evidence of macrophage activation and monokine production is also prominent in known antigen-specific T-cell driven chronic inflammatory sites[35,149]. In addition, even a few CD4 T-cells secreting small amounts of cytokines can be responsible for a marked inflammatory response and CD4 T-cells may also be the source of GM-CSF, TNFα and IL6 (see table 1.VI)[149]. Impaired T-cell activation and low levels of T-cell cytokines in the synovial compartment may be due to the inhibitory effects of macrophage products, including prostaglandins and TNFα [240,241].

FIGURE 1.8

Oncogene activation in synoviocytes may be the result of retroviral infection and contribute to local tissue destruction through the effects of collagenase and cathepsin L.

Figure reproduced from [34]



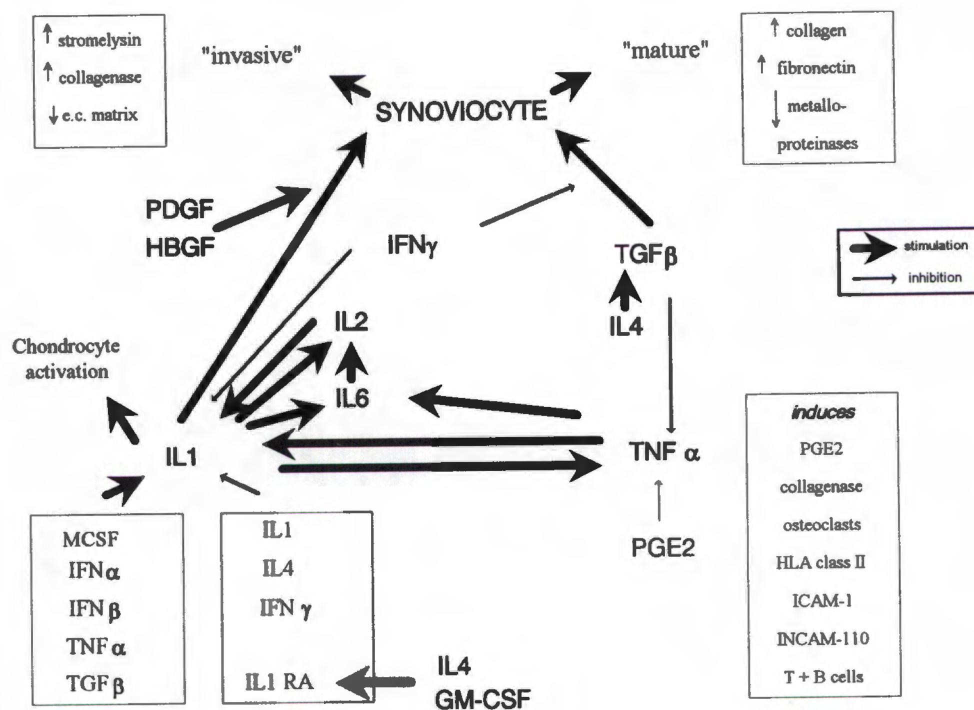
5.3. Cytokines

A wide range of cytokines, growth factors and their inhibitors have been identified within rheumatoid joints, either in the protein form or at the mRNA level (see table 1.I). It is likely that these factors play an important role in the pathogenesis of RA by a variety of mechanisms. A complex network of synergistic and antagonistic interactions between cytokines may determine their final effects on fibroblast-derived synoviocytes and local inflammatory processes in rheumatoid joints (FIGURE 1.9).

FIGURE 1.9

Cytokine network in rheumatoid arthritis. Complex synergistic and antagonistic interactions of cytokines in the synovial compartment may combine to promote invasive transformation or, conversely, maturation of fibroblast-derived (type B) synoviocytes.

Figure derived from [242,243,244,245,246]



IL1 and TNFα are probably the most important cytokines with regard to cartilage and bone destruction in RA[247]. Intra-articular injection of either of these cytokines into rabbit knees results in acute inflammatory arthritis with leukocyte infiltration and cartilage damage, the latter being more marked with IL1 than TNFα[248,249]. IL1 (α or β) upregulates adhesion

molecule expression on endothelial cells and also stimulates chondrocytes[247]. Correlation of IL1 levels, DR expression and X-ray evidence of joint damage has been found in RA[250]. In addition to IL1-like effects, TNFα activates osteoclasts and upregulates class I and class II MHC expression on macrophages and synoviocytes[247,251]. IL1 and TNFα stimulate the release of each other and are found together in several other inflammatory arthritides; a hierachy of cytokine production in which TNFα induces production of IL1, IL6, IL8 and GM-CSF has been suggested[247,252,251]. Genetic variations in the promoter region of IL1 and TNFα genes may influence the magnitude of the cytokine response to a particular stimulus and increase the risk of autoimmunity[128].
IL6 in RA is mainly produced by synovial macrophages, although synovial T and B-cells sources have also been identified[253]. It has been suggested that IL6 is a mediator of the acute phase response; in support of this hypothesis correlation between serum levels of IL6 and C-reactive protein in RA have been reported[254]. Given the immunoglobulin-inducing activity of IL6, it has also been suggested that this cytokine may be an important stimulus for rheumatoid factor production[255].

IL8 is a potent chemoattractant that is present in synovial fluid in RA and correlates with the number of neutrophils present[256,257]. Several growth factors, including epidermal growth factor (EGF), acidic and basic fibroblast growth factors (aFGF and bFGF) and platelet-derived growth factor (PDGF) have been detected in rheumatoid joints (see Table 1.I). EGF may play a role in tissue remodelling and bone resorption[258,259]. PDGF is a potent synoviocyte stimulant that may be relevant to the development of synovial hypertrophy and pannus formation[260].

The paucity of classical T-cell cytokines in RA synovial fluid and membrane has previously been noted. However, soluble IL2 receptor (sIL2-r, p55 chain) is readily detectable in RA and has been shown to be a good monitor of disease activity[261]. The *in vitro* inhibitory effects of IFNγ on rheumatoid synoviocyte production of PGE2 and collagenase provided the rationale for use of this cytokine in the treatment of RA, with variable results[262,263,264,265]. IL4 is not readily demonstrable in rheumatoid synovial fluid but has been shown to have marked inhibitory effects on rheumatoid synoviocyte proliferation in vitro[213]. In addition, *in vitro* inhibitory effects of IL4 on rheumatoid synoviocyte spontaneous production of pro-inflammatory cytokines (IL1 and TNF) and immunoglobulin have also been observed[214]. Recently it has been demonstrated that IL10 produced by T cells and monocytes in the rheumatoid synovium may inhibit macrophage production of TNFα and IL1β, and T-cell production of IFNγ[266].

Cytokine inhibitors present in rheumatoid joints include a third member of the IL1 gene family, the IL1 receptor antagonist, which has been shown to have anti-inflammatory properties[267]. In

addition shed/soluble forms of cytokine membrane-bound receptors, including sIL2-R and TNF receptors, may function as specific cytokine antagonists[268,269].

5.4. B cells and rheumatoid factor

RA synovium contains significant amounts of activated B-cells and plasma cells that spontaneously produce large quantities of rheumatoid factor *in vitro*[270]. Rheumatoid factor was first identified nearly 50 years ago and provided the initial indication of an immunological process in RA[271]. Rheumatoid factor may be detectable in the serum of up to 80% of RA patients and high titres of IgM rheumatoid factor may be associated with severe disease and extra-articular manifestations[27]. However, rheumatoid factor is not specific for RA and may occur in a variety of inflammatory conditions and in a significant percentage of healthy normal individuals[272]. Although IgG and IgM antibodies that recognise antigenic determinants on Fc fragments of IgG are the most common forms of rheumatoid factor, IgA, IgD and IgE isotypes have also been reported[273]. Investigation of rheumatoid factor genes has yielded evidence of somatic mutation indicative of a T-cell dependent antigen-driven process[274]. The role of rheumatoid factor in the pathogenesis of RA is uncertain. Polyclonal IgM rheumatoid factor and immune complexes containing rheumatoid factor can fix and activate the classical complement pathway and thereby initiate an inflammatory process[275,276]. However, the fact that RA can occur in patients with congenital agammaglobulinaemia has raised doubts about the pathogenetic significance of rheumatoid factor[277].

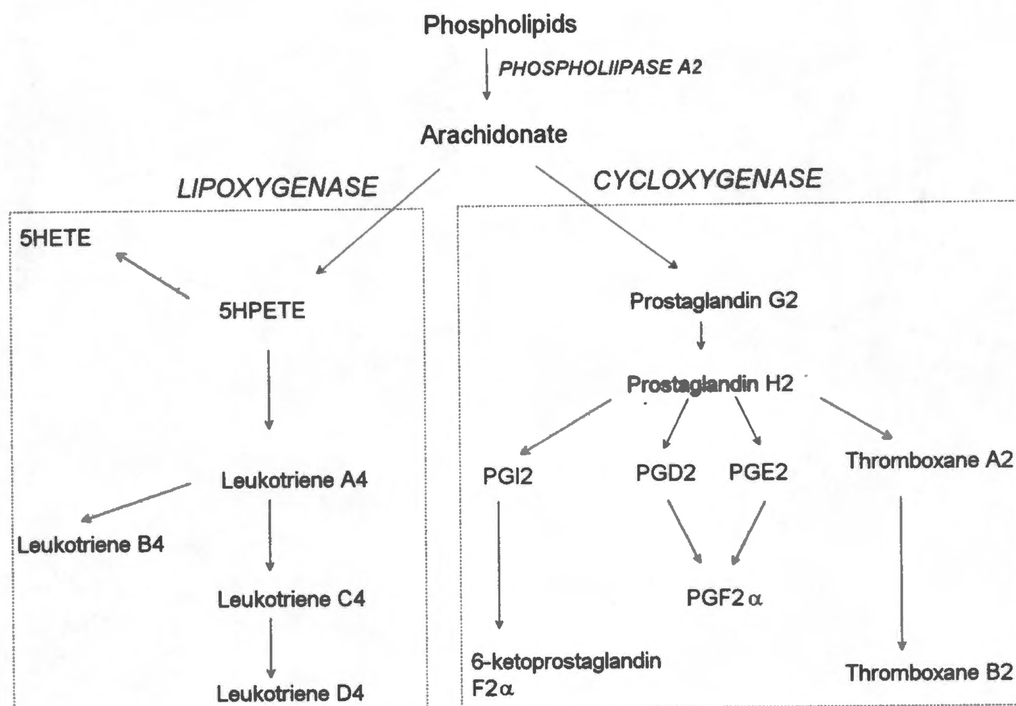
5.5 Arachidonic acid metabolites

Arachidonic acid is released from phospholipids of cell membranes by the action of phospholipase A2 and further metabolised by two main pathways (FIGURE 1.10). Products of both cyclooxygenase (predominantly E-series prostaglandins, PGE) and lipoxygenase (predominantly leukotriene B4, LTB4) metabolism of arachidonic acid (AA) are readily demonstratable at sites of active rheumatoid synovitis[278].

Sources of eicosanoids include neutrophils, macrophages, fibroblasts and follicular-dendritic cells; although lymphoid cells, including T-cells, B-cells and NK cells, have cyclooxygenase and lipoxygenase metabolic potential, their role in the production of eicosanoids is controversial[279,280,281,282,283,284,285]. In general, prostanoids (cyclooxygenase products of AA metabolism) have diverse immune suppressive and anti-inflammatory properties, and lipoxygenase products, in particular LTB4, are immune stimulatory and pro-inflammatory (TABLES 1.VIIa and 1.VIIb).

FIGURE 1.10

Arachidonic acid metabolism



HPETE - hydroperoxyeicosatetraenoic acid; HETE - hydroxytetraenoic acid; PG(x) - prostaglandin (x)

TABLE 1.VIIa. Immune modulatory effects of Prostaglandin E2 and other products of cyclooxygenase metabolism of arachidonic acid.

TARGET CELL	EFFECT	PGE2	OTHER PROSTANOIDS	REF.
PBMNC	↓TNF (α and β); ↓IFNγ	✓	PGE1, PGE3, MISO, PGA2, PGF1α, PGF2α	286
	↓IFNγ	✓		287
	↓MLR prolif, ↓HLA-DR, ↓IL2-R	✓	MISO and ENISO	288
	↓GM-CSF		MISO	289
CD4 T-CELL	↓Th1 (murine) TNFα	✓		290
	↓Th1/Th0 (human) IFNγ	✓	MISO	240
	↓Th1/Th0 (murine) GM-CSF	✓		207
	↑Th2 (human) IL4	✓	MISO	240
CD8 T-CELL	↓Cytotoxicity	✓	PGE1	228,291
	↓Generation of effectors	✓		292
NK CELL	↓Cytotoxicity	✓		293,294
B CELL	↓Prolif, ↓activation, ↓IgM	✓		295
	↑IL4 - induced IgE production	✓		295
MONOCYTE / MACROPHAGE	↓IL1, ↓TNFα	✓		296,297
	↑IL6		MISO	286
	↓class II MHC	✓		298
PMN	↓activation, ↓O2 release, ↓LTB4	✓		299,300

MISO - misoprostol; ENISO - enisoprost; PBMNC - peripheral blood mononuclear cells; PMN - polymorphonuclear neutrophil.

TABLE 1.VIIb. Immune modulatory properties of leukotriene B4 (LTB4)

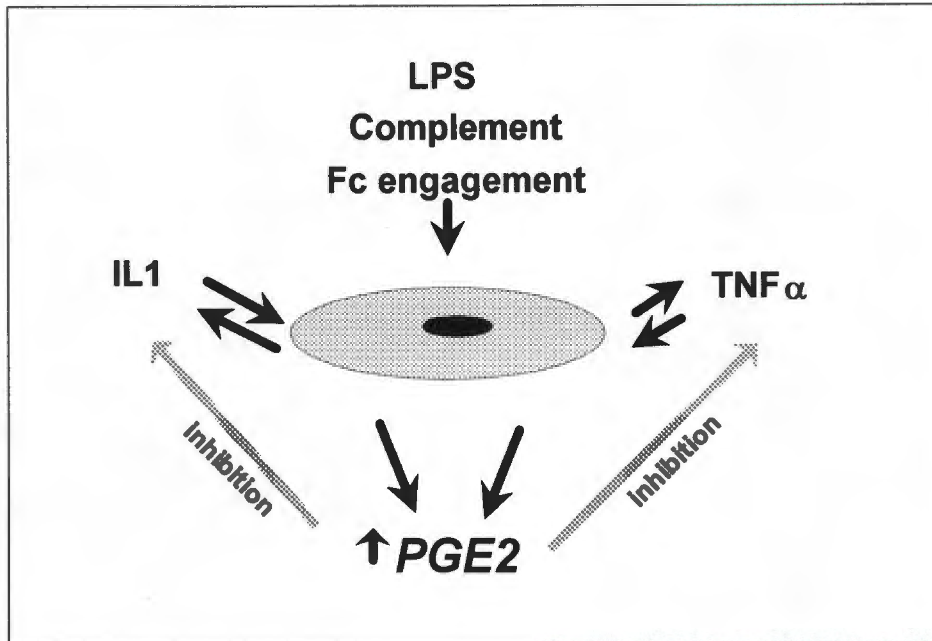
TARGET CELL	EFFECT	REF.
PBMNC	↑ IFNγ	301
	Necessary for pha-induced proliferation	302
CD4 T-CELL	↑ IFNγ, ↑IL2	303
CD8 T-CELL	↑IL2-Rβ	304
	↑PHA-induced responses	305
	↑CD8 expression	306
NK CELL	↑cytotoxicity	304,307,308
	↑IL2-Rβ	304
B CELL	↑IL4 - induced activation	309
	↑antibody production	309
MONOCYTE / MACROPHAGE	↑IL6	310
	↑TNFα (IL2 - induced)	311
	↑IL1 and TNFα	312
PMN	↑Chemotaxis and chemokinesis	313

5.5.1. Prostaglandin E

PGE has historically been viewed as harmful in RA, mainly due to its potentially deleterious effects of vasodilatation, hyperalgaesia and bone resorption[299]. Recently, however, the significance of these potential deleterious effects and their role in the pathogenesis of RA has been questioned[299]. Conversely, growing evidence indicates a potential beneficial role of PGE in RA (see TABLE 1.VIIa above). Prostaglandin E2 (PGE2) production by macrophages and macrophage-lineage synoviocytes is increased by a variety of stimuli, including IL1, TNFα, lipopolysaccharide (LPS), components of the complement cascade and Fc receptor engagement[314,315,316,317,318]. The potent inhibitory effects of PGE2 on IL1 and TNFα production indicate that PGE2 may function as a negative feedback inhibitor of these pro-inflammatory cytokines (FIGURE 1.11). It has been suggested that modulation of T-cell functions by PGE1-3 and misoprostol is mediated by binding to a common cell surface receptor resulting in increased intracellular cyclic AMP[286]. PGE2 has several other potential beneficial effects in RA, including inhibition of lymphocyte adhesion to endothelial cells, inhibition of transendothelial migration of T lymphocytes, suppression of synoviocyte collagenase production and inhibition of LTB4 production [319,320,321,300]. PGE2 mediated inhibition of *in vivo* and *in vitro* models of acute and chronic inflammation, and beneficial therapeutic effects on adjuvant arthritis have also been reported[322,323].

FIGURE 1.11

Function of PGE2 as a negative feedback inhibitor of proinflammatory cytokines following macrophage activation



5.5.2 Leukotriene B4

An important role for LTB4 in the pathogenesis of RA has been indicated by the effectiveness of reduction of this eicosanoid (pharmacological and dietary) in the treatment of the disease. In contrast to PGE, LTB4 is a potent stimulator of diverse immune functions (TABLE 1.VIIb) which may be mediated by binding to a cell surface receptor with resultant increase in intracellular cGMP, or by its action as a calcium ionophore[324,325,326]. LTB4 may be important in the activation of NK, B and T-cells[285]. In addition to exogenous sources of LTB4, T-cells may provide their own source of LTB4 by LTA4-hydrolase metabolism of macrophage-derived LTA4[327,328]. Further evidence of the role of LTB4 in T-cell activation is indicated by the fact that inhibition of PHA-induced proliferation of T-cells by a 5 lipoxygenase inhibitor and, most interestingly, hydrocortisone, can be abrogated by the addition of exogenous LTB4[302,329]. It has been suggested that LTB4 stimulation (indirectly via IFNγ production or directly by another mechanism) of PGE2 release may represent an important feed-back regulatory mechanism[285]. This hypothesis may account for the apparent contradiction in earlier reports that noted LTB4, HPETE and HETE actually suppressed mitogen-induced proliferation of mixed T-cell and monocyte populations, while in the presence of a cycloxygenase inhibitor LTB4 enhanced proliferation[330,331].

In addition to LTB4, other 5 lipoxygenase products may be involved in the pathogenesis of RA. It has recently been demonstrated that leukotrienes C4 and D4 stimulate angiogenesis via

a receptor-mediated interaction, and thus may contribute to the development of pannus[332]. Immune modulatory properties of 5 HPETE and HETE may also be significant[330,333].

Lysophosphatidylcholine and platelet activating factor are additional products of phospholipase A_2 activity that have potent proinflammatory and immune modulatory functions that may be relevant at the site of pathology in RA[334,335].

6. THERAPY

6.1 Current treatment

Historically a conservative approach to the treatment of RA has been followed, fuelled by the perception that the disease was not life-threatening, and was self-limiting in the majority of cases. This approach is exemplified by the "therapeutic pyramid" in which non-steroidal anti-inflammatory drugs (NSAIDs) are used exclusively in the early stages of the disease, while the so-called disease modifying anti-rheumatic drugs (DMARDs) were reserved for patients in whom NSAIDs failed. In practice this has meant that the time from diagnosis to the use of DMARDs has been 7 years or longer[336]. With the growing realisation that RA is a severe disease with a predictably poor outcome, early and more aggressive therapeutic intervention has been recommended[336]. The goal of such intervention is to induce disease remission early, and thereby prevent erosive joint damage, preserve function and improve life expectancy. The safety and efficacy of current therapies in achieving these goals is questionable[337].

6.1.1 Non steroidal anti-inflammatory drugs

NSAIDs are some of the most widely-prescribed pharmacological agents, and are used extensively in the treatment of all forms of arthritis. Aspirin, the prototype NSAID, was the cornerstone of RA treatment for many years. Numerous NSAIDs belonging to several different chemical classes are now available and share a common inhibitory effect on cyclooxygenase metabolism of arachidonic acid, resulting in reduction of prostaglandins in the synovial compartment[338]. Although inhibition of prostaglandin synthesis has been suggested as the mechanism of the anti-inflammatory action of these agents, their definitive mode of action remains unclear[339]. Indeed, there is increasing evidence that prostaglandins themselves, particularly PGE2, have extensive anti-inflammatory and immune suppressive effects (see Table 1.VIIa). Scavenging of oxygen radicals and effects on leukocyte function have been been proposed as alternative mechanisms of NSAID action[340,341]. Systemic inhibition of prostaglandin E (PGE) does, however, appear to be responsible for many NSAID-induced side-effects[299].

Inhibition of arachidonic acid metabolism by conventional NSAIDs at standard doses is generally confined to the cyclooxygenase pathway, and may result in a compensatory increase in lipoxygenase activity[342]. Products of this pathway, in particular leukotrienes, have pro-inflammatory and immune stimulatory properties (see Table 1.VIIb). NSAID therapy has been associated with increased accumulation of neutrophils at chronic inflammatory sites *in vivo*, possibly as a result of the chemotactic effects of LTB4[343]. Interestingly, combined inhibition of cycloxygenase and lipoxygenase by benoxaprofen, a NSAID that is no longer available, resulted in reduced migration of leukocytes, particularly monocytes, into sites of experimental inflammation[344]. The disease modifying properties of Tenidap, a new NSAID that inhibits both cycloxygenase and lipoxygenase pathways of arachidonic acid metabolism, may be due to combined inhibitory effects on leukotrienes and pro-inflammatory cytokines[345].

While often used to achieve symptomatic improvement in RA, NSAIDs acting only on the cycloxygenase pathway of arachidonic acid metabolism do not beneficially affect disease progression[346]. It has also been suggested that therapeutic reduction in PGE levels may prevent remission induction by gold therapy[347]. NSAID inhibition of proteoglycan synthesis in cartilage of osteoarthritis patients has been demonstrated and may have implications for RA[348]. In addition, the significant morbidity and mortality associated with NSAID side-effects would suggest that these agents are not as safe as previously thought, and rival the side effect profiles of several DMARDs and corticosteroids[349].

Given the accumulated evidence, a reappraisal of the role of cycloxygenase inhibitors in the treatment of RA has been suggested[336]. Apart from a theoretical reduction in systemic side effects, new experimental cycloxygenase inhibitors that specifically inhibit inducible isoforms of the enzyme (cycloxygenase-2) do not offer additional benefits in the treatment of RA[350]. As has been suggested for other chronic inflammatory diseases, inhibition of lipoxygenase, alone or in combination with cycloxygenase inhibition, may be preferable[351]. It has also been suggested that the sole use of cycloxygenase inhibitors in the treatment of early RA, as promoted by the therapeutic pyramid, is no longer tenable[336].

6.1.2 Corticosteroids

Corticosteroids have a lengthy historical association with the treatment of RA[352]. Initial enthusiasm for their use diminished once the adverse side effects of high dose corticosteroid therapy became apparent. The recent resurgence of interest in corticosteroid therapy has resulted from reappraisal of data obtained during early clinical trials in RA, which demonstrated a significant reduction in the development of new joint erosions using comparatively low dose regimens[353,354].

The precise mechanism of action of corticosteroids in the potential modification of the disease process in RA is unknown. Corticosteroids exert a wide range of effects on inflammatory mediators and immune functions and, given the fact that they are also endogenously produced, represent an important group of natural anti-inflammatory and immune suppressants. Steroid receptors can be found in virtually all cell types in every organ system[355]. Following binding of these cytoplasmic receptors, steroids are transported to the nucleus where they can affect transcriptional, translational and post-translational processes concerned with protein synthesis, processing and secretion, and cell growth, division and apoptosis[356]. Some of these effects may be modulated by lipocortins or annexins, a group of steroid-inducible proteins[357].
Corticosteroids have diverse effects on immunological cells, including inhibition of IFN-γ, IL2, IL3 and IL6 production by T-cells and suppression of GM-CSF, IL1 and TNF-α production by monocytes[358,359,360]. Inhibition of cytolytic mediators, granzymes and perforins, within cytotoxic lymphocytes has also been described[361]. Differential effects of corticosteroids on Th1 and Th2 subsets of CD4 T-cells may be responsible for some of the prior observations[143,144]. Corticosteroids reduce arachidonic acid metabolites by inhibiting phospholipase, thereby impeding arachidonic acid release from phospholipids[362]. This effect thus occurs at a level distinct from that exerted by NSAIDs, and results in reduction of products of both cycloxygenase and lipoxygenase pathways, although predominant inhibitory effects on lipoxygenase products have been described in a short term study[338].

6.1.3 Disease modifying anti-rheumatic drugs

The so-called disease modifying anti-rheumatic drugs (DMARDs) constitute a diverse group of agents used empirically to modify the outcome of RA. These drugs include gold, penicillamine, anti-malarials, sulphasalazine and methotrexate; more recently, immune suppressants and cytotoxic drugs including cyclophosphamide, azothiaprine and cyclosporin have been added to this list[57,363,364,365,366,367,368]. At present there is no consensus whether these agents actually influence disease progression in RA[369]. Thus, despite growing knowledge of some of their effects, including modulation of inflammatory and immune parameters, it is difficult to identify any disease-relevant processes modulated by DMARD therapy that could account for a mechanism of action in RA[370,371,372,373].

A reappraisal of outcome measures in RA has shifted the focus from short term control of inflammation and symptoms, to long term preservation of function and retardation of joint erosions; viewed from this perspective, it has been suggested that methotrexate is the only DMARD to have significant beneficial effects[374,375]. The reasons for failure of other DMARDs may relate to side-effects and lack of efficacy leading to high attrition rates, and the practice of reserving their use for well-established disease[374]. Although methotrexate may be

effective in modifying the outcome in RA, its precise mechanism of action remains unclear. It has been suggested that with weekly dose regimens used in the treatment of RA, inhibition of its target enzyme, dihydrofolate reductase, is never complete[376]. Inhibition of other folate-dependent enzymes, particularly aminoimidazole carboxamide riboside transformylase and, secondarily, adenosine deaminase, by polyglutamyl derivatives of methotrexate may be relevant to its cytostatic and neutrophil suppressive effects[377]. Modulation of purine biosynthesis by methotrexate has several other effects potentially relevant to its action in RA, including inhibition of leukotriene synthesis and IL1 secretion[378,379]. Additional mechanisms of action of methotrexate defined in animal models that may have implications for RA include increased concentrations of adenosine in inflamed murine air pouches and markedly reduced leukocyte accumulation[380].

6.1.4 Non-pharmacological therapy

Several studies have demonstrated a short- to medium-term beneficial effect of dietary supplementation with omega-3 fatty acids in rheumatoid arthritis[381,382]. Omega-3 fatty acids may competitively inhibit the formation of 2-series prostaglandins and 4-series leukotrienes from arachidonic acid, resulting in the formation of compounds with altered and reduced biological activity, and indicate a possible mechanism of their action in RA[383].

6.2 Immunotherapy

The growing understanding of the immunopathogenesis of RA has led to attempts to intervene in the disease process using a variety of methods. The goal of such therapy is to modulate a specific pathogenetic process, with minimal side-effects, and induce lasting disease remission or cure[384]. While immunotherapy in RA represents probably the first use of rational therapy in the treatment of the disease, efficacy and safety profiles of these new generation anti-rheumatics vary widely. In addition, most clinical trials to date have been open-label and included small numbers of patients with long-standing disease, and focused on short-term effects.

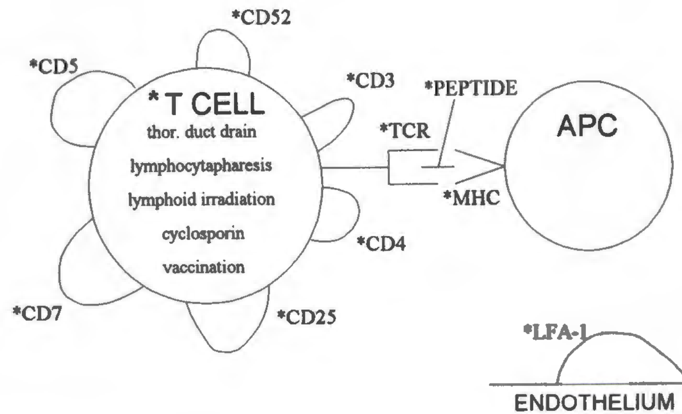
6.2.1. Targets for immunotherapy

The CD4 T-cell / antigenic peptide / HLA/ antigen-presenting cell complex has been the focus of most immunotherapeutic regimens to date (FIGURE 1.12). Given that specific suppression of pathogenetic mono- or oligo-clonal CD4 T-cell populations requires identification of the antigenic peptide, current targets fall short of the goal of immunotherapy in RA. However, despite such failings important insights into the pathogenesis of RA may arise from these studies.

FIGURE 1.12

Targets for immunotherapy in rheumatoid arthritis

(References indicated in text)



* Immunotherapeutic target in RA

6.2.2. Monoclonal antibody therapy

The majority of therapies directed at the targets indicated above have used monoclonal antibodies, of murine origin, specific for the target antigen. Binding of the antibody to its specific ligand may, in the case of T-cell surface antigens, result in (i) inhibition of T-cell - APC interaction, (ii) partial/inappropriate activation of the T-cell leading to anergy or apoptosis, (iii) complement fixation and cell depletion and (iv) receptor blockade[385,386,387,388].

Anti-CD4 therapy with a range of different monoclonal antibodies is the most extensively studied antibody therapy in RA at present, and was based on successful prevention of animal models of autoimmune disease[389,390]. Although early phase I studies yielded promising clinical and laboratory results, more recent controlled trials of anti-CD4 therapy have failed to show significant efficacy[391,392,385,393]. Prolonged depletion of circulating CD4 +ve cell populations has been noted with chimeric antibody therapy, and there appears to be no correlation between CD4 cell numbers and therapeutic efficacy, perhaps due to the persistence of small numbers of pathogenetic cells sequestered in the joints[394,395]. Modulation of monocyte/macrophage cell populations that also express CD4 antigen may result from anti-CD4 therapy[385]. In addition to CD4 depletion, side-effects of anti-CD4 therapy include a cytokine release syndrome immediately following antibody infusion, the development of human anti-mouse antibodies (considerably reduced with chimeric or humanised antibodies, although anti-idiotypic responses may still limit prolonged therapy), and opportunistic infections[396,385,394]. The long-term risk of malignancy in patients with prolonged CD4 T-cell depletion has not been defined. Given the present difficulties of anti-CD4 treatment in RA, alternative treatment protocols have been proposed, including targeting patients with early disease and the use of non-depleting

antibodies to induce a state of prolonged immune tolerance, as has been achieved in murine transplant models[397].

Other monoclonal antibodies directed against cell surface antigens and used in the treatment of RA include anti-CD5, anti-CD7, anti-CD25 (IL2-R), and anti-CDw52 (Campath-1H); as with anti-CD4 therapy, the studies reported to date have included comparatively small numbers of patients over short time periods and have yielded variable results[398,399,400,388,401]. Improvement on standard monoclonal antibodies has included an immunoconjugate that combines a monoclonal antibody with a toxin to increase depleting efficiency[398,399]. Targeting activated cells with increased expression of IL2-R is the rationale behind the use of anti-CD25 therapy; a novel technique to achieve the same goal has been the use of an IL2 fusion toxin that couples sequences of human IL2 to components of diptheria toxin and thus avoids the problems associated with monoclonal antibody therapy[402].

Monoclonal antibodies against cytokines have also been used to treat RA. Anti-TNFα monoclonal antibody therapy has been effective in collagen induced arthritis and, more recently, in early phase studies in RA[403,239,404]. Significant clinical improvement (swollen joint count and painful joint count) associated with reduction in acute phase reactants has been noted in the clinical trials of anti-TNFα monoclonal antibody therapy, but the requirement of shorter dosing intervals on retreatment may limit the application of this form of therapy[405]. Reduction of IL1, GMCSF and IL8 has been shown to be an *in vitro* effect of anti-TNFα, suggesting a pivotal role for TNFα in the hierachy of cytokines in RA[251]. A combination of anti-TNFα and anti-CD4 monoclonal antibody therapies has a synergistic effect in the treatment of collagen induced arthritis, and may have implications for RA[395]. Additional strategies to inhibit cytokines in RA may include the use of cytokine receptor antagonists and soluble cytokine receptors or, in the case of TNFα, metalloproteinase inhibitors that prevent the release of biologically active cytokine from the cell membrane [406,407,408,409,410,411].Recently, a phase I study in patients with RA using a recombinant human TNFα-receptor (p75) linked to the Fc portion of a human IgG1 antibody was completed, demonstrating considerable improvement in inflammatory markers and clinical parameters (Moreland L, Annals of Rheumatic Diseases 1995 (in press)). Further trials using similar strategies to inhibit TNFα in patients with RA are currently in progress.

6.2.3. T-cell vaccination

T-cell vaccination has been shown to be effective in the prevention and treatment of animal models of autoimmunity[412,413]. The technique involves the isolation and *in vitro* expansion of known pathogenetic T-cells, followed by activation, attenuation and subsequent subcutaneous vaccination of an aliquot of these cells. The vaccine activates anti-clonotypic (presumably

anti-T-cell receptor) and anti-ergotypic (anti-activated) T-cells of both CD4 and CD8 phenotype that may regulate the original pathogenetic T-cell population[414,415,416]. T-cell vaccination in RA has been essentially unsuccessful, largely due to the difficulty in identifying pathogenetic T-cells for use in the vaccine[417,418]. In the absence of a defined auto-antigen, non-specific expansion of cells from the site of pathology has been utilised[419]. However, given the non-specific nature of the T-cell accumulation at chronic inflammatory sites, it is likely that such techniques result in only modest enrichment of pathogenic T-cell populations, and may cause potentially harmful responses to protective memory T-cells included in the vaccine. Additional constraints include the labour- and facility-intensive nature of T-cell vaccination, making it impractical for general clinical application[417].

6.2.4. Peptide therapy

Peptide immunotherapy may inhibit APC-T-cell interaction and subsequent activation by three distinct mechanisms: (i) activation of regulatory T-cell networks by peptides of disease-associated TCR families, (ii) induction of selective T-cell anergy or tolerance by modified antigenic epitopes with partial TCR agonist or antagonist properties, and (iii) MHC blockade using non-antigenic peptide sequences that displace autoantigenic and other epitopes and inhibit all T-cell interactions with the particular MHC haplotype[190,420,421]. Potential problems confound all peptide therapy techniques and include the finding that vaccination with disease associated TCR peptides may aggravate the disease[190]. Determinant spreading during an autoimmune response may nullify therapeutic attempts using single peptides, and short half-lives of infused peptides make prolonged MHC blockade extremely difficult[420]. Given the latter aspect of MHC blockade, TCR antagonism may have an advantage as a therapeutic option and suggest a rational approach to antigen-specific immuno-intervention[422]. Apart from early phase studies of TCR peptide vaccination in patients with multiple sclerosis, *in vivo* experience of peptide immunotherapy has been confined to animal models of autoimmunity[423,424]. For specific peptide therapy in RA identification of autoantigenic epitopes and clarity on disease-associated TCR Vβ families is required[190].

6.2.5. Mucosal tolerance

It has been recognised for some time that encountering an antigen at a mucosal surface can lead to the development of specific immune tolerance[425]. In the case of orally ingested myelin basic protein in animals, it has been suggested that antigen-specific suppressor T-cells activated in the small intestine release TGFβ on re-encountering their specific antigen in the central nervous system, resulting in suppression of local immune responses[426,427,428,429,430]. Inhaled peptides may induce tolerance as a result of failure to activate pulmonary dendritic cells and local inflammatory responses[190]. Oral tolerance therapy has proved successful in the prevention and treatment of animal models of autoimmune arthritis[431,432,433]. Safety and a

degree of efficacy of oral tolerance therapy has also been demonstrated in humans with multiple sclerosis (using myelin basic protein) and RA (type II chicken collagen), although both studies included comparatively small patient numbers studied over short periods[434,435].

6.2.6. Antimicrobials

Based on impressions that RA is the result of an infectious agent, and the degree of success achieved in the treatment of lyme disease and some cases of reactive arthritis with antibiotics, several studies have investigated the therapeutic effects of antimicrobials in RA[436,437,438]. In general, significant efficacy has not been demonstrated, even with prolonged treatment. In addition, in isolated cases where a therapeutic effect has been observed using antimicrobials, this may be a consequence of antiinflammatory and immune modulatory properties of the agents, rather than a direct antimicrobial effect[439].

7. CONCLUSIONS

Recognition of the severity of rheumatoid arthritis, its impact at many levels, and the lack of efficacy of most conventional therapeutic agents provides ample incentive for the development of new therapeutic regimens. The increasing understanding of the immunopathogenesis of the disease permits, for the first time, the rational targeting of specific cells, products or processes thought to be involved in the disease process. The evidence in support of a role for CD4 T-cells in the initiation and perpetuation of RA would suggest that these cells are involved in proximal events in the pathogenesis of the disease, and thus may provide important therapeutic potential. Several aspects of CD4 T-cell function in RA remain largely unknown, including the nature of the inciting 'rheumatoid antigen', the role and regulation of CD4 T-cell mediated cytotoxicity at the site of pathology, and the potential modulatory effects of eicosanoids, present in the synovial compartment, on T-cell effector functions. The aims and objectives of this thesis, as detailed below, encompass investigation of several of these aspects of the immunopathogenesis of RA. The findings may have implications for novel therapeutic interventions in the immunopathogenetic processes of the disease.

8. AIM AND OBJECTIVES OF THIS STUDY

8.1. Aim

To investigate the effector functions and regulation of CD4 T-cells from the synovial compartment of patients with rheumatoid arthritis, and identify novel targets for the therapeutic modulation of CD4 T-cell functions.

8.2. Objectives

(i) To investigate the proliferative responses and cytotoxic effector function of CD4 T-cells from the site of pathology in patients with RA and other arthritides, and correlate this function with clinical parameters.

(ii) To determine the potential modulatory effects of eicosanoids on the *in vitro* functions of CD4 T-cells.

(iii) To define the cellular regulation of CD4 T-cell mediated cytotoxicity and its association with other cell functions.

(iv) To investigate the specificity of mycobacterial antigen-reactivity of synovial T-cells in patients with RA drawn from a population with high environmental exposure to *Mycobacterium tuberculosis*.

(v) To identify the source of the T-cell stimulus in the synovial compartment in RA.

Each of these objectives are addressed in the five chapters that follow (chapters 2 - 6).

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Chapter 2

Prostanoid Modulation of Synovial Antigen-Specific CD4+ T- Cell Cytotoxic Function in Rheumatoid Arthritis

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INTRODUCTION

The pathology of rheumatoid arthritis (RA) is characterised by an infiltration of activated CD4+ T-lymphocytes in synovial membrane and synovial fluid[1,2]. These cells have been thought to have a central function in the immunopathogenesis of the disease, mediated primarily by their cytokine stimulation of B-cells and the mesenchymal reaction[3]. This paradigm of RA has recently been disputed, largely due to the absence of T-cell derived cytokines at the site of pathology[4]. However, the demonstration that a subset of synovial CD4+ lymphocytes has cytolytic potential has suggested an alternative role for these cells in the pathogenesis of RA[5]. CD4+ T-cell mediated cytolysis of target cells presenting a class II HLA-restricted antigen in the synovial compartment may be an important effector mechanism of joint destruction. Factors that modulate this function may have therapeutic relevance.

Although products of cyclooxygenase metabolism of arachidonic acid (prostanoids) have been associated with several potentially deleterious effects in RA, their therapeutic reduction by non steroidal anti-inflammatory drugs (NSAIDs) does not impede disease progression[6]. There is evidence from early studies that cyclooxygenase inhibition as the sole form of treatment in RA accelerates erosive joint damage[7], suggesting that synovial prostanoids may mediate local protective effects. It is now well established in both *in vivo* and *in vitro* models, that prostanoids have anti-inflammatory and immune modulatory properties[8,9,10]. In particular, E-series prostaglandins (PGEs) suppress natural killer (NK) and CD8 T-cell cytotoxic function[9,10]. The potential role of prostanoids in modulating antigen-specific class II HLA-restricted synovial fluid mononuclear cell (SFMNC) cytotoxic function in RA has not previously been described.

The results of this study of 20 seropositive RA patients indicate heterogeneity in SFMNC antigen-specific cytotoxicity that correlates with therapy. Furthermore, evidence is provided that both synthetic prostaglandin E2 (PGE_2) and prostanoids produced by macrophage-lineage synoviocytes inhibit antigen-specific CD4+ T-cell cytotoxic function *in vitro*. Taken together the data may indicate a role for synovial prostanoids in suppressing T-cell mediated antigen-specific cytolysis at the site of pathology in RA.

MATERIALS AND METHODS

1. Patients

20 patients with seropositive RA, as diagnosed according to the revised ARA criteria[11], were included in this study. Apart from one newly-diagnosed patient who was untreated, all patients were receiving either NSAIDs, disease modifying anti-rheumatic drugs (DMARDs) or combinations thereof, at the time of study (TABLE 2.I). Paired samples of synovial fluid and venous blood, collected into heparinized tubes, were obtained at the time of clinically-indicated arthrocentesis and processed simultaneously. Mononuclear cells (MNC) were isolated from samples by Ficoll-Hypaque density centrifugation (Lymphaprep - Nycomed, Oslo, Norway) as previously described[12]. Cells were washed three times in phosphate buffered saline (PBS) and resuspended at 1 x 10^6/ml in tissue culture medium. Tissue culture medium used throughout these studies was RPMI 1640 (Flow Laboratories, Mclean, VA) containing 100U/ml of penicillin, 100μg/ml of streptomycin and 10% pooled AB human serum. PBMNC from 13 age-matched healthy laboratory staff were used as normal controls.

2. Proliferation and cytotoxicity assays

PBMNC and SFMNC proliferation in response to mitogen and antigen was analysed as previously described[12]. MNC (10^5 per well) were stimulated in triplicate wells of a 96-well U-bottomed sterile tissue-culture plate (Flow Laboratories, Irvine, Scotland) with the mitogen phytohaemagglutin (PHA, Wellcome Research Laboratories, Beckenham, England) and purified protein derivative of *Mycobacterium tuberculosis* (PPD, Central Veterinary Laboratory, Weybridge, Surrey, England) in a final volume of 200 μl of culture medium. Wells containing cells without antigen or mitogen were used for determining background proliferation. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days (mitogen) or 7 days (antigen) and tritiated thymidine (2μCi/well) incorporation (Amersham International, Buckinghamshire, England; specific activity 185 μCi/mmol) over the final 18 h of incubation measured.

Antigen-specific cytotoxicity was measured in a 15 h ^{51}chromium (^{51}Cr) release assay as previously reported[12]. PPD-specific effector cells were generated from PBMNC and SFMNC by culturing 6 -10 x 10^6 MNC at 10^6/ml in medium with 3μg/ml PPD in 25cm^2 tissue culture flasks (Becton Dickinson Labware, Lincon Park, NJ) for 6 days, without the addition of interleukin 2 (IL2). Target cells were autologous adherent peripheral blood MNC that were plated at the initiation of the assay. On day 5 of culture, target cells were pulsed with the specific antigen, PPD (10μg/ml); an irrelevant antigen, streptokinase-streptodornase (1:10 dilution) (SK-SD, Lederle Laboratory, Wayne, NJ - 250u/ml streptokinase, 62.5u/ml streptodornase), or medium alone, and labelled with 6μCi/well ^{51}Cr for 24 h. After extensive washing, effectors were added to give final effector:target ratios of 10:1. 3:1 and 0.3:1.

Adherent target cell numbers were estimated as 10% of mononuclear cells plated. Wells containing target cells and medium only were used for the determination of spontaneous ^{51}Cr release. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 15 h, following which the total content of each well was transferred to a disposable counting tube and 100µl of 5% Triton X added to the remaining adherent cells for the determination of maximum release. After 3 h incubation at 37°C the total volume of Triton X was transferred to similar tubes and radioactivity counted. The percentage specific cytotoxicity for the mean of triplicate wells was calculated as follows: percentage specific lysis = [mean test cpm/ (mean test + mean cpm after Triton X treatment of the same triplicate wells)] x 100% - percentage spontaneous release. The percentage spontaneous release was calculated as follows: mean cpm in spontaneous release wells/ (mean cpm in spontaneous release wells + mean cpm after Triton X treatment of the same triplicate wells) x 100%, and was always less than 20%. For the PPD-specific cytotoxicity reported in this chapter, anomalous cytotoxicity (targets with irrelevant antigen or without antigen) was subtracted.

Positive and negative selection of $CD4^{+ve}$ and $CD8^{+ve}$ subsets of effector MNC was performed with antibody coated T-25 tissue culture flasks, used according to the manufacturer's directions (AIS Microcellector, Applied Immune Sciences Inc, Menlo Park, CA). Antigen-specific cytotoxicity of the selected cell subsets was measured in a standard 15h ^{51} Cr release assay. Phenotypic analysis of unseparated and selected effector MNC subsets was performed by flow cytometry on an Epics Profile II (Coulter Electronics, Hialeah, Florida) using the following monoclonal antibodies: CD3-RD1, CD4-FITC, CD8-FITC, and NKH1-RD1 (anti-CD56) (all from Coulter Immunology, Hialeah, Fl.). The flow cytometry methodology is as described in detail below (section 3 of methods), except that lymphocytes were bitmapped visually to exclude monocytes and debris on histogram 1.

The *in vitro* effects of cholorquin (Sigma Chemical Company, St Louis, Mo), sulphasalazine (Salazopyrine, Keatings Pharmaceuticals, Johannesburg, South Africa), methotrexate (Lederle Laboratory, Wayne, NJ) and prostaglandin E_2 (PGE_2, Sigma Chemical Company, St Louis, Mo) on proliferation and antigen-specific cytotoxic function of PBMNC from 5 normal subjects were investigated. Effects on proliferation were determined by adding the agents at the initiation of the culture period. The effects of chloroquin and PGE_2 on both the generation, and effector function of cytotoxic cells, were investigated by adding the agents at the initiation of culture of the effector cells, or during the 15h incubation period of the ^{51}Cr release assay.

3. PGE_2 production by CD14+ synoviocytes

Thawed SFMNC (2x 10^6/ml) were adhered to 24 well tissue culture plastic plates (Falcon 3047, Becton Dickinson Labware) for 3 h. The percentage of viable cells expressing CD14 in

unseparated and non-adherent fractions was determined by dual parameter flow cytometric analysis performed on an Epics Profile II (Coulter) using FITC-conjugated anti CD14 (Mo2-FITC, Coulter Immunology, Hialeah, Fl) and propidium iodide (Sigma Chemical Company). Histograms generated were (i) forward scatter (FS) vs log side scatter (LSS), (ii) log fluorescence 1 (LF1) vs log fluorescence 2 (LF2), (iii) and (iv) single parameter histograms of LF1 and LF2. Monocytes were bitmapped on histogram 1 to visually exclude lymphocytes and debris. Non-specific antibody binding was evaluated using the relevant mouse isotypic control, allowing for 2% false positives. Histogram data were generated for 10^4 events. Together, these data allowed quantitation of numbers of viable adherent CD14+ synoviocytes, which were found to be in the range of 1.8×10^5 to 5.28×10^5 cells per well.

Supernatants were collected from the unstimulated adherent cells after 18h incubation in the presence or absence of 10^{-5}M indomethacin in 1 ml of culture medium. For measurement of PGE_2 concentrations, aliquots of supernatants underwent standard solid phase extraction and derivation of PGE_2 to the methyl oximate form prior to freezing[13]. Samples were stored at -70°C for no longer than 3 days prior to assay. PGE_2 concentrations were measured by commercial RIA (Prostaglandin E_2 (I^{125}) assay system, code RPA 530, Amersham International) according to the manufacturer's directions. For comparative purposes, concentrations of PGE_2 were corrected for numbers of viable adherent $CD14^{+ve}$ cells[14].

4. Cytokine assays

The supernatants of SFMNC (3×10^6/ml in culture medium), stimulated by PHA (5.75×10^{-3} mitogenic units/ml) in 24 well tissue culture plates, were collected after 48 h. Samples were stored at -70°C until measured. Interferon-γ (IFN-γ) concentration in the supernatants was measured by commercial RIA (IFN-γ IRMA, Medgenix Diagnostics, Fleurus, Belgium) and interleukin 4 (IL4) concentration determined by commercial ELISA (IL-4 EASIA, Medgenix Diagnostics), according to the manufacturers' directions.

5. Cytotoxic CD4+ T-cell clone

Clone TS7.2E was obtained by standard limiting dilution cloning[15] of synovial membrane mononuclear cells from a patient with acute gout, using PPD as the antigen, as detailed in chapter 7 (pg. 177). This clone was maintained in culture by weekly re-stimulation with PPD, irradiated (4000 rads) autologous PBMNC and recombinant interleukin 2 (rIL2 - 100 iu/ml) (Cetus, Emeryville, CA - kindly provided by Dr Eli Kedar, Hadassah Medical School, Jerusalem, Israel). Cultures were supplied with additional fresh medium and rIL2 (100iu/ml) 3 - 4 days after re-stimulation. Phenotypic and functional analyses have demonstrated that TS7.2E is a Th1 CD4+ve T-cell clone, with PPD-specific proliferative and cytotoxic responses

(see chapters 5 and 7). Cytotoxic function of the clone was measured 2 days after stimulation with feeders and antigen, in a standard 15 h ^{51}Cr release assay.

6. Statistical analysis

Regression analysis was performed using the software program Statgraphics (STSC Inc, Rockville, MD). Krukskal Wallis non-parametric analysis of variance, and where appropriate the Mann-Whitney-U test, were employed for statistical comparison using the program Instat (GraphPad Software, San Diego, CA). Unless stated otherwise, two-tailed p values are reported; p values less than 0.05 were considered significant.

RESULTS

1. Dissociation of SFMNC proliferative and cytotoxic responses in a sub-group of patients with RA

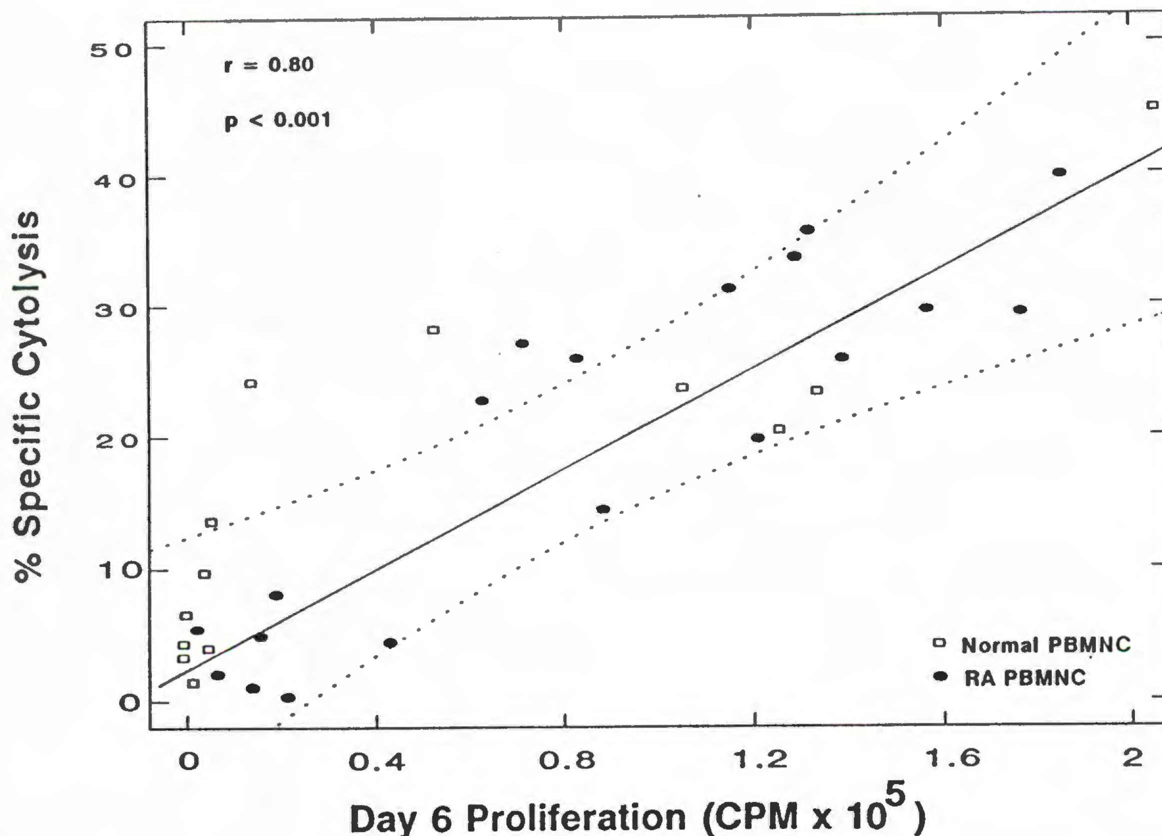
A significant positive linear relationship was found between PPD-induced proliferation and PPD-specific cytotoxicity, at an effector:target ratio of 3:1, for PBMNC from 13 healthy laboratory normals and 20 patients with seropositive RA ($r = 0.80$; $p < 0.001$) (Figure 2.1). A similar correlation was found for SFMNC from the RA patients ($r = 0.82$; $p < 0.001$) (Figure 2.2). In addition, similar correlations were also found for the PBMNC (patients and normals) and SFMNC at two additional effector:target ratios (10:1 and 0.3:1) (data not shown). Non-specific cytotoxicity, against targets with no antigen and targets pulsed with an irrelevant antigen, streptokinase-streptodornase, was in the range of 2-15%. These data confirm previous reports that have indicated a positive linear relationship between MNC antigen-induced proliferation in primary *in vitro* culture, and levels of cytolysis against antigen-pulsed autologous macrophages[16]. However, there was some heterogeneity in the responses of SFMNC allowing the categorization of the patients into three distinct sub-groups of similar numbers, which have been termed groups A, B and C (Figure 2.2). Group A consisted of 6 patients who demonstrated a marked dissociation between PPD-induced proliferation and PPD-specific cytotoxicity, with significantly lower levels of cytotoxicity than predicted by the amount of proliferation; group B comprised 7 patients with significant levels of proliferation (defined as $\Delta cpm > 20000$) and predicted levels of cytotoxicity; group C included 7 patients with low proliferative (defined as $\Delta cpm < 20000$) and predictably low cytotoxic responses to PPD.

The impaired antigen-specific cytotoxicity of patients in group A was confined to cells from the synovial compartment, and not associated with an impairement of antigen-induced proliferation. These patients demonstrated significantly lower cytotoxic function in their SFMNC than their PBMNC (effector:target ratio 10:1 : mean PBMNC cytotoxicity $41.4 \pm 7.7\%$; mean SFMNC cytotoxicity $19.5 \pm 3.2\%$, $p < 0.05$; effector:target ratio 3:1 : mean

PBMNC cytotoxicity 28.4 ± 4.6% ; mean SFMNC cytotoxicity 10.7 ± 2.7%, $p < 0.01$). Patients in group B had high levels of cytotoxicity in both compartments, with significantly higher SFMNC cytotoxicity than patients in group A (group B mean SFMNC cytotoxicity 41.9 ± 6.2% at effector:target ratio of 10:1, $p < 0.01$ compared to group A SFMNC; and 29.0 ± 4.4% at an effector:target ratio of 3:1, $p < 0.01$ compared to group A SFMNC). Box and whisker plots of PPD-specific cytotoxicity at an effector to target ratio of 3:1 for patient groups A and B are shown in figure 2.3. Low levels of both PBMNC and SFMNC cytotoxicity were found in patients in group C (at an effector:target ratio of 10:1 : mean PBMNC cytotoxicity 5.0 ± 1.9% ; mean SFMNC cytotoxicity 4.5 ± 2.1%). Cytotoxicity data at 3 effector:target ratios, for an individual representative patient from group A and B, are displayed in chapter 5, figure 8 (pg. 141). This figure also illustrates the antigen-specificity of the findings.

Figure 2.1

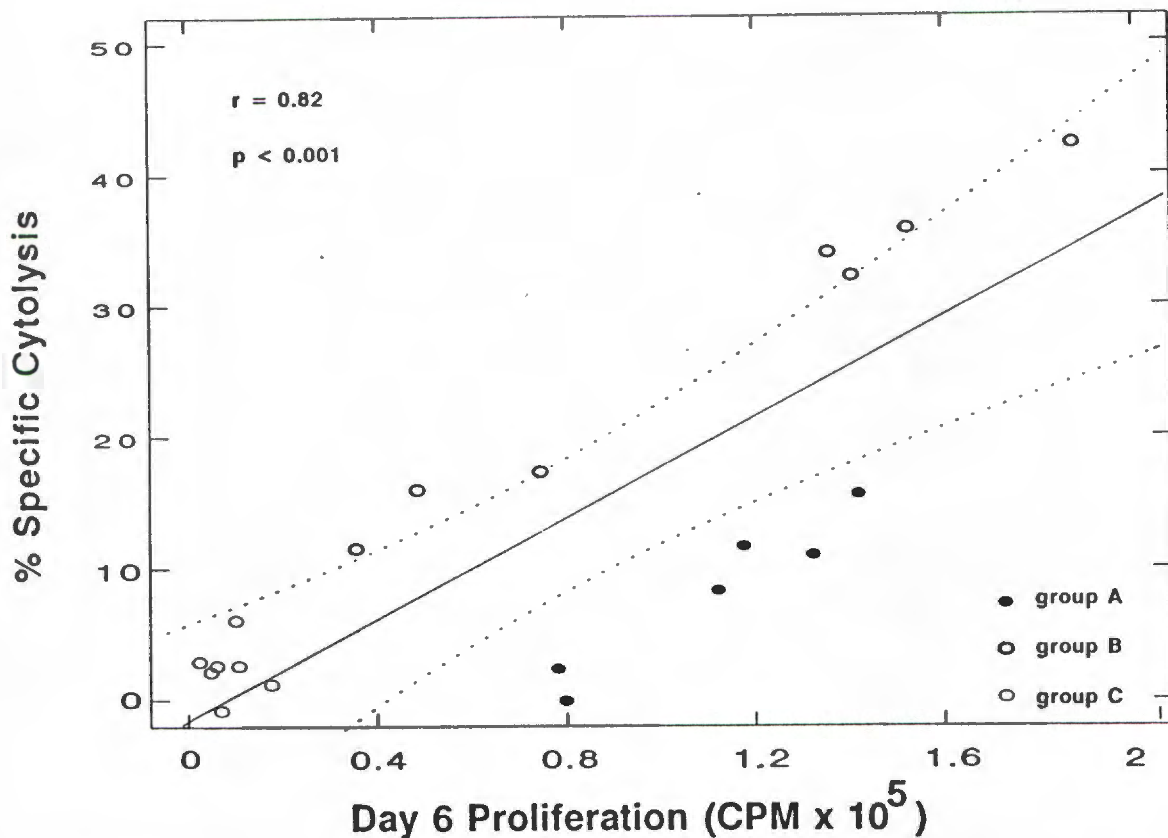
Correlation of cytotoxicity, at an effector:target ratio of 3:1, and proliferation to PPD of PBMNC from 15 healthy normals and 20 seropositive RA patients.*



*Dotted lines indicate 95% confidence limits.

Figure 2.2

Correlation of cytotoxicity, at an effector:target ratio of 3:1, and proliferation to PPD of SFMNC from 20 seropositive RA patients*.

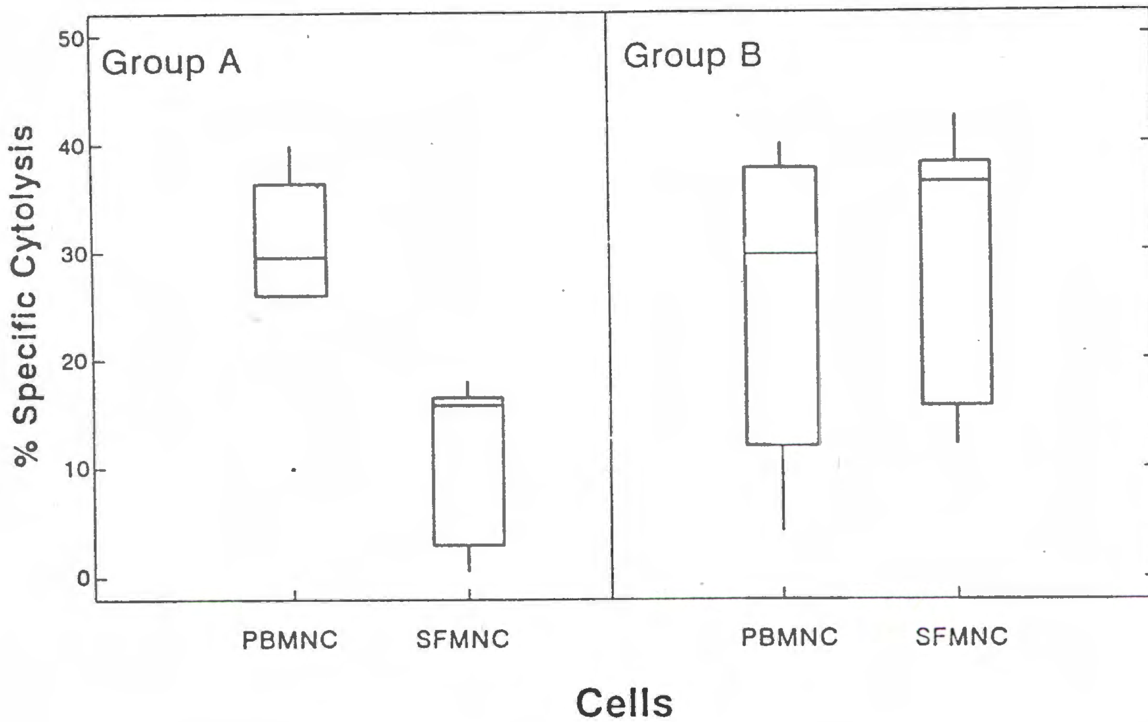


*Patients can be grouped into 3 categories according to their SFMNC responses. Group A: 6 patients whose SFMNC demonstrate significantly lower levels of cytotoxicity than predicted by the amount of proliferation; Group B: 7 patients with significant levels of proliferation (Δcpm > 20000) and predicted levels of cytotoxicity; Group C: 6 patients with low proliferative (Δcpm < 20000) and cytotoxic responses. Dotted lines indicate 95% confidence limits.

The levels of SFMNC and PBMNC proliferation to PPD were not significantly different between patients in groups A and B. Patients in group C had significantly lower proliferative responses to PPD in both PBMNC and SFMNC than patients in groups A and B ($p < 0.005$). SFMNC from all patients, including those in group C with poor proliferation to PPD, had significant proliferative responses to the mitogen PHA (mean day 3 Δcpm: 60236 $\pm$ 8325). There was no significant difference between the groups in the amount of SFMNC proliferation to mitogen, but when compared as a whole, the SFMNC responses were significantly lower than PBMNC ($p < 0.005$).

Figure 2.3

Box and whisker plots of PPD-specific cytolysis, at an effector:target ratio of 3:1, of PBMNC and SFMNC from patients with RA with significant proliferative responses (Δcpm > 20 000)*.



*Impaired cytotoxic function is confined to the synovial compartment of patients in Group A and is significantly lower than PBMNC from the same group ($p < 0.05$) and SFMNC of group B ($p < 0.01$). The interquartile range (75% - 25%) is indicated by the upper and lower borders of the "box", the median by the horizontal line within the "box" and the population range by the vertical "whiskers".

Confirmation that PPD-specific cytolysis measured in the 15h ^{51}Cr release assay is mediated by CD4+ T-cells was obtained using effector PBMNC positively or negatively selected for CD4+ or CD8+ cells. Depletion of CD4+ effector MNC resulted in almost complete abrogation of PPD-specific cytotoxicity (control/unseparated effectors: 22% and 12 % PPD-specific cytolysis at effector:target ratios of 10:1 and 3:1 respectively; CD4 depleted effectors: 1.2% and 0% PPD-specific cytotoxicity at corresponding effector:target ratios), while depletion of CD8+ effector MNC did not significantly affect PPD-specific cytolysis (22% and 15% at effector:target ratios of 10:1 and 3:1). Conversely, positively selected CD4+ effector

MNC were capable of higher PPD-specific cytotoxicity than unseparated PBMNC effectors (38% and 26% at effector:target ratios of 10:1 and 3:1), while CD8+ enriched MNC effectors did not demonstrate PPD-specific cytolysis at any of the effector:target ratios tested. Efficiency of effector subset selection was investigated by phenotypic analysis. CD4+ depleted effectors: 11% CD4+ and 42% CD8+ (unseparated effectors: 62% CD4+ and 20% CD8+); CD4+ enriched effectors: 88% CD4+ and 1% CD8+; CD8+ depleted effectors: 71% CD4+ and 3% CD8+; CD8+ enriched effector: < 1% CD4+ and 90% CD8+. CD4+ depleted effectors also showed an increase in CD56+ cells (unseparated 20%, CD4+ depleted 42%) which was not observed in other selected effectors.

2. SFMNC proliferative and cytotoxic responses correlate with therapy

In order to identify whether classification of patients according to SFMNC *in vitro* proliferative and cytotoxic responses had clinical significance, analysis of patient clinic records was performed. An association between therapy, disease duration and SFMNC proliferative and cytotoxic responses was noted (Table 2.I). Specific impairment of cytotoxicity confined to the synovial compartment (group A) was associated with DMARD therapy at the time of study in 5 of 6 patients. Of note, while three of these patients were also receiving variable doses of NSAIDs, three patients in this group were not on NSAID therapy at all. One patient was newly-diagnosed and untreated. Conversely, high levels of SFMNC cytotoxicity in a group of 7 patients was associated with NSAID monotherapy (group B). The absence of a significant PPD-proliferative response in both PBMNC and SFMNC (group C) occurred in 7 patients with significantly longer disease duration ($p < 0.01$). There were no significant differences in the age or sex distribution between the three patient sub-groups. The stability of this classification of RA patients with respect to time and changes in therapy was not examined as recurrent synovial effusions were not observed in any of the patients during the course of this study; monitoring the effects of changes in therapy and increased disease duration on SFMNC function in RA may provide additional insight into the pathogenetic significance of our findings.

3. PGE_2 production by CD 14+ve synoviocytes and association with SFMNC function

As E series prostaglandins suppress cytotoxic function of NK cells and CD8+ T-cells[9,10,17], we theorised that these cyclooxygenase products may mediate similar suppressive effects on SFMNC CD4+ T-cell cytotoxicity. The correlation of therapy with SFMNC function may thus have been the result of therapeutic modulation of synovial prostaglandins. Prostaglandin E_2 production by CD14+ synoviocytes was significantly higher in patients from group A (DMARD ± NSAID), whose SFMNC demonstrated a specific impairment in cytotoxicity, than in patients from group B (NSAID monotherapy) where intact SFMNC cytotoxic function was found ($p < 0.05$) [Figure 2.4].

Table 2.I.

*Clinical parameters of 20 seropositive RA patients classified according to SFMNC proliferative and cytotoxic responses to PPD**.

PATIENT GROUPS	AGE (yrs)	SEX M:F	Duration (yrs)	TREAT MENT DMARD	NSAID
A	47.6 ± 5.5	2 : 4	5.2 ± 2.7	SSZ (2), CHQ (2), MTX (1)	INDO (2), DS (1)†
B	51.8 ± 4.0	1 : 6	2.5 ± 0.9	NIL	NAP (2), INDO (4) DS (3)#
C	61.6 ± 4.7	2 : 5	16.0 ± 1.8§	SSZ (1), CHQ (2)	NAP (1), INDO (3) DS (2)‡

*Group A: significant proliferation but impaired cytotoxicity; Group B: significant proliferation and predicted levels of cytotoxicity; Group C: impaired proliferation and cytotoxicity.

† One patient was newly diagnosed and untreated; 3 patients were receiving combinations of DMARDs and NSAIDs. (n) = number of patients.

Two patients were receiving combinations of indomethacin and diclofenac sodium; § ($p < 0.01$)

‡ 3 patients were receiving combinations of DMARDs and NSAIDs.

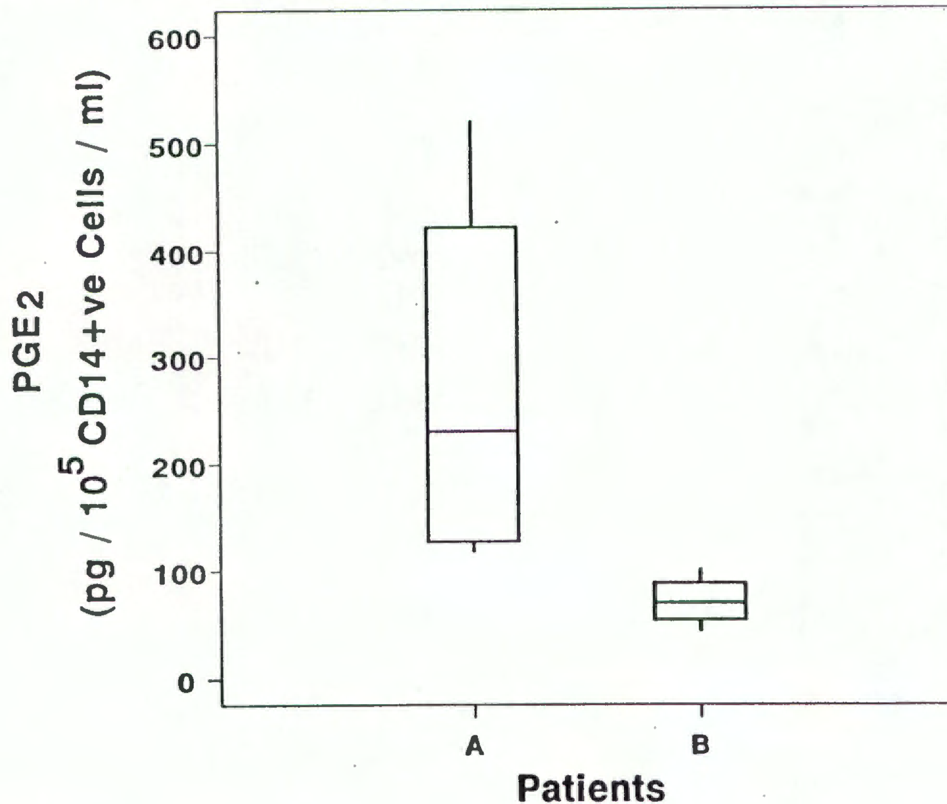
SSZ: sulphasalazine; CHQ: chloroquin; MTX: methotrexate; NAP: naproxen; INDO: indomethacin; DS: diclofenac sodium;

4. SFMNC cytokine production

The majority of cytotoxic CD4+ T-cell clones belong to the Th_1 and Th_0 phenotypes (predominantly IFN-γ producing) and a dissociation between proliferative and cytotoxic responses at the clonal level is commonly observed in antigen-specific Th_2 CD4+ clones (IL4 producing)[18]. In addition, recent reports have indicated that PGE_2 favours the emergence of Th_2 clones[19]. To determine whether the observed dissociation between proliferation and cytotoxicity in group A was the result of a predominantly Th_2 population in the SFMNC, 48h supernatants of mitogen stimulated cells from 3 patients in group A and 2 in group B were assayed for IFN-γ and IL4 levels. SFMNC from all patients tested produced moderate to high levels of IFN-γ with low or undetectable levels of IL4 [Table 2.II], indicating a Th_1- like cytokine profile in both patient groups, and not the distinct Th_2 profile of high levels of IL4 (> 80 pg/ml) and low or absent IFN-γ production (< 5 iu/ml)[20]. Thus the impaired cytotoxic function in patients of group A was not due to the presence of predominantly Th_2 non-cytolytic T-cells, suggesting a direct suppression of cytotoxic effector function, possibly by PGE_2, or other products of cyclooxygenase metabolism of arachidonic acid .

Figure 2.4

Box and whisker plot of PGE_2 concentration in 18 h supernatants of CD14+ve synoviocytes from 4 RA patients with impaired SFMNC cytotoxic function (group A) and 4 patients with high levels of SFMNC cytotoxic function (group B).*



*PGE_2 production is expressed as pg / 10^5 CD14+ synoviocytes / ml supernatant. Impaired SFMNC cytotoxicity was associated with significantly higher PGE_2 production ($p < 0.05$).

Table 2.II.

*SFMNC mitogen-induced cytokine production from 5 RA patients with significant PPD proliferation**

GROUP	PATIENT	SFMNC PPD RESPONSES	IFN-γ (iu/ml)	IL4 (pg/ml)
A	1	Significant proliferation;	165.6	< 0.8
	2	impaired cytotoxicity	308.5	2.32
	3		1048.0	5.77
B	1	Significant proliferation	359.2	< 0.8
	2	and cytotoxicity	66.9	5.62

* SFMNC (3 x 10^6/ml) were stimulated *in vitro* for 48 h using the mitogen PHA. IFN-γ and IL4 concentrations in supernatants were determined by RIA and ELISA. Patients were grouped according to SFMNC proliferative and cytotoxic responses to PPD.

5. PGE_2 mediates dissociation of *in vitro* PBMNC proliferative and cytotoxic functions

The *in vitro* effects of PGE_2 on normal PBMNC proliferative and cytotoxic function were investigated. Significant inhibition of PPD-specific cytotoxicity, but not proliferation, was found with PGE_2 at a final concentration of 1 μM (one-sided $p < 0.05$) (Table 2.III). This dissociation in functions was similar to that found in the SFMNC from patients with high CD14+ synoviocyte PGE_2 production (group A). In contrast to PGE2, specific inhibition of cytotoxicity with sparing of proliferation was not a feature of the DMARDs tested; inhibition of cytotoxicity by chloroquin was associated with a marked dose-dependent inhibition of proliferation, which was also observed with methotrexate and sulphasalazine (Table 2.III). The finding that chloroquin inhibited proliferation more than cytotoxicity may be a result of chloroquin being present in the proliferation assay for considerably longer than the cytotoxicity assay (7 days compared to 15h).

Table 2.III.

The in vitro effects of prostaglandin E_2, chloroquin, methotrexate and sulphasalazine on PPD-induced proliferation and PPD-specific cytotoxicity of PBMNC from 5 healthy individuals.

AGENT*	FINAL CONCENTRATION	PROLIFERATION† (% Inhibition)	CYTOTOXICITY† (% Inhibition)
PGE2	1.0 μM (352 pg/ml)	-3.3	29.6‡
CHLOROQUIN	0.4 μg/ml	36.1	3.2
	2.0 μg/ml	58.2	6.6
	10 μg/ml	99.2	22.4
	25 μg/ml	99.3	30.1
METHOTREXATE	3.2 μM	99.4	NT
	16 μM	99.6	NT
	80 μM	99.9	NT
SULPHASALAZINE	9 μg/ml	38.7	NT
	18 μg/ml	62.7	NT
	36 μg/ml	64.1	NT

* Effects on proliferation and cytotoxicity were determined by adding the agents at the initiation of the 6 day culture or for the duration of the cytotoxicity assay. Final concentrations of the DMARDs were in the range of pharmacological levels *in vivo*[21,22]. PPD-induced proliferation and PPD-specific cytotoxicity were determined *in vitro* using standard tritiated thymidine incorporation and ^{51}Cr release assays.

†Values shown represent the mean of at least triplicate determinations (SEM < 25% for proliferation assays and < 10% for cytotoxicity assays). PPD-induced proliferation of PBMNC in the absence of drugs was in the range Δ cpm 98 000 to 150 000 and control PPD-specific cytotoxicity between 35 - 55%.

‡one-tailed $p < 0.05$

NT - not tested

6. Effects of CD 14+ve synoviocyte supernatants on a cytotoxic CD4+ve clone

To test the hypothesis that prostanoids produced by macrophage lineage synoviocytes have a suppressive effect on CD4+ T cell cytotoxic function, the effect of supernatants generated from CD14+ synoviocytes on the function of a cytotoxic CD4+ T-cell clone were evaluated. A CD4+ T-cell clone was used in preference to PBMNC in order to examine specific effects on CD4+ T-cell cytotoxicity in isolation, thereby excluding potential effects on cytotoxic cells of other lineages. CD14+ synoviocyte supernatants were generated in the presence or absence of 10^{-5} M indomethacin for 18h. Supernatants from 2 patients who demonstrated impaired SFMNC cytotoxicity and high levels of PGE_2 production per CD14+ synoviocyte (group A), and 2 patients who had high levels of SFMNC cytotoxicity and low PGE_2 production per CD14+ synoviocyte (group B), were added to the 15h ^{51}Cr release assay (final concentration 50% v/v) of clone TS7.2E against PPD-pulsed target macrophages (Table 2.IV). Supernatants from all 4 patients generated in the absence of indomethacin contained PGE_2 (110 - 1650 pg/ml) and suppressed the cytotoxic function of the clone. However, significant abrogation of this suppressive effect by indomethacin was confined to the 2 patients from group A, and associated with a marked reduction of PGE_2 concentration in the supernatants (>70% inhibition). This abrogation was not due to the presence of indomethacin in the assay (see control, Table 2.IV), and suggests a role for products of the cyclooxygenase pathway being responsible for suppression of cytotoxic CD4+ effector function in these patients. Addition of indomethacin to the CD14+ synoviocytes of the two patients from group B resulted in minimal reduction in PGE_2 concentrations (< 30% inhibition), and failure to abrogate the observed suppressive effect on cytotoxicity, indicating that the function of cyclooxygenase had been inhibited *in vivo* by NSAID therapy.

Table 2.IV.

*Effect of CD14+ve synoviocyte supernatants from 4 patients with RA, on the cytotoxic function of a CD4+ve T-cell clone**.

		CD14 +VE SYNOVIOCYTES		CYTOTOXIC CD4+ve CLONE (TS7.2E)		
		PGE_2 IN 18h SUPERNATANT (pg /ml)§		Effect of CD14+ supernatants on CYTOTOXIC ACTIVITY† (% Specific Cytolysis)		% Increase by indomethacin
Patient Group#	Patient	Control	Indomethacin 10^{-5}M (% Inhibition)	Control supernatant	Indomethacin supernatant	
A	1	1650	54 (96.7)	10.0	17.0	70.0
A	2	135	38 (71.8)	8.9	12.6	42.0
B	1	110	93 (15.4)	8.3	9.4	13.2
B	2	115	83 (27.8)	8.5	9.0	6.3
	CONTROL‡			15.2	12.5	

* Supernatants were generated for 18h in the presence or absence of indomethacin (10^{-5}M), and added to a 15h cytotoxicity assay of clone TS7.2E. PGE_2 levels of the supernatants were quantified by RIA. Significant abrogation of the suppressor effects of the supernatants by indomethacin was confined to patients in group A, and was associated with marked reduction in PGE_2 concentration.

Patients were grouped according to SFMNC proliferative and cytotoxic responses to PPD. Group A had significant levels of proliferation but impaired cytotoxicity; Group B had high levels of proliferation and cytotoxicity.

§ Absolute PGE_2 concentration (not corrected for CD14+ cell numbers)

† Effector:target ratio 3:1

‡ Medium alone or medium + 10^{-5} M indomethacin added to assay for 15 h; cytotoxicity of non-antigen pulsed targets or targets pulsed with SK-SD was less than 0.5%.

DISCUSSION

The data presented provide evidence of antigen-specific cytotoxic function of SFMNC from patients with RA previously inferred by observations of cytolytic mediators in these cells[5,23], and highlight an effector function of CD4+ T-cells that may be relevant to the pathogenesis of the disease. A consequence of cytotoxic T-cell activity, even in an infectious disease, may be tissue damage[24]. In a chronic autoimmune disease such as RA, it is possible that on-going cytolysis of target cells bearing self or foreign antigenic epitopes, sequestered in the synovial compartment, may contribute to joint destruction[25]. The specific aetiological antigen in RA is not known and thus investigation of T-cell cytotoxic function requires the use of an alternate antigenic stimulus. PPD was chosen for this purpose because, although mycobacterial reactivity is not specific for RA[26], SFMNC from many RA patients do in fact proliferate in response to these antigens[27]. PPD-specific cytolysis is HLA class II-restricted and almost entirely due to the function of cytotoxic CD4+ T-cell effectors[12,28,29]. *Mycobacterium tuberculosis* has been invoked in the pathogenesis of RA[30] and it also has previously been suggested that cytolysis of mycobacterial antigen-expressing target cells in synovial membrane by cytotoxic T-cells may be an important pathogenetic mechanism[31].

As T-cell proliferation is required to generate CD4+ cytotoxic effectors[16], low levels of antigen-specific cytotoxicity found in association with impaired antigen-induced proliferation (group C PBMNC and SFMNC) were as expected. Given that BCG vaccination and environmental exposure to mycobacteria was uniform for all patients studied, impaired proliferation to PPD (and two additional recall antigens - data not shown) may reflect a state of anergy in this group of patients[32]. It is interesting to note that these patients had significantly longer histories of disease, suggesting that MNC proliferative and cytotoxic function may be relevant earlier in the pathogenesis of RA when maximum erosive damage occurs[33]. Impaired PPD-specific cytotoxicity of MNC from patients in group A was associated with adequate proliferative responses to PPD and confined to cells from the synovial compartment. This implies a different mechanism from that responsible for the low MNC cytotoxicity of patients in group C, and suggests the presence of modulators of T-cell cytotoxic function at the site of pathology in patients in group A.

Previous reports have indicated that DMARD suppression of lytic mediators in T-cells may be an important mechanism of action of these agents[5,23]. The data from this study suggest that modulation of SFMNC cytotoxic function cannot be accounted for by the effects of DMARD therapy alone, but may also mediated by the effects of NSAID therapy on synovial prostanoid production. Evidence in support of this hypothesis includes: (i) the association of specific impairment of SFMNC cytotoxicity with significantly higher PGE_2 production by

macrophage-lineage synoviocytes (group A), and intact SFMNC cytotoxic function with NSAID monotherapy and lower CD14+ synoviocyte PGE_2 production (group B); (ii) abrogation of the suppressive effect of CD14+ supernatants from patients with elevated PGE_2 production, on the CD4+ cytotoxic clone, by the generation of the supernatants in the presence of indomethacin; (iii) *in vitro* suppression of normal PBMNC antigen-specific cytotoxicity by PGE_2, with sparing of proliferation, and (iv) inhibition of cytotoxicity with sparing of proliferation was not an *in vitro* effect of the DMARDs tested. Given the comparatively small size of the patient population investigated, conclusions should, however, remain guarded; these findings need to be confirmed in a larger study.

High concentrations of arachidonic acid metabolites have previously been described in the synovial compartment of patients with RA[34], and originate mainly from activated neutrophils and macrophage lineage synoviocytes[35,36]. The *in vitro* concentrations of PGE_2 used in these studies is, however, considerable higher than found *in vivo*, and thus represent pharmacological concentrations[37]. However, as has been suggested by others, prostaglandins are essentially locally acting hormones, often involving cells that are in direct contact where concentrations approaching pharmacological levels may be achieved[38]. Although PGEs have been traditionally viewed as having harmful effects in RA, there is increasing evidence to the contrary. In a study reported 25 years ago, the beneficial effects of PGE_2 on adjuvant arthritis, an animal model of RA, were noted[39]. Misoprostol, a synthetic PGE_1 analogue that is increasingly used to treat NSAID-associated gastropathy in patients with RA, does not appear to aggravate synovitis[40]. Anti-inflammatory effects on *in vivo* models of acute and chronic inflammation have also been demonstrated[8]. Suppression of cytotoxic function of NK and CD8 cells by PGE is mediated by increased intracellular cAMP, resulting in reduced recycling of lytic mediators[10].

In conclusion, the data suggest that heterogeneity in SFMNC antigen-specific cytotoxicity may be accounted for by prostanoid modulation *in vivo*. Release of prostanoids by activated macrophage-lineage cells may provide a negative feedback signal that controls the extent and duration of cytotoxic T-cell effector function. In an autoimmune disease like RA, NSAID monotherapy with reduction of synovial prostanoids early in the disease may remove this local protective effect.

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Chapter 3

Eicosanoid regulation of human cytotoxic CD4+ T-cell effector functions

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INTRODUCTION

Evidence from the patient-based study reported in chapter 2 indicates that CD4+ T cells from the synovial compartment of patients with RA are capable of effecting antigen-specific cytotoxicity and that impairment of this function in a subset of these patients may have been mediated by synoviocyte-derived prostanoids. As the rheumatoid synovial compartment is characterised by the accumulation of CD4+ T cells and the presence of significant levels of eicosanoids, including products of both cycloxygenase and lipoxygenase metabolism, further elucidation of the effects of these products on CD4+ T cell functions may have pathogenetic and therapeutic relevance[1,2].

As discussed in chapter 1 (pg. 25), leukotrienes (predominantly LTB4 and LTC4) and prostanoids (essentially E-series prostaglandins) exert reciprocal modulatory effects on a broad spectrum of immune functions; in general leukotrienes have stimulatory effects and prostanoids are inhibitory. With particular relevance to cell mediated cytotoxicity, reciprocal modulation of natural killer (NK) function by leukotrienes and prostaglandins has been described[3,4,5]. Previous reports of the effects of eicosanoids on CD4+ T-cell effector functions have been confined to PGE2 modulation of cytokine production, noting differential effects on cytokine secretion by Th1 and Th2 CD4+ clones[6,7]. In addition, recent evidence from animal studies has highlighted novel pathways of cytotoxicity of CD4+ T cells that may be distinct from NK cytotoxic mechanisms[8,9].

The data reported in this chapter have been obtained using in vitro models of CD4+ T cell effector functions. The effects of eicosanoids on polyclonal and monoclonal CD4+ T cell populations were examined and attempts made to define the underlying mechanisms responsible for the observed effects. The findings extend previous observations of reciprocal influences of leukotrienes (B4 and C4) and PGE on immune functions to include CD4+ T-cell mediated cytotoxicity and, in certain instances, cytokine production. Marked inhibition of cytokine production was a common effect of the prostanoids on both Th1 and Th0 cytotoxic clones, indicating similar signalling pathways in these two CD4+ T-cell subsets. Modulation of CD4+ T-cell cytotoxicity by the eicosanoids tested was mainly due to effects on calcium-dependent pathways of cytotoxicity. In addition, the data indicate that PGE suppression of cytotoxicity by CD4+ T-cell clones is a result of modulation of both effector to target binding and post-binding events. The implications of these observations for the treatment of RA, and the approach to the further definition of the mechanisms of eicosanoid modulation of CD4+ T cell functions are discussed.

MATERIALS AND METHODS

1. Leukocyte separation and T-cell clones

Modulation of CD4+ T-cell functions by PGE was examined in both bulk (unseparated) mononuclear cell populations and at the clonal level; examination of the effects of leukotrienes on CD4+ T cell function was limited to CD4+ T cell clones. For investigation of effects on peripheral blood mononuclear cells (PBMNC), venous blood was obtained from 5 healthy volunteers known to have significant mycobacterial antigen reactivity. PBMNC were isolated from samples by Ficoll-Hypaque density centrifugation (Lymphoprep - Nycomed, Oslo, Norway) as described in chapter 2 (pg 60). Tissue culture medium used throughout these studies was RPMI 1640 (Flow Laboratories, Mclean, VA) containing 100U/ml of penicillin, 100μg/ml of streptomycin and 10% pooled AB human serum.

CD4+ T-cell clones were obtained from the site of pathology of three patients with inflammatory synovitis, and PBMNC from one healthy laboratory worker. A panel of 11 clones were used in the studies reported in this chapter. Details of the clones are provided in chapters 7 and 5. Briefly, seven of the clones were obtained by limiting dilution cloning of IL2-dependent T cell lines from synovial explants of patients with inflammatory synovitis (TS7.2E, TS7.5E and TS8.5A (gout), VS1.3D and VS2.5A (inflammatory OA) and AP2.3E and AP2.3C (RA)), and 4 clones from the circulation of a healthy individual (PL 2.2B, PL3.2D, PL18.1B and PL19.5D). Apart from the VS clones (SK-SD reactive) and clones PL18.1B and PL19.5D (tetanus toxoid reactive) all clones demonstrated PPD-specific reactivity. Limiting dilution cloning was performed using either a mitogenic stimulus (AP and VS clones - PHA) or antigenic stimulus (TS and PL clones - generated with PPD with the exception of PL18.1B and PL19.5D [tetanus toxoid]). Clones were maintained by weekly stimulation with antigen or mitogen, irradiated (4000 rads) autologous PBMNC feeders and recombinant interleukin 2 (rIL2 - 100 iu/ml) (Cetus, Emeryville, CA - kindly provided by Dr Eli Kedar, Hadassah Medical School, Jerusalem, Israel), and fed with additional fresh medium and rIL2 (100 iu/ml) every 3 - 4 days.

2. Reagents

Prostaglandin E_2 (PGE_2), forskolin (FSK, a cyclic AMP agonist) and indomethacin (cycloxygenase inhibitor) were purchased from Sigma Chemical Company (St Louis, Missouri). Misoprostol, a synthetic PGE1 analogue, was kindly donated by Dr R Marks, GD Searle and Co, Skokie, Il. Agents were solubilised in dimethyl sulphoxide (DMSO) (BDH Chemicals, Poole, England), and diluted in culture medium so that final concentrations of DMSO were always less than 0.1%; as a vehicle control DMSO 0.1% was added to all relevant control cultures and assays. Stock solutions were stored at -70°C in light-protected

containers. Leukotriene B4 (LTB4) and leukotriene C4 (LTC4) were purchased from Sigma, stored at -20°C and diluted in tissue culture medium immediately prior to use in the assays. Ethylene glycol bis - (β-amino ethyl ether) N,N,N',N' - tetraacetic acid (EGTA, Sigma) was solubilised in RPMI, and pH adjusted to 7.4.

3. Cytotoxicity assays

Antigen-specific cytotoxicity of PBMNC and CD4+ T-cell clones was measured in a 15 h ^{51}chromium (^{51}Cr) release assay as described in chapter 2 (pg. 60). Cytotoxic function of PBMNC was measured after 6 days of antigen exposure and of the clones 2 days after re-stimulation with feeders and antigen. Adherent autologous MNC were used as target cells following an optimal interval of 4-7 days culture at 37°C in 100μl complete medium in 96-well U-bottomed tissue-culture plates (Flow Laboratories). Target cells were pulsed with PPD (10μ g/ml), SK-SD (1:10 dilution), tetanus toxoid (Lederele Laboratories, American Cyanamid Co, Pearl River, NY) (1:10 dilution), or medium alone, and labelled with 6μCi/well ^{51}Cr for 24h. Effectors were added to give final effector:target ratios of 10:1. 3:1 and 0.3:1. The percentage specific cytotoxicity for the mean of triplicate wells was calculated as detailed in chapter 2 (pg. 61) .The percentage spontaneous release was always less than 20%. Lectin-dependent cytotoxicity of the T-cell clones was measured by the addition of PHA (Wellcome, final concentration of 5.75 x 10^{-3} mitogenic units/ml) to the 15h assay, using ^{51}Cr-labelled autologous mononuclear cells without antigen as targets[10]. The effects of the eicosanoids on cytotoxicity were investigated by adding the agents at the initiation of the 15h incubation period of both the antigen-specific and lectin-dependent ^{51}Cr release assays. Effects of PGE2 on the generation of cytotoxic effectors in PPD-stimulated PBMNC were determined by adding the agent at the initiation of the 6 day effector culture period.

To determine the extracellular calcium ($[Ca^{2+}]_{ext}$) dependency of antigen-specific cytotoxicity mediated by the CD4+ T cell clones, T cell clones (5 x 10^{5}/ml in complete medium) were incubated on anti-CD3 (OKT3, Jannsens Pharmaceuticals, Johannesburg, South Africa) coated 96 well flat-bottomed plates (Flow Laboratories) for 3h at 37°C in a humidified atmosphere containing 5% CO_2. Plates were centrifuged at 200g for 5 minutes prior to incubation. Anti-CD3 (5μg/ml) was adhered to the tissue culture plates by overnight incubation at room temperature in PBS; the antibody coated plates were washed 3 times prior to use. Following stimulation by plastic adherent anti-CD3, T cell clones were resuspended and washed and used in 15h ^{51}Cr release assays in the presence or absence of MgEGTA (3mM $MgCl_2$ and 6mM EGTA), as previously described[11]. This concentration of EGTA was calculated to be sufficient to chelate all free Ca^{2+} present in complete medium. Viability of effector cells cultured in the presence of MgEGTA for 15h was determined by trypan blue exclusion. The effects of MgEGTA on spontaneous release of ^{51}Cr by target cells were

investigated by culturing ^{51}Cr-labelled target cells in the presence or absence of MgEGTA for 15h (without the addition of antigen or effector cells), and measuring spontaneous and maximum ^{51}Cr release as in the cytotoxicity assay.

4. Phenotypic analysis

Dual parameter flow cytometric analysis of clones and PPD-stimulated PBMNC was performed on an Epics Profile II (Coulter Electronics, Hialeah, Florida). Monoclonal antibodies used were CD3-RD1, CD4-FITC, CD8-FITC, NKH1-RD1 (anti-CD56), IL2-R1-FITC, I2-FITC (anti-HLA-DR), B4-FITC (anti-CD19) and T11-RD1(anti-CD2) (Coulter Immunology, Hialeah, Florida). Histograms generated were (i) forward scatter (FS) vs log side scatter (LSS), (ii) log fluorescence 1 (LF1) vs log fluorescence 2 (LF2), (iii) and (iv) single parameter histograms of LF1 and LF2. Lymphocytes were bitmapped on histogram 1 to visually exclude monocytes and debris. Non-specific antibody binding was evaluated using the relevant mouse isotypic control, allowing for 2% false positives. Histogram data were generated for 10^4 events. With this technique it is possible to determine (i) percentage of cells positive for a particular surface phenotypic marker, and (ii) the fluorescence intensity of the phenotypic marker. The latter parameter is measured as the mean channel number of fluorescence, and is an indicator of the density of expression of the cell surface marker. The units of expression of mean channel number are arbitrary, and comparable only for an individual antibody.

5. Cytokine assays

CD4+ T-cell clones (2 x 10^6/ml in culture medium) were re-stimulated using either feeders and antigen, feeders and mitogen, or plastic-adherent anti-CD3 (OKT3) and rIL2, in the presence or absence of varying concentrations of PGE2, misoprostol, LTB4 and LTC4 for 48h. Supernatants were collected and stored at -70°C until assayed. Interferon-γ (IFN-γ) concentration in the supernatants was measured by commercial RIA (IFN-γ IRMA, Medgenix Diagnostics, Fleurus, Belgium) and interleukin 4 (IL4) concentration determined by commercial ELISA (IL-4 EASIA, Medgenix Diagnostics), according to the manufacturers' directions.

6. Data analysis

All cytotoxicity assays of the T cell clones were performed in triplicate on at least 2 separate occasions. The paired students t test was employed for statistical comparison using the program Instat (GraphPad Software, San Diego, CA). Unless stated otherwise, two-tailed p values are reported; p values less than 0.05 were considered significant.

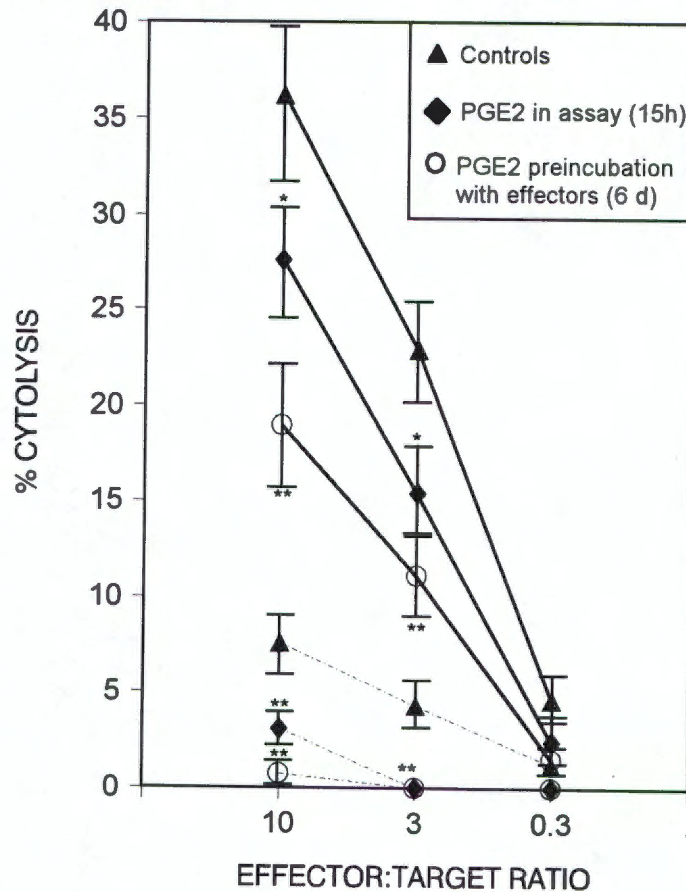
RESULTS

1. PGE2 inhibits antigen-specific and non-specific cytotoxicity of PPD-stimulated PBMNC.

PGE2 (10^{-6}M) caused significant inhibition of PBMNC cytotoxicity. Both the generation of cytotoxic effectors as well as the cytotoxic function of these effectors were inhibited (Figure 3.1). Inhibition of both antigen-specific (against PPD-pulsed targets) and non-specific cytotoxicity (targets without antigen or with irrelevant antigen, SK-SD) was found and was maximal when PGE2 was added at the initiation of the effector culture (Figure 3.1).

Figure 3.1

The in vitro inhibitory effects of PGE2 on antigen-specific and non-specific cytotoxic functions of PPD-stimulated PBMNC.



PBMNC from 5 healthy individuals were primed *in vitro* for 6 days with PPD (triangles). PGE2 (10^{-6}M) was added either at the initiation of the effector culture period (open circles) or for the duration of the 15h ^{51}Cr release assay only (diamonds). Solid lines indicate antigen-specific cytotoxicity and dotted lines show non-specific cytotoxicity. Mean ± standard error are shown. * $p < 0.05$; ** $p < 0.005$

2. Modulation of effector PBMNC phenotypic expression by PGE2

Phenotypic analysis of effector PBMNC indicated differential modulation of cytotoxic cell populations by PGE2 (Table 3.I). A significant reduction in the percentage of CD56+ MNC (NK/LAK) ($p < 0.05$) and a trend to lower percentages of CD8+ lymphocytes was found in MNC stimulated with PPD in the presence of PGE2, and contrasts with the significant increase in the percentage of CD4+ lymphocytes in these effectors ($p < 0.005$). As PPD-specific cytotoxicity measured in the ^{51}Cr release assay is mediated almost entirely by CD4+ T-cells[12,13], the finding of increased percentages of CD4+ T-cells in effectors generated in the presence of 10^{-6}M PGE2 suggests a functional rather than quantitative inhibitory mechanism on cytotoxicity. This contrasts with the parallel reduction in the percentage of CD56+ cells and the impairment in anomalous cytotoxicity of the PBMNC effector cells stimulated in the presence of PGE2. Although PGE2 resulted in a relative increase in the percentage of CD4+ T-cells in response to PPD, these cells were less activated, with a reduced percentage of CD4+ cells expressing HLA-DR when compared with control effectors(Table 3.I). In addition, reduction in cell surface expression of CD4 was indicated by a significant decrease in CD4 mean channel number or brightness of fluorescence (Table 3.I) (Figure 3.2). Marked reduction of HLA-DR expression on CD4- cells was also observed as an effect of PGE2 (Fig 3.2).

TABLE 3.I

Phenotypic analysis of PPD-stimulated peripheral blood mononuclear effector cells cultured in the presence or absence of 10^{-6}M PGE2 for 6 days†

	PERCENTAGE POSITIVES		MEAN CHANNEL NUMBER	
	CONTROL	PGE2	CONTROL	PGE2
CD3	73.7 ± 3.6	79.8 ± 4.5	6.17 ± 0.37	5.63 ± 0.69
CD4	53.2 ± 2.6	65.7 ± 1.7**	4.79 ± 0.40	3.42 ± 0.39**
CD8	21.7 ± 3.0	18.9 ± 3.7	5.11 ± 0.42	6.27 ± 1.82
CD56	20.87 ± 3.3	7.9 ± 1.3*	14.76 ± 3.95	5.84 ± 1.03
%CD4 IL2R#	16.5 ± 6.3	11.9 ± 2.6	2.22 ± 0.33	2.64 ± 0.33
%CD4 DR‡	32.0 ± 6.7	20.87 ± 4.4*	5.07 ±0.91	4.01 ± 0.58
IL2R	19.7 ± 6.5	9.2 ± 1.8	2.14 ± 0.33	2.37 ± 0.47
DR	35.3 ± 5.4	21.2 ± 2.0*	5.77 ±0.45	4.14 ± 0.38

† Peripheral blood mononuclear cells were obtained from 3 healthy PPD-reactive volunteers. Dual parameter flow cytometric analysis of PPD-stimulated effector cells cultured in the presence or absence of PGE2 (10^{-6}M) was performed as described in materials and methods.

Percentage of CD4+ cells positive for IL2 receptor expression

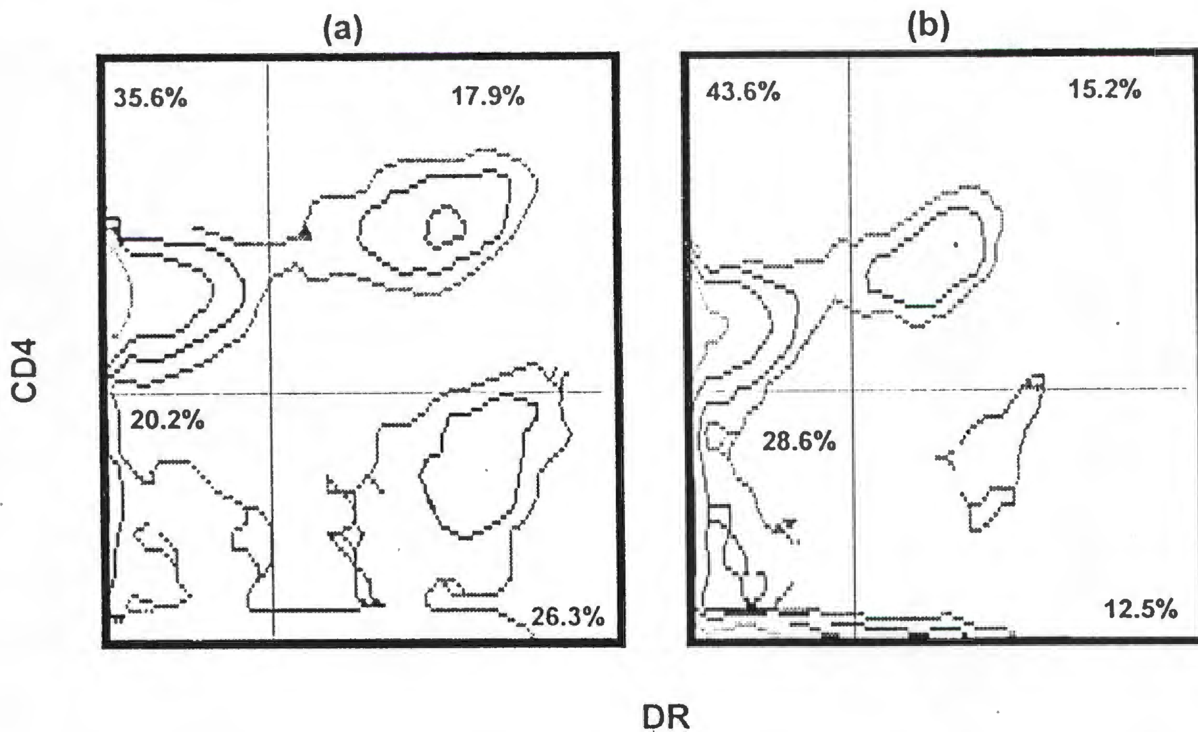
‡ Percentage of CD4+ cells positive for HLA-DR expression. *$p < 0.05$ **$p < 0.005$

3. PGE2, misoprostol and forskolin inhibit cytotoxicity of CD4+ T-cell clones.

The mechanism of prostaglandin suppression of CD4+ T-cell cytotoxicity was further investigated by determining the effects of PGE2, misoprostol and forskolin on the cytotoxic effector function of a panel of seven CD4+ T-cell clones. PGE2, misoprostol and forskolin, alone or in combination, suppressed cytotoxicity when added to the ^{51}Cr release assay of these clones (Table 3.II). Representative cytotoxicity data at multiple effector:target ratios for clone TS 7.2E is shown in figure 3.3. In addition, significant inhibition of cytotoxicity was found with PGE2 and misoprostol at final concentrations of 10^{-7}M and 10^{-5}M; forskolin was only weakly inhibitory at concentrations less than 10^{-5}M (Table 3.II). PGE2 suppression of cytotoxicity was not titratable (unlike misoprostol), displaying a dose response with peak inhibition at 10^{-6}M.

FIGURE 3.2

Dual parameter flow cytometric analysis of PBMNC cultured for 6 days with PPD in the absence (a) or presence (b) of PGE2.



* Analysis was performed as described in materials and methods. Although PGE2 resulted in increased percentages of CD4+ cells, the mean channel number of CD4 on these cells was lower than controls (see Table 3.I). PGE2 caused reduced expression of HLA-DR on CD4+ and CD4- cells. Figures shown represent the percentage of cells in each quadrant.

TABLE 3.II

*Percentage inhibition of cytotoxic effector function of CD4+ T cell clones by PGE_2 and other cAMP agonists**

CLONE	PGE_2 10^{-5}M	PGE_2 10^{-6}M	PGE_2 10^{-7}M	MISO 10^{-5}M	MISO 10^{-6}M	MISO 10^{-7}M	FSK 10^{-5}M	FSK 10^{-6}M	PGE2+ FSK†
TS7.2E	26.9	55.9	25.4	58.8	57.8	42.8	38.6	8.3	59.8
TS7.5E	29.2	44.2	30.1	49.1	45.6	29.8	nd	nd	nd
TS8.5A	nd	32.6	nd	48.5	22.3	18.4	20.5	6.3	nd
PL2.2B	16.4	48.2	22.3	52.3	18.9	12.2	17.9	nd	63.8
PL3.2D	10.6	51.9	35.6	48.8	nd	nd	22.3	7.7	57.9
VS2.5A	nd	89.3	nd	nd	nd	nd	nd	nd	nd
VS1.3D#	nd	23.9	nd	nd	nd	nd	nd	nd	nd

*Data shown as percentage inhibition of antigen-specific cytotoxicity (unless indicated otherwise), at an effector to target ratio of 10:1, by the presence of the agent for the duration of the 15h ^{51}Cr release assay. Cytotoxicity assays were performed in triplicate on a least two occasions. Control (maximum) cytotoxicity: TS7.2E - 44%, TS7.5E - 25%, TS8.5A - 48%, PL2.2B - 36%, PL3.2D - 30%, VS2.5A - 28%, VS1.3D - 29%; with the exception of the lectin dependent assay of clone VS1.3D, non-specific cytolysis of targets without antigen, or targets with irrelevant antigen was less than 2%.

† PGE2 10^{-6}M and forskolin 10^{-5}M

Lectin dependent cytotoxicity

PGE - prostaglandin E; MISO - misoprostol; FSK - forskolin; nd - not done

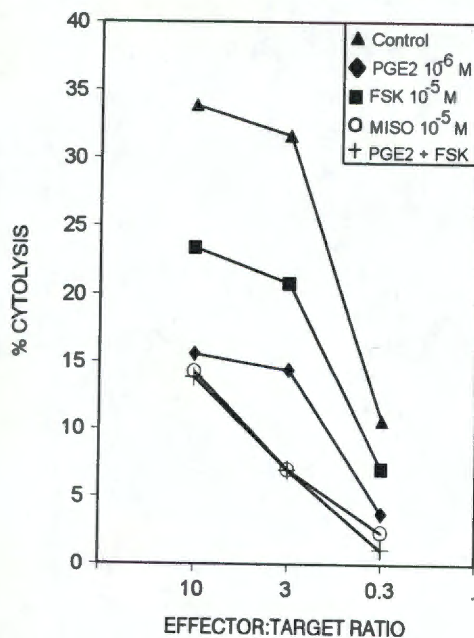


FIGURE 3.3

The inhibitory effects of cyclic AMP agonists on the PPD-specific cytotoxic effector function of a CD4+ T cell clone, TS7.2E.

The agents were added for the duration of the 15h ^{51}Cr release assay. Data represent the mean of triplicate determinations performed under the same experimental conditions. Similar results were found with this clone on a further two occasions and with 6 other CD4+ T cell clones.

FSK - forskolin; MISO - misoprostol; PGE2 + FSK - PGE2 10^{-6}M and forskolin 10^{-5}M.

4. Modulation of CD4+ T cell mediated lectin dependent cytotoxicity by the presence of cAMP agonists in the assay.

CD4+ T-cell mediated cytolysis may be mediated by granule exocytosis and fas/fas ligand interactions (see Ch1 pg 20). Both mechanisms of cytotoxicity involve effector-to-target binding that initiates a series of events leading to target cell death. We undertook to define whether PGE2 and forskolin exert their suppressive effects on CD4+ T-cell cytotoxicity at effector-to-target binding or post-binding events. Effects on binding were investigated in a lectin-dependent cytotoxicity assay in which PHA was used as a non-specific adhesive agent, thereby overcoming any inhibition of effector-to-target binding[14]. Both PGE2 (10^{-6}M) and forskolin (10^{-5}M) inhibited lectin-dependent cytotoxicity of clone TS7.2E if present during the assay, although the levels of inhibition were lower than that observed on antigen-specific cytotoxicity: PGE2 mean inhibition of antigen specific cytotoxicity : 56.6 ± 4.4% and mean inhibition of lectin dependent cytotoxicity : 29.7 ± 2.7%; forskolin mean inhibition of antigen specific cytotoxicity : 24.3 ± 3.6% and mean inhibition of lectin dependent cytotoxicity : 13.5 ± 2.1%; (n=3, 10:1 effector:target ratio). Representative lectin dependent cytotoxicity data of clone TS7.2E at multiple effector to target ratios is shown in Figure 3.4. The reason for the plateau in cytolysis between 3:1 and 10:1 effector to target ratios is not clear; this may reflect saturation of available targets at a ratio of 3:1, or be a function of the incubation period.

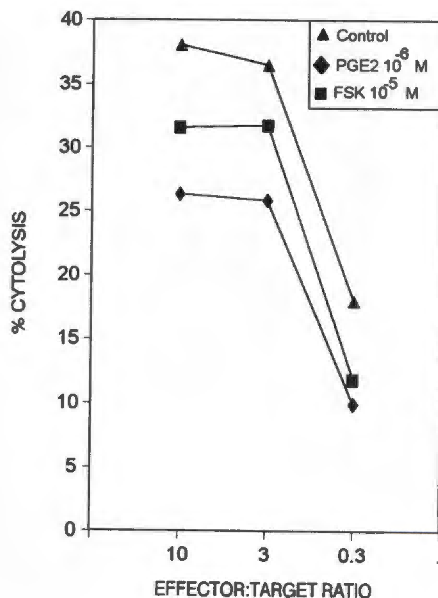


FIGURE 3.4 *The inhibitory effects of cyclic AMP agonists on the lectin dependent cytotoxic effector function of a CD4+ T cell clone, TS7.2E.*

The agents were added for the duration of the 15h ^{51}Cr release assay in the presence of PHA (5.75 x 10^{-3} mitogenic units/ml). Similar results were found with this clone on a further two occasions.

FSK - forskolin.

5. Pre-incubation of CD4+ T cell clones with PGE2 inhibits antigen-specific but not lectin dependent cytotoxicity.

To investigate the effects of PGE2 on the generation of cytolytic potential, CD4+ T-cell clones were pre-incubated in the presence of 10^{-6} M PGE2 for periods of between 48h and 3 weeks. For the longer pre-incubation periods (> 1 week), T cell clones were cultured on plastic adherent anti-CD3 in complete medium supplemented with rIL2 (50 iu/ml) and PGE2 or 0.1% DMSO in RPMI (control), fed every 3-4 days with fresh medium containing PGE2 or control solution, and passaged weekly. Cytotoxicity assays were performed after extensive washing of effector cells, without the addition of PGE2 to the assay. Inhibition of antigen-specific but not lectin-dependent cytotoxicity was a consistent finding. Representative data obtained with clone PL3.2D following a 3 week pre-incubation in the presence of PGE2 are shown in Figure 3.5; similar results were observed with this clone on two additional occasions (pre-incubation periods of 48h and 1 week) and with clones PL2.2B and TS7.2E following a 48h pre-incubation with PGE2. These findings thus indicate that pre-incubation with PGE2 had no direct effect on the generation of cytolytic potential in the CD4+ T cell clones, as demonstrated in the lectin dependent assays. The suppression of antigen-specific cytotoxicity under the same experimental conditions was an interesting observation, suggesting sustained modulation of effector-target cell interaction, despite the absence of exogenous PGE2 in the assay.

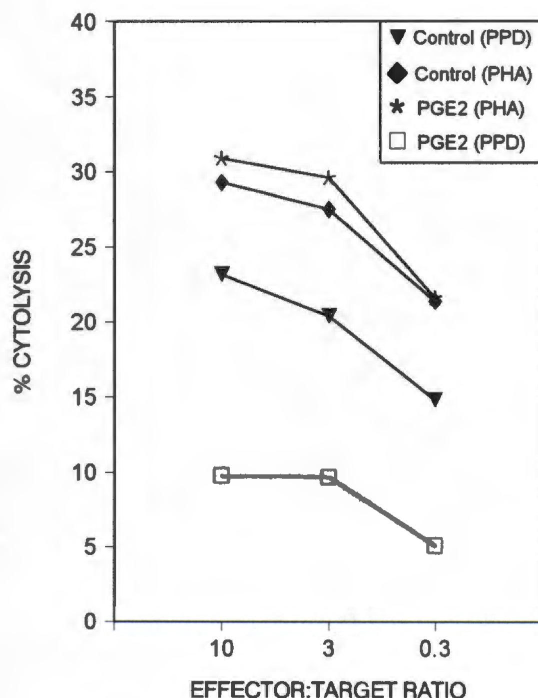


FIGURE 3.5

Pre-incubation with PGE2 (10^{-6}M) inhibits antigen-specific, but not lectin-dependent cytotoxic function of a CD4+ T-cell clone.

Clone PL3.2D was passaged for 3 weeks using plastic-adherent anti-CD3 and recombinant IL2 (50 iu/ml), in the presence or absence of PGE2. Effectors were washed extensively before measuring cytotoxicity in a standard 15h ^{51}Cr release assay, without the addition of PGE2 to the assay.

Control (PPD) - control effectors (antigen-specific); Control (PHA) - control effectors (lectin-dependent); PGE2 (PHA) - PGE2 pre-incubated effectors (lectin dependent); PGE2 (PPD) - PGE2 pre-incubated effectors (antigen-specific).

6. Effects of PGE2 on phenotypic expression of CD4+ T cell clones

Phenotypic analysis of CD4+ T-cell clones stimulated with feeders and antigen in the presence of 10^{-6}M PGE2 for 48 h indicated no significant modulation of CD4, CD28 or CD29 when compared to controls. Pooled data obtained with clones TS7.2E, TS7.5E and PL 2.2B are shown in Table 3.III. Thus the observed functional inhibition of cytotoxicity cannot be attributed to effects on these antigens. Interestingly, differential modulation of activation markers on CD4+ clones by PGE2 was observed, with significant reduction in HLA-DR expression ($p < 0.05$) and a trend to increased IL-2 receptor expression ($p = 0.06$, ns) (Table 3.III).

TABLE 3.III

Phenotypic analysis of 3 antigen-specific CD4+ T-cell clones cultured in the presence or absence of 10^{-6}M PGE2 for 48 h†

	PERCENTAGE POSITIVES		MEAN CHANNEL NUMBER	
	CONTROL	PGE2	CONTROL	PGE2
CD4	91.5 ± 1.0	91.0 ± 1.4	19.28 ± 6.41	21.95 ± 7.18
IL2R	15.4 ± 1.6	25.3 ± 5.4	10.57 ± 2.70	11.32 ± 3.32
DR	53.5 ± 13.3	48.2 ± 16.5	34.59 ± 6.69	25.61 ± 9.62*
CD29	45.0 ± 4.7	39.6 ± 6.2	2.78 ± 0.70	3.68 ± 0.20
CD28	36.2 ±3.0	37.8 ± 4.6	2.13 ± 0.31	1.68 ± 0.07

†Clones were re-primed using irradiated autologous feeders and antigen at optimal concentrations. Indomethacin (10^{-5} M) was used to inhibit *de novo* prostaglandin synthesis. Dual parameter flow cytometric analysis was performed as described in materials and methods. *$p < 0.05$

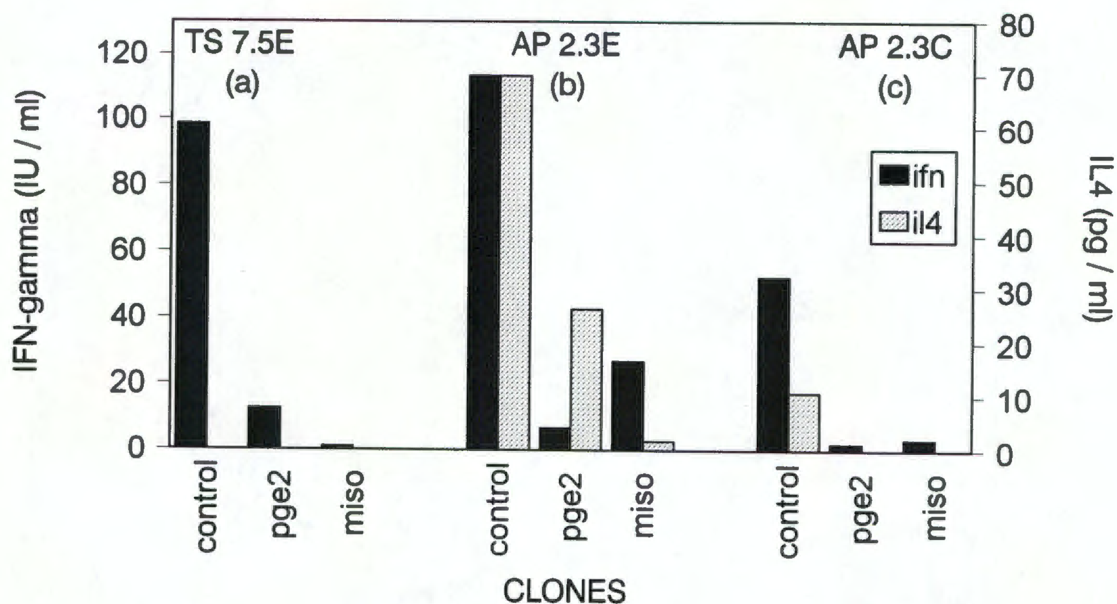
7. PGE2 and misoprostol inhibit cytokine production by Th1 and Th0 cytotoxic CD4+ T-cell clones.

The effects of PGE2 and misoprostol on cytokine production by a panel of six cytotoxic CD4+ T-cell clones were investigated. Cytokines were measured in supernatants of cells stimulated for 48 h, in the presence or absence of PGE2 or misoprostol, with either autologous feeders and antigen, autologous feeders and mitogen or immobilised anti-CD3 and rIL2, without feeders. Indomethacin (10^{-5}M) was used to inhibit *de novo* prostaglandin production by feeders. IFN-γ and IL4 were quantified using commercial kits (Medgenix). Marked inhibition (60-95%) of IFN-γ production by 3 Th1 clones and IFN-γ and IL4 production by 3 Th0 clones

was caused by both PGE2 and misoprostol, irrespective of the mode of stimulus. Representative data from 3 clones is shown in Figure 3.6. PGE2 and misoprostol inhibition of IL4 production by Th0 clones occurred even in the presence of high concentrations of recombinant IL2 (100 iu/ml) used in combination with solid phase anti-CD3 to stimulate cytokine production (Fig 3.6c).

FIGURE 3.6

*Prostanoid inhibition of cytokine production by cytotoxic CD4+ T cell clones**



*PGE2 and misoprostol inhibit IFN-γ production by a Th1 CD4+ clone stimulated with feeders and antigen (a), and IFN-γ and IL4 production by Th0 cytotoxic CD4+ clones stimulated with feeders and mitogen (b) or with plastic adhered anti-CD3 and rIL2 (100iu/ml) (c). Supernatants were harvested after 48 h and assayed for IFN-γ and IL4 using commercial kits. Similar results were found for an additional Th0 clone and 2 Th1 clones.

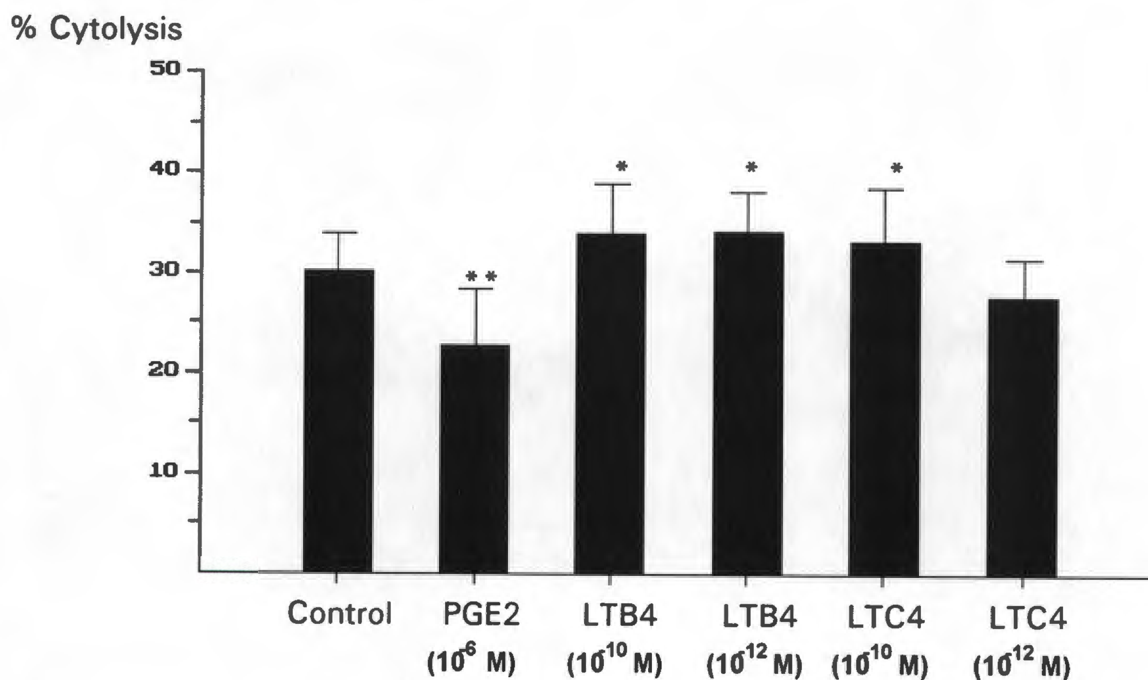
8. Leukotriene B4 and C4 augment cytotoxic effector function of CD4+ T cell clones

The effects of LTB4 and LTC4 on CD4+ T cell mediated antigen-specific cytotoxicity were investigated by adding the agents to the 15h ^{51}Cr release assay of a panel of 3 CD4+ T cell clones (PL2.2B, PL18.1B and TS7.2E). Indomethacin (10^{-5}M) was used to inhibit *de novo* prostaglandin synthesis, known to be stimulated by leukotrienes[15]. In addition, the effects of PGE2 (10^{-6}M) on cytotoxicity were assayed under the same experimental conditions. Pooled cytotoxicity data, at an effector to target ratio of 10:1, for the 3 clones on at least 2 separate

occasions is shown in figure 3.7. Both LTB4 (10^{-10}M and 10^{-12}M) and LTC4 (10^{-10}M only) resulted in moderate but significant increases in antigen specific cytotoxicity ($p < 0.05$). Under the same experimental conditions, PGE2 caused significant suppression of cytotoxic effector function ($p < 0.01$). Increase in cytotoxicity was in the range 12.3 ± 3.5% (LTB4 10^{-10}M) to 17.3 ± 4.8% (LTC4 10^{-10}M). Pooled data from experiments performed under the same conditions indicated that PGE2 resulted in mean inhibition of cytotoxicity of 31.9 ± 8.2%.

Figure 3.7

Modulation of cytotoxic effector function of a panel of 3 CD4+ T cell clones by leukotrienes B4 and C4, and PGE2#.



#Leukotrienes and PGE2 were added for the duration of the 15h ^{51}Cr release assay. Data shown represent the mean ± standard error of antigen specific cytotoxicity (effector:target ratio 10:1) of a panel of 3 CD4+ T cell clones assayed on a total of 7 occasions. * $p < 0.05$; **$p < 0.01$

9. Modulation of CD4+ T cell cytokine production by leukotrienes.

The effects of LTB4 and LTC4 on cytokine production by a panel of three cytotoxic CD4+ T-cell clones were investigated. Cytokines were measured in supernatants of cells stimulated for 48 h by PHA and autologous feeders, in the presence or absence of the agents. Indomethacin (10^{-5}M) was used to inhibit *de novo* prostaglandin production by feeders. Some diversity in the effects of LTB4 and LTC4 on cytokine production was noted (Table 3.IV). High levels of IFN γ production by a Th1 clone were essentially unaffected by the presence of exogenous LTB4 and LTC4. However, low levels of IFN γ production by a Th0 and an additional Th1 clone were increased in the presence of LTC4 (both clones) and LTB4 (Th1 clone only). IL4 production was not influenced by the leukotrienes; levels of IL4 remained undetected in the supernatants of Th1 clones stimulated in the presence of LTB4 and LTC4, and high levels of production of IL4 by the Th0 clone was unaltered in the presence of the leukotrienes.

Table 3.IV

*Effects of leukotrienes B4 and C4 on cytokine production by CD4+ T cell clones**

	TS 7.2E		PL 18.1B#		PL 19.5D#	
	IFN γ (iu/ml)	IL4 (pg/ml)	IFN γ (iu/ml)	IL4 (pg/ml)	IFN γ (iu/ml)	IL4 (pg/ml)
CONTROL	195	< 16	34	> 1358	15	< 16
LTB4 10^{-8}M	187 (-4.1)	< 16	33 (-2.9)	> 1358	25 (66.7)	< 16
LTB4 10^{-10}M	197 (1.0)	< 16	33 (-2.9)	> 1358	23 (53.3)	< 16
LTB4 10^{-12}M	198 (1.5)	< 16	36 (5.9)	> 1358	25 (66.6)	< 16
LTC4 10^{-8}M	178 (-8.7)	< 16	44 (29.4)	> 1358	24 (60)	< 16
LTC4 10^{-10}M	179 (-8.2)	< 16	38 (11.8)	> 1358	27 (80)	< 16
LTC4 10^{-12}M	190 (-2.6)	< 16	34 (0)	> 1358	31 (106)	< 16

* T cell clones were stimulated in vitro with PHA and autologous irradiated PBMNC as feeder cells. Leukotrienes were added at the initiation of the culture period. Supernatants were harvested after 48 h and assayed for IFN-γ and IL4 using commercial kits. Values in parentheses are percentage change in cytokine concentration.

#Clones PL18.1B and PL19.5D were obtained by limiting dilution cloning of normal PBMNC and have tetanus toxoid-specific proliferative responses.

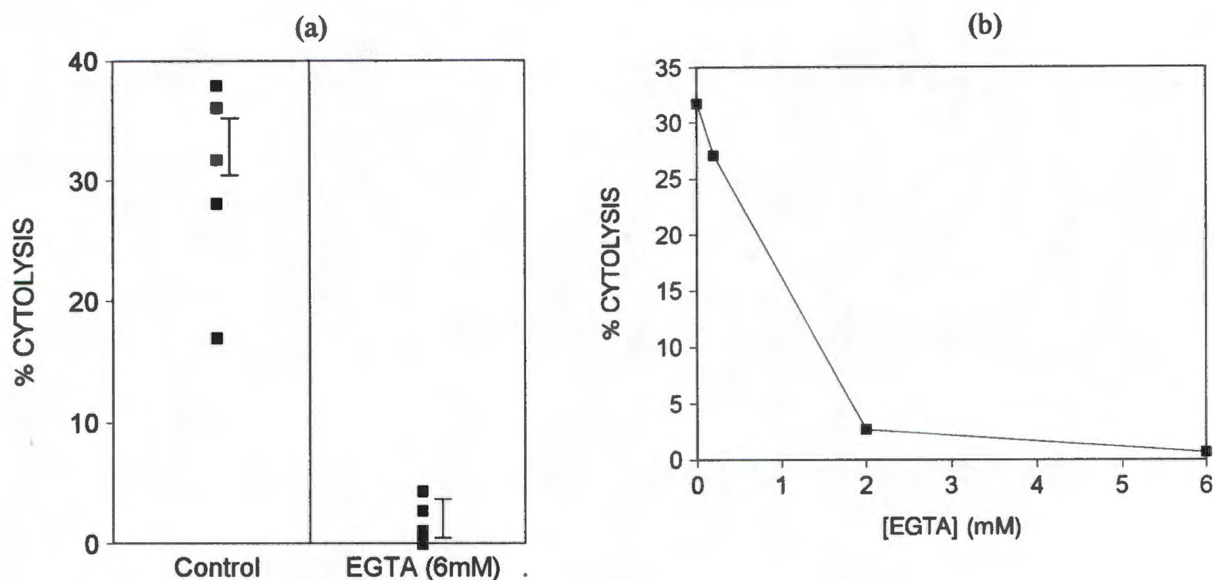
10. Extracellular calcium ($[Ca^{2+}]_{ext}$) dependency of antigen-specific cytotoxicity mediated by CD4+ T cell clones

Antigen-specific cytotoxicity of a panel of 5 clones used in the above experiments was measured under $[Ca^{2+}]_{ext}$ free conditions following pre-activation by plastic adherent anti-CD3 stimulation for 3h. Parallel assays performed in standard culture conditions, after a similar pre-activation procedure, served as controls. In the presence of MgEGTA (3mM $MgCl_2$ and 6 mM EGTA) cytotoxic effector function of all clones tested was markedly inhibited; control cytotoxicity: 33.4 ± 2.2%, $[Ca^{2+}]_{ext}$ free cytotoxicity: 2.19 ± 0.83% at an effector to target ratio of 10:1 ($p < 0.001$) (Figure 3.8a). Both effector cell viability and levels of spontaneous ^{51}Cr release by target cells were unaffected by the presence of the chelating agent at the concentrations used in the cytotoxicity assays. Effector cell viability after 15h culture in the presence of MgEGTA was greater than 95% (determined by trypan blue exclusion for each of the 5 clones); spontaneous:maximum ^{51}Cr release of target cells incubated in the presence of MgEGTA for 15h was always less than 20% (determined for each cytotoxicity assay), and was similar to controls.

Clone TS7.2E was pre-activated as described above and cytotoxicity assays performed in the presence of variable concentrations of MgEGTA. A dose-dependent inhibition of cytotoxic effector function was observed, with almost complete suppression of cytotoxicity at a final EGTA concentration of 6mM (Figure 3.8b).

Figure 3.8

Extracellular calcium-dependency of antigen specific cytotoxicity mediated by CD4+ T cell clones



(a) Pooled cytotoxicity data (effector:target ratio 10:1) of 5 clones following preactivation on plastic adherent anti-CD3, under standard conditions (control) or $[Ca^{2+}]_{ext}$ free conditions (6mM EGTA). Data points represent the mean of triplicate determinations for each clone and error bars indicate mean ± standard error for the pooled data.

(b) Dose dependent inhibition of cytotoxic effector function of clone TS7.2E by MgEGTA. Antigen-specific cytotoxicity at an effector to target ratio of 10:1 is shown. Data points represent the mean of triplicate determinations; variation was less than 10% for each triplicate.

DISCUSSION

In the studies reported in this chapter the role of eicosanoids in the *in vitro* regulation of CD4+ T cell effector functions has been examined and the mechanisms of their actions addressed. Within the limitations of the *in vitro* studies, the findings provide evidence suggestive that leukotrienes (B4 and C4) and prostaglandins have opposite modulatory effects on CD4+ T-cell effector functions. As current paradigms of the immunopathogenesis of RA support a central role for synovial CD4+ T cells in the initiation and perpetuation of the disease, the potential regulatory effects of eicosanoids suggested in the current study may be of importance at the site of pathology in RA[16,17].

A relative increase in the *in vitro* expansion of CD4+ PBMNC in response to soluble antigen was observed in the presence of PGE2. However, these cells were found to have impaired cytotoxic function. As antigen-specific cytotoxicity of the T cell clones demonstrated in the current study was mainly calcium dependent, inhibition of the granule exocytosis pathway of cytotoxicity by cAMP agonists is suggested, in common with previous studies of CD8+ T cells and NK cells[17,5]. PGE2 inhibition of antigen-specific cytotoxicity mediated by the T cell clones displayed an unusual titration effect, with maximal inhibition observed at a final concentration of 10^{-6} M (table 3.II). Recently a similar bi-phasic inhibitory phenomenon of T cell mediated cytotoxicity has been described with the cAMP analog 8-chlorophenylthio-cAMP, and may be related to activation status of effector cells[18]. It is possible that a similar mechanism may account for the findings of the current study, given that PGE2 is a potent stimulator of intracellular cAMP. The difference in the titration of the inhibitory effects on cytotoxicity of PGE2 and misoprostol may be due to the relative potency of these agents, with the misoprostal titration curve to the left of that for PGE2[19].

The inhibitory mechanisms of cAMP agonists on CD4+ T cell mediated cytotoxicity were further investigated using a lectin dependent cytotoxicity assay. Suppression of antigen-specific but not lectin-dependent cytotoxicity by pre-incubation of T cell clones with PGE2 is indicative of inhibitory effects on effector to target cell binding[14]. In addition, inhibitory effects of PGE2 and other cAMP agonists on post-binding events are implied by the suppression of lectin dependent cytotoxicity when the agents were present in the assay; similar effects on post-binding events have previously been demonstrated in a cytotoxic CD8 T cell clone and attributed to inhibition of intracellular cytolytic granule delivery mechanisms[17]. The maintenance of lectin dependent cytotoxicity despite long-term culture of a CD4+ T cell clone in the presence of high concentrations of PGE2 suggests that PGE2 does not modulate the generation of cytolytic machinery, and thus inhibits T cell cytotoxicity at a level distinct from cyclosporin and corticosteroids[20,21].

Inhibition of effector to target binding by PGE2 could not be attributed to effects on the cell surface antigens studied. In particular, modulation of CD28, a structure thought to be relevant to the activation and function of cytotoxic T-cells, was not an effect of PGE2 or misoprostol[22,23]. While PGE2 inhibition of class II MHC expression is well-established, and confirmed in the present study, this does not account for impairment of effector to target binding, as (i) antigen-specific cytotoxicity of T cell clones pre-incubated in the presence of PGE2, but without PGE2 in the assay, was inhibited - thus inhibition of target cell HLA-DR expression cannot be invoked in this setting, and (ii) as demonstrated in the next chapter, HLA-HLA-DR expression of cytotoxic CD4+ T cell clones does not necessarily correlate with cytotoxic function[24]. It is possible that inhibition of binding is due to effects of cAMP agonists on other surface antigens or, as has been described in a cytotoxic CD8+ T-cell clone, may be due to modulation of dynamic cytoskeletal function[17].

The role of leukotrienes in T cell function is somewhat controversial, as discussed previously (Ch1 pg. 29). It has been demonstrated that T cells are capable of converting macrophage-derived LTA4 to LTB4 by the action of LTA4 hydrolase and that LTB4 stimulates T cell proliferation[25,26]. The current study provides a novel demonstration that LTB4 and LTC4 have moderate but significant stimulatory effects on CD4+ T cell cytotoxicity. However, the effects of LTB4 were not titratable, which may question the reliability of the finding. As indicated above, these effects were mainly on the calcium dependent cytotoxic function of the CD4+ T cell clones and thus may be the result of similar mechanisms as have been described for leukotriene augmentation of NK function[3].

The mechanisms of T cell cytotoxicity have been discussed previously (Ch1 pg. 20). Based on animal studies it has been suggested that a $[Ca^{2+}]_{ext}$ independent pathway, mediated by fas/fas-ligand binding, is the major mechanism of CD4+ T cell mediated cytotoxicity[11]. However, recent evidence indicates that cytotoxic CD4+ and CD8+ T cells mediate both fas- and perforin ($[Ca^{2+}]_{ext}$ dependent) pathways of cytotoxicity, and that considerable variation with respect to the relative importance of either pathway exists within each subset[27]. In the present study, antigen-specific cytotoxicity mediated by a panel of 5 human CD4+ T cell clones of diverse origin was demonstrated to be almost entirely $[Ca^{2+}]_{ext}$ dependent. It is possible that this finding may reflect a difference between murine and human CD4+ T cell cytotoxicity mechanisms, or as previously reported, may be a function of the target cells used in the assays[11]. In addition, although $[Ca^{2+}]_{ext}$ independent cytotoxicity may be mediated by fas/fas ligand binding, upregulation of fas ligand on effector T cells and apoptosis (the result of fas binding) of target cells may be $[Ca^{2+}]_{ext}$ dependent, and consequently inhibited under $[Ca^{2+}]_{ext}$ free conditions[28,29]. Furthermore, fratricidal killing by human CD4+ T cell clones, a

function demonstrated to involve apoptosis, was reported to be almost completely inhibited in the presence of 1mM MgEGTA[30].

Cytotoxic T cell-induced apoptosis of target cells may be mediated by fas/fas ligand binding or the constituents of cytotoxic granules, particularly granzyme B; regulation of fas-mediated apoptosis within the target cell may be controlled by the bcl-2 gene product[31,32,33,34,35]. To date, reports of the effects of eicosanoids on apoptosis have been limited to PGE modulation of apoptosis induced by mechanisms other than fas binding, with conflicting results. Although PGE2-augmented apoptosis of thymocytes and other cells has been demonstrated, a recent report has indicated no effect on human mononuclear cells[36,37]. In addition, increased cAMP promotes a cell survival response and retards apoptosis[38].

It is now well established that human CD4+ T-cell clones can be divided into three populations according to their pattern of cytokine production (see chapter 1, pg. 19). Differential modulation of Th1 and Th2 cytokine production by PGE has been demonstrated, with inhibition of Th1 production of IFN-γ, but either stimulation or no effect on Th2 production of IL4[6,7]. In addition, misoprostol has been shown to have similar effects[7]. Inhibition may be the result of suppression of cytokine mRNA expression[7]. PGE2 modulation of cytokine production by Th0 clones appears to reflect heterogeneity of this cell population, with both Th1 and Th2-like responses[39]. The findings reported in this chapter that PGE2 and misoprostol cause marked inhibition of both IFN-γ production by Th1 clones, and IFN-γ and IL4 production by Th0 cytotoxic T-cell clones, supports the recent suggestion that modulation of cytokine production by cAMP agonists is a property of the cell type and not the cytokine[40]. This data thus indicates that signalling pathways in the cytotoxic Th0 clones studied resemble those of Th1 CD4+ clones.

Although PGE2 caused marked suppression of IFN-γ production by cytotoxic CD4+ T-cell clones, long term passaging of a clone in the presence of high concentrations of PGE2 did not affect the cytolytic potential of this clone, as demonstrated in a lectin dependent cytotoxicity assay. This differential modulation by PGE2 of lectin-dependent cytotoxicity and IFN-γ production provides interesting insight into the association of CD4+ T-cell cytotoxicity and IFN-γ production. Several lines of evidence have indicated a relationship between IFN-γ production and cytotoxic function of CD4+ T-cells: cytotoxicity is almost exclusively confined to CD4+ T-cell clones that produce IFN-γ (Th1 and Th0 subsets); levels of cytotoxicity correlate with the amount of IFN-γ production, and IFN-γ drives Th1 differentiation and increases cytolytic activity of T-cell lines[10,41]. The findings reported here indicate that the maintenance of cytolytic potential of CD4+ T-cell clones occurs independently of IFN-γ

production and supports a previous suggestion that IFN-γ is not responsible for CD4+ T-cell mediated cytotoxicity[10].

LTB4 augmentation of IFN-γ and IL2 secretion by polyclonal CD4+ T cell populations has previously been reported[42]. However, the effect of leukotrienes on cytokine production by CD4+ T cell clones has not previously been described. In the present study, LTB4 and LTC4 augmentation of IFN-γ production by CD4+ T cell clones was found, but restricted to clones with low control levels of IFN-γ production. In addition, the effects of the leukotrienes showed unusual titration patterns which may question the reliability of the data. The lack of effect of the leukotrienes on the high IFN-γ production by a Th1 clone may indicate that maximal levels of secretion had been achieved. The finding that LTC4 but not LTB4 stimulated IFN-γ production in a Th0 clone is of interest and warrants further investigation. Differential modulation of IFN-γ and IL4, as previously reported for PGE2, was not found to be an effect of leukotrienes in this study as no direct modulation of IL4 production was demonstrated. Nevertheless, the data provide additional suggestive evidence of the immunostimulatory properties of LTB4 and LTC4, especially given the role of IFN-γ in cellular immune functions[10].

Having provided functional evidence suggestive of the role of eicosanoids in the modulation of CD4+ T cell effector functions, the studies reported in this chapter raise several issues about the mechanism of action of these agents that require further investigation. In particular, the effects of eicosanoids on fas- and perforin-dependent pathways of T cell cytotoxicity may be further defined by investigation of their effects on fas expression of target cells, and fas ligand and intracellular granzyme and perforin expression of effector T cells using cytofluorimetric techniques. Given the recent awareness of the role of apoptosis in animal models of autoimmunity and the potential role in immune regulation, investigation of the effects of eicosanoids on apoptotic pathways of cell death (both fas-dependent and independent) may have relevance to RA[43,44,45,30].

There is increasing understanding of the role of cytotoxic CD4+ T-cells in a broad spectrum of clinical conditions in addition to RA, including the immune response to infectious diseases, tumours, and transplant rejection[46,47,20]. The *in vitro* studies reported here indicate a potential target for novel therapeutic modulation of CD4+ T-cell effector functions in these settings; specific inhibition of 5 lipoxygenase metabolism or antagonism of LTB4 and LTC4 at chronic inflammatory sites (including the rheumatoid synovial compartment) may reduce the stimulatory effects of leukotrienes on CD4+ T cell helper and cytotoxic functions. In addition, the inhibition of CD4+ T cell functions by the PGE1 analogue, misoprostol, may contribute to

its beneficial effects *in vivo* on graft survival, and indicates the therapeutic potential of this agent in RA[48].

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Chapter 4

Dissociation of functions of CD4+ T-cell clones: post-activation Th1 and Th0 clones retain their cytolytic potential

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INTRODUCTION

A series of cellular events occur following the interaction of a CD4+ T cell with its specific antigenic epitope in association with the MHC molecule of an antigen-presenting cell. This stimulus transmitted by the T cell receptor/CD3 complex activates intracellular signalling pathways that promote transcription of cytokine and other genes leading to upregulation of a variety of cell surface receptors, cytokine secretion with autocrine and paracrine effects and progression into the cell cycle with associated blastogenesis and cellular proliferation[1,2,3,4]. In the case of a cytotoxic CD4+ T cell (Th1 and Th0 subsets), these events may be accompanied by killing of the antigen-presenting cell[5]. Given the possibility that cytotoxic effector function of CD4+ T cells in the rheumatoid joint may be involved in the pathogenesis of the disease (see Ch2 pg. 58), defining the cellular regulation of CD4+ T cell mediated cytotoxicity and its relationship with other cell functions may be of importance.

In a polyclonal T cell population, a positive linear relationship between levels of *in vitro* antigen-induced proliferation and antigen-specific cytotoxicity has previously been demonstrated and was confirmed in the studies of peripheral blood and synovial fluid mononuclear cells from patients with RA reported in chapter 2 [6]. It has also previously been shown that the kinetics of proliferation following an antigenic stimulus closely parallel the kinetics of antigen-specific cytotoxicity in polyclonal T cells from tuberculous effusion[7]. However, it is possible that the relationship between *in vitro* proliferation and cytotoxicity of CD4+ T cells observed in polyclonal populations noted above is due to clonal expansion of specific effector cells, and do not reflect the interaction of these functions at a single cell or monoclonal level.

Separation of *in vitro* proliferation and cytokine production from cytotoxicity in murine CD4+ T cell clones has been demonstrated with the use of peptide substitution involving critical T cell receptor binding residues[8]. These findings thus suggest different pathways of control of effector functions in CD4+ T cells and are supported by the description of similar dissociations in cellular functions of anergic murine CD4+ T cell clones that retain cytotoxicity in the absence of proliferation or IL2 secretion[9]. Separation of murine CD4+ T cell proliferation from cytotoxicity has also been demonstrated as an *in vitro* effect of cyclosporin and in cells activated through CD2 and using different monoclonal antibodies as ligands[10]. Further evidence indicating independent regulation of T cell proliferation and cytotoxicity includes the finding that senescent human CD8 T cell cultures retain cytolytic function in the absence of proliferative responses[11].

In this chapter the *in vitro* relationship between human CD4+ T cell functions at the clonal level have been addressed by investigating the kinetics of activation marker expression, proliferation, cell cycle progression, cytokine production and cytotoxicity following a single initiating stimulus. A panel of human Th1 and Th0 clones, including clones generated from rheumatoid synovium, were studied. The findings demonstrate dissociation in CD4+ T cell functions in that post-activated clones retained cytolytic potential, irrespective of levels of proliferation, cytokine secretion or activation marker expression. Physiological, pathophysiological and therapeutic relevance of these findings are discussed.

MATERIALS AND METHODS

1. T cell clones.

A total of 6 CD4+ T-cell clones were included in these investigations. A brief description of the clones is included in Table 4.I; detailed descriptions of cloning techniques and functional properties of the clones are included in chapters 7 and 5 (pgs. 177 and 126). Tissue culture medium used throughout these studies was RPMI 1640 (Flow Laboratories, Mclean, VA) containing 100U/ml of penicillin, 100μg/ml of streptomycin and 10% pooled AB human serum. T cell clones were maintained by weekly passaging with irradiated autologous PBMNC feeder cells, antigen or mitogen at optimal concentrations, and rIL2 (100 iu/ml), and fed with additional fresh medium and rIL2 (100 iu/ml) every 2 to 3 days. Experiments were timed such that the cells had not received additional rIL2 for 3 days and feeders and antigen/mitogen for 7 days at the commencement of study.

For investigation of kinetics of responses, T cell clones (10^6/well) were re-stimulated with irradiated autologous PBMNC (5×10^5/well), antigen and rIL2 (100 iu/ml) in 24 well tissue culture plates (Costar) in a final volume of 1ml of culture medium. A series of experiments was also performed without supplemental rIL2. Cells and their supernatants were harvested after 24h (day 1), 48h (day 2), 96h (day 4) and 168h (day 7), and analysed as described below. During this culture period the cells were resuspended regularly to disrupt clumping, but were not provided with additional medium or rIL2. Cell viability was determined by trypan blue exclusion.

2. Cytotoxicity assays.

Antigen-specific and lectin-dependent cytotoxic functions were determined at each time point in a 15h ^{51}Cr release assay as described previously (chapter 3 pg. 82). Autologous MNC target cells were plated between 4 and 6 days prior to use at each time point to ensure uniformity in target cell maturity.

3. Proliferation.

Proliferative responses of CD4+ T-cell clones were assayed in triplicate in microculture as previously described[12]. Cloned T cells (5 x 10^4 per well) were stimulated with PPD (Central Veterinary Laboratory, Weybridge, Surrey, England) at a final concentration of 3µg/ml, and irradiated autologous PBMNC (4000 rads) as feeder cells (10^4/well) in a final volume of 200 µl in the presence or absence of rIL2 (100iu/ml). ^{3}H thymidine incorporation was measured over 15h at the termination of the incubation period for each time point.

4. Cytokines.

Cell supernatants were collected and stored at -70°C until assayed. Interferon-γ (IFN-γ) concentration in the supernatants was measured by commercial RIA (IFN-γ IRMA, Medgenix Diagnostics, Fleurus, Belgium) and interleukin 4 (IL4) concentration determined by commercial ELISA (IL-4 EASIA, Medgenix Diagnostics), according to the manufacturers' directions.

5. Monoclonal antibodies.

Monoclonal antibodies used were T4-RD1 (CD4), I2-FITC (HLA-DR), IL-2R1-FITC (IL2 receptor, CD25) and 4B4-FITC (memory, CD29), all from Coulter Immunology (Hialeah Fl. USA), and CD28-FITC (Immunotech, Westbrook, Maine). Corresponding isotypic controls were used in all cases to set cursors to allow 2 % false positives. Antibodies were used at the concentrations suggested by the manufacturer.

6. Flow cytometry.

An Epics Profile II flow cytometer was used (Coulter Electronics) to perform immunophenotyping and cell cycle analysis. Histograms were analysed using Cytologics Software (Coulter). Histograms were gated on the lymphocyte population by forward scatter (FS) versus log side scatter (LSS), and data generated for 10^4 events.

Changes in cell morphology following stimulation were investigated by analysis of the dual parameter histograms of forward scatter (FS) vs log side scatter (LSS), where FS is a measure of cell size and LSS reflects cell granularity[13]. Gates were set to include the whole cell population and exclude debris. Single parameter histograms were projected from the gated area of the dual parameter histogram and analysed using Cytologics Software.

7. Cell cycle analysis.

T-cell clones were permeabilised, fixed and stained with propidium iodide using DNA Prep Kit (Coulter). Gating was performed as described for immunophenotyping and in addition doublets

were excluded from the analysis by gating on the histogram, fluorescence 3 peak (FL3P) vs fluorescence 3 (FL3). Single parameter histograms of FL3, showing G0/G1, S and G2/M, were analysed using Cytologic software (Coulter) to calculate proliferative index (PI = S + G2/M). The method of broadened rectangles was used to analyse DNA histograms[14]. Cells in G0/G1 phase of the cell cycle have the normal diploid amount of DNA (DNA index (DI) = 1). Tetraploid amounts of DNA are found in cells in the G2/M phase (DI = 2). Intermediate amounts of DNA are present in cells in the S phase of the cell cycle (2 > DI > 1).

8. Intracellular IFN-γ and PCNA.

Detection of intracellular antigens was performed using a modified technique of Jung et al[15]. Antibodies used were anti-interferon-γ (Genzyme, Cambridge, MA), anti-PCNA (Coulter) and GAM-FITC (Cappel, Organon Teknika, Turnhout, Belgium). Cells were washed twice with RPMI 1640 to remove protein. The cell pellet was resuspended in 100μl RPMI. Cells were fixed and permeabilised by adding methanol (stored at -20°C) dropwise while vortexing. Dehydration was allowed to continue on ice for 30 minutes. Cells were washed twice with 1 % AB serum in RPMI and resuspended in 100 % AB serum at a cell concentration of 4 x 10^6 / ml. Antibodies were added to V-bottom microtitre wells (4μg of each antibody / well). A negative control of 4μg mouse IgG was included. A 50μl aliquot of cells was added to each well and incubated on ice for 60 minutes. Cells were washed 3 times with 1 % AB serum in RPMI at 4°C and resuspended in 50μl 100 % AB serum. GAM-FITC was added to a final volume of 100μl at a 1:100 dilution. This was incubated on ice for 60 minutes and then washed 3 times as above. The cells were resuspended with a pipette between washes and finally resuspended in 50μl 1 % AB serum in RPMI and 100μl 1 % paraformaldehyde in PBS. Flow cytometry was performed on an Epics II flowcytometer (Coulter). Cells were gated on forward scatter (FS) and log side scatter (LSS). FITC labelling was measured as integrated fluorescence on a log scale and a linear scale as well as peak fluoresence.

9. Statistics.

Statistical analysis were performed using the program Instat (GraphPad Software, San Diego, CA). Repeated measures analysis of variance (nonparametric) was used to analyse data obtained from kinetics experiments; p values less than 0.05 were considered significant.

RESULTS

1. Kinetics of cytotoxicity

The cytotoxic function of a panel of 6 Th1 and Th0 clones was assayed at four time points (day 1, day 2, day 4 and day 7) following stimulation with irradiated autologous PBMNC, antigen and rIL2. No significant changes in cytotoxicity were noted over the seven day test period (Table 4.I). Comparable kinetics of cytotoxicity were observed in antigen specific and lectin-dependent assays, with moderate levels of cytotoxicity observed at each time point in all 6 clones. Similar results were obtained using clone PL2.2b on a further two occasions (Table 4.I). The mean and standard error of antigen-specific cytotoxicity at each time point is shown in figure 4.1. Lower (but not significant) cytotoxicity on day 1 following stimulation may have been the result of residual contamination of effector cells by irradiated feeder cells.

Table 4.I

*Kinetics of antigen-specific and lectin-dependent cytotoxicity of a panel of 6 CD4+ T cell clones**

		KINETICS OF CYTOTOXICITY (% Cytolysis)							
		D1[#]		D2		D4		D7	
CLONE	DESCRIPTION	Agen	PHA	Agen	PHA	Agen	PHA	Agen	PHA
PL2.2B (Exp. 1)	Source: normal PBMNC; cloned with PPD; Th0	31.9	29.2	34.4	49.1	27.6	37.6	36.9	29.7
PL2.2B (Exp. 2)	as above	32.6	24.2	39.2	20.3	48.4	38.7	45.2	53.5
PL2.2B (Exp. 3)	as above	31.3	22.3	44.4	30.5	46.9	43.7	48.0	nd
PL2.2B (mean ± se)	mean of 3 experiments	*31.9 ±0.3*	*25.2 ±1.7*	*39.3 ±2.4*	*33.3 ±6.9*	*41.0 ±5.5*	*40.0 ±1.5*	*43.4 ±2.7*	*41.6 -*
PL 7.2E	Source: normal PBMNC; cloned with PPD; Th1	11.5	34.2	23.2	53.9	21.6	40.5	20.9	27.4
PL 18.1B	Source: normal PBMNC; cloned with TT; Th0	nd	35.9	nd	55.8	nd	38.0	nd	34.8
AP 2.3C	Source: RA synovium; cloned with PHA; Th0	nd	21.3	nd	nd	nd	28.1	nd	36.3
AP 2.5E	Source: RA synovium; cloned with PHA; Th?	nd	23.5	nd	nd	nd	28.6	nd	31.3
AP 3.7F	Source: RA synovium; cloned with PHA; Th1	nd	25.1	nd	nd	nd	23.1	nd	27.5
Mean ± se	POOLED DATA	*26.8 ±4.4*	*27.0 ±1.8*	*35.3 ±3.9*	*41.9 ±6.3*	*36.1 ±5.9*	*34.8 ±2.4*	*37.8 ±5.3*	*34.4 ±3.2*

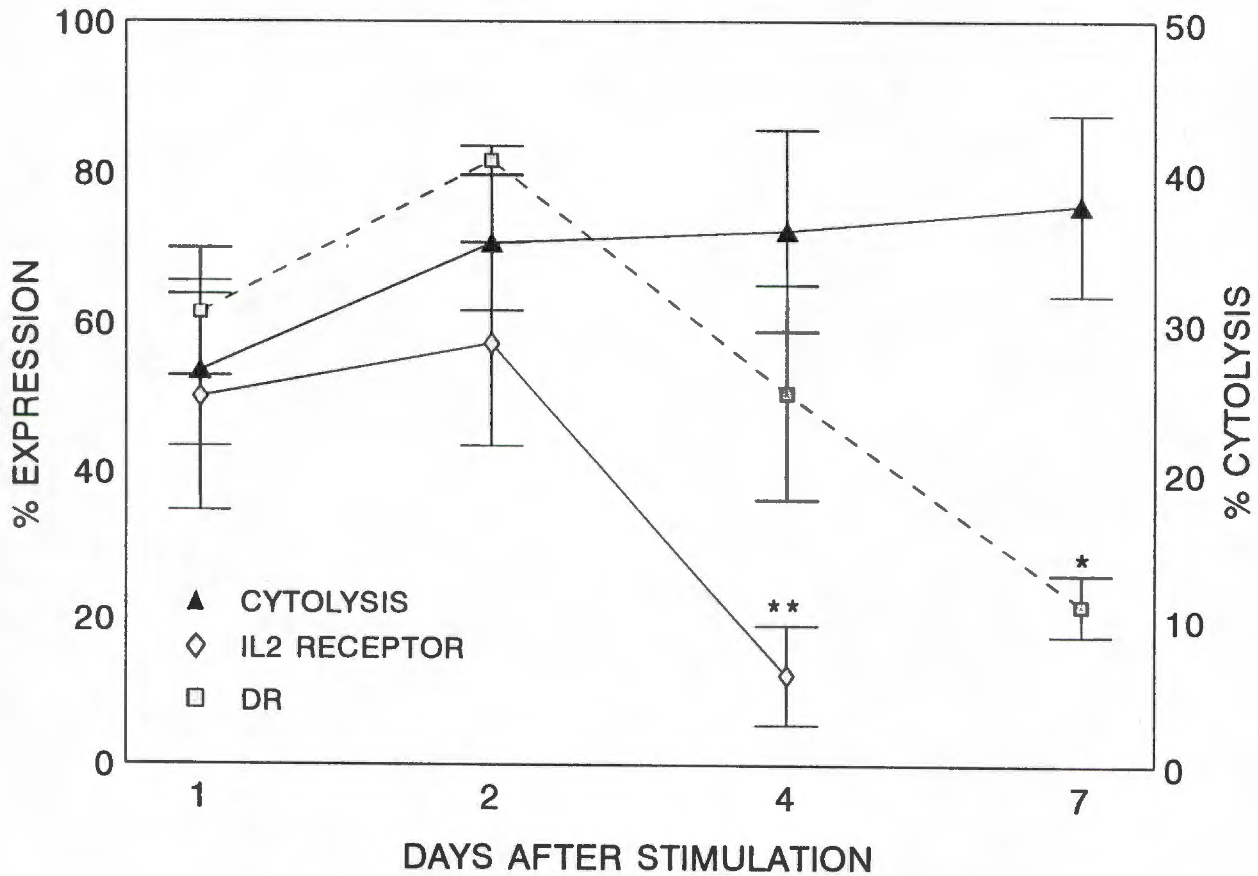
* Cytotoxicity was measured in a 15h 51Cr release assay at four time points following a single activating stimulus of the clones (effector to target ratio 10:1)

[#] Days following stimulus.

Agen - antigen; TT - tetanus toxoid; nd - not done; se - standard error

Figure 4.1

Kinetics of antigen-specific cytotoxicity and activation marker expression of a panel of 4 CD4+ T cell clones



Assays were performed at four time points following a single antigenic stimulus. Data shown represents mean ± standard error for 4 clones.

* $p < 0.05$; ** $p < 0.01$

2. Surface immunophenotyping and cell cycle analysis

Flowcytometric analysis of a panel of four CD4+ clones following stimulation was performed at the same time points as for the cytotoxicity assays. The percentage of cells expressing IL2-R and HLA-DR peaked 2 days after stimulation and then declined to significantly lower levels on day 4 (IL2-R) ($p < 0.01$) and day 7 (HLA-DR) ($p < 0.05$). The mean and standard errors for the pooled data from the four clones are shown in figure 4.1.

Modulation in the cell surface density of activation markers and other surface antigens was indicated by changes in mean channel number or brightness of fluorescence[16]. Representative one parameter histograms from clone PL2.2b are shown in figure 2. Peak surface density of HLA-DR (fig 4.2a) and IL2-R (fig 4.2b) occurred on day 1 following stimulation and declined at subsequent time points; the bimodal HLA-DR expression observed on day 1 may have been the result of contaminating feeder cells (DR^{dim}), or dual populations of activated (DR^{bright}) and pre-activated (DR^{dim}) clones. A modest increase in mean channel number of CD28 expression (fig 4.2c) was found on days 4 and 7 following stimulation, while CD29 cell density remained essentially unchanged (fig 4.2d). Interestingly, cell surface density of CD4 was modulated following stimulation (fig 4.2e). Peak CD4 brightness was observed on day 1 and declined to a nadir on day 4, with a return to near peak levels on day 7.

Cell cycle analysis was performed concurrently with surface immunophenotyping in clones PL2.2b, PL7.2e and PL18.1b. As was found for activation marker expression, the peak proliferative index (percentage of cells in G2M and S phase) was observed on day 2 following stimulation, and declined at subsequent time points (Table 4.II). Representative DNA histograms from clone PL18.1b are shown in figure 4.3.

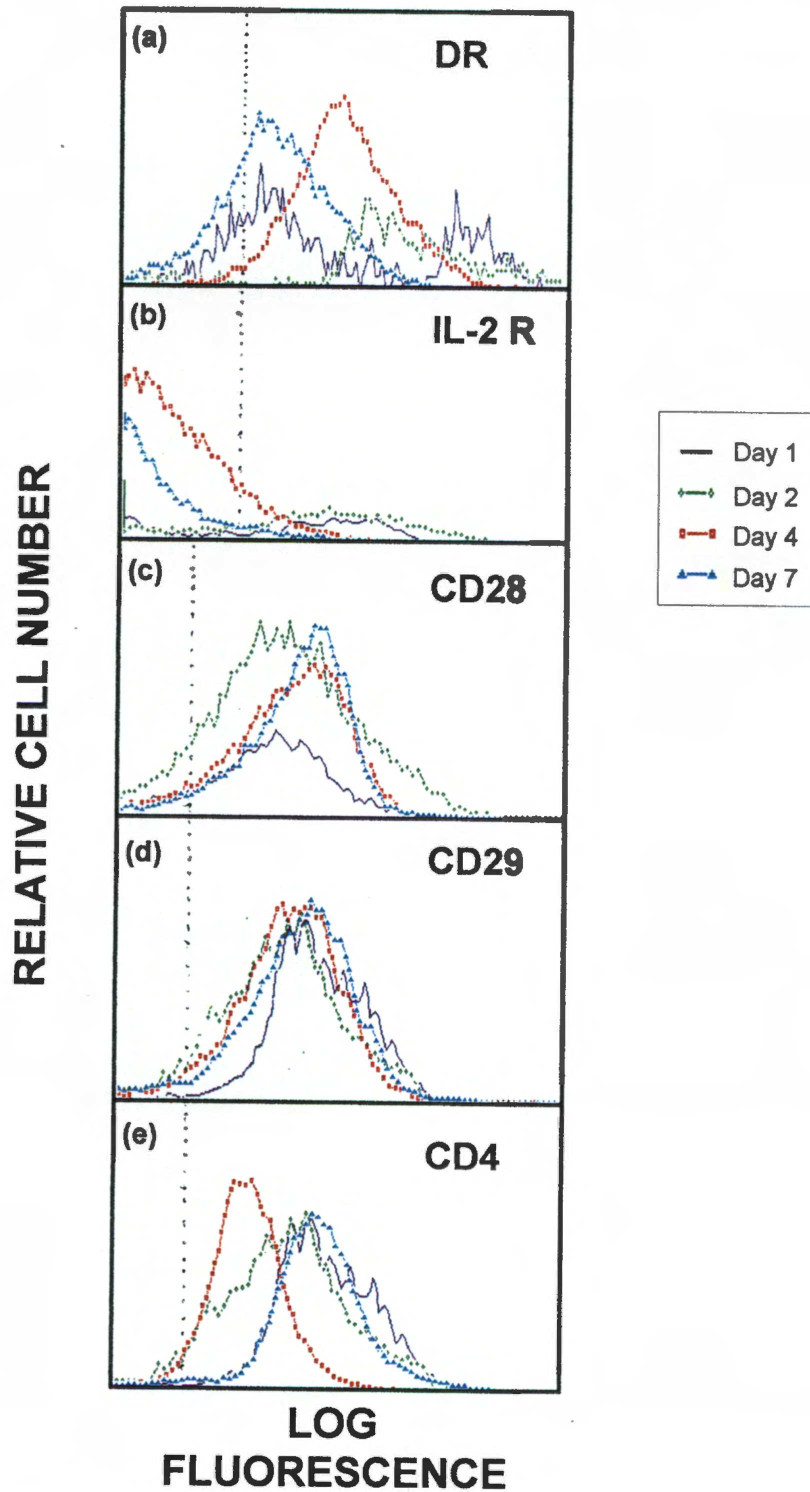
Table 4.II

*Changes in proliferative index following stimulation of 3 CD4+ T cell clones**

CLONE	Day 1	Day 2	Day 4	Day 7
PL2.2B	11.5	21.6	16.0	15.9
PL7.2E	6.3	15.4	15.8	6.1
PL18.1B	5.9	35.4	3.8	3.0

* Proliferative index (percentage of cells in G2M and S phase of the cell cycle) was calculated as described in materials and methods.

FIGURE 4.2. *Modualtion in phenotypic expression of clone PL2.2b following stimulation**



*Immunophenotyping was performed by flow cytometry at 4 time points following stimulation of the clone with irradiated autologous PBMNC, antigen and recombinant IL2. Similar results were obtained with 3 additional clones. Data is displayed as overlayed one parameter histograms. Dotted line indicates 2% cut off of isotypic controls.

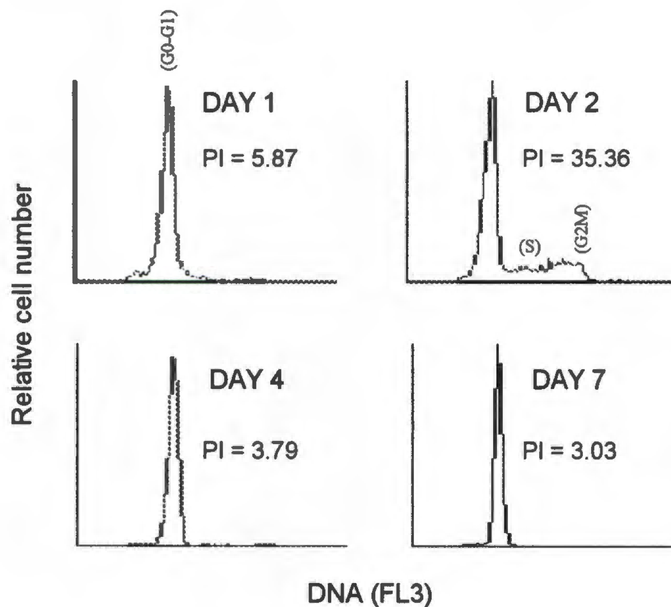


Figure 4.3

Cell cycle analysis of clone PL18.1B at 4 time points following stimulation.

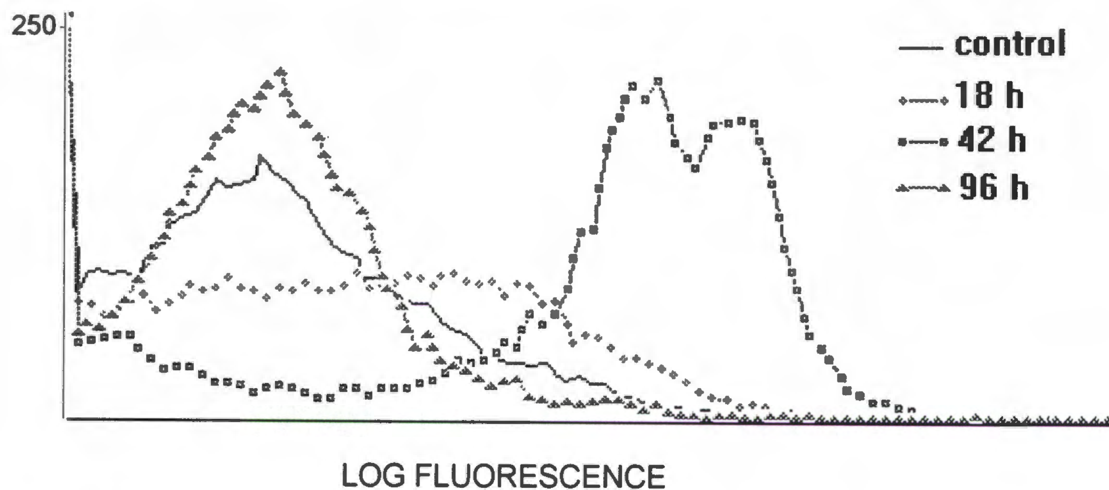
Stages of the cell cycle are indicated in parentheses in the upper panels

PI - proliferative index. FL3 - red fluorescence.

As an additional indicator of cell cycle activity, the kinetics of intracellular expression of proliferating cell nuclear antigen (PCNA) or cyclin[17,18] were investigated in 3 clones. Maximal percentage of cells with detectable intracellular PCNA was found on day 2 following stimulation in each clone, and declined to pre-stimulated levels by day 4. Representative single parameter histograms for clone 18.1B are shown in figure 4.4

Figure 4.4

*Modulation of intracellular PCNA expression of clone PL18.1B following stimulation**



*Intracellular detection of PCNA was performed by flow cytometry at each time point, following permeabilization and fixation of cells as described in materials and methods.

Control - isotypic antibody control

3. Cell size and granularity

Significant changes in cell size but not granularity were observed after stimulation of clones PL2.2b, PL7.2e, PL18.1b and AP2.5e. The mean channel number of forward scatter, a reflection of cell size, was significantly greater on day 2 than day 7 after stimulation ($p < 0.01$) (Table 4.III). Representative single parameter histograms for clone AP2.5e are shown in figure 4.5a (cell size) and 4.5b (granularity). Similar results were obtained if log side scatter histograms were converted to a linear scale.

Table 4.III

Changes in cell size and granularity of a panel of 4 CD4+ T cell clones following a single antigenic stimulus[#]

	DAY 1	DAY 2	DAY 4	DAY 7
Cell size (FS) (MCN ± se)	33.5 ± 0.32	35.4 ± 1.0*	29.6 ± 1.8	25.4 ± 1.1*
Granularity (LSS) (MCN ± se)	32.5 ± 0.56	33.1 ± 0.15	32.8 ± 0.72	31.9 ± 0.6

[#]Changes in cell morphology of clones PL2.2b, PL7.2e, PL18.1b and AP2.5e were determined by flow cytometry as described in materials and methods.

FS - forward scatter; LSS - log side scatter; MCN - mean channel number; se - standard error.

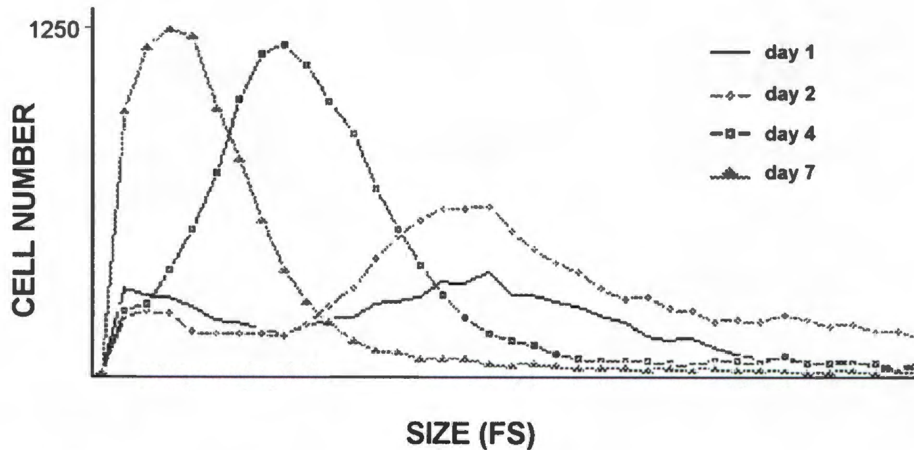
*$p < 0.01$

4. Cytokine production

Concentrations of IFN-γ and IL4 in cell supernatants were assayed as a function of time following stimulation of clones PL2.2b, PL7.2e and PL18.1b. Peak concentrations of IFN-γ in the supernatants of the Th1 clones and IL4 in the supernatant of the Th0 clone were found 24h after stimulation (Table 4.IV). In general, IFN-γ concentrations remained near peak levels at subsequent time points, whereas IL4 levels in the supernatants of the Th0 clone declined rapidly. It is interesting to note that while the IL4 levels in the supernatant of this Th0 clone declined rapidly between day 1 and day 2, IFN-γ concentrations remained constant.

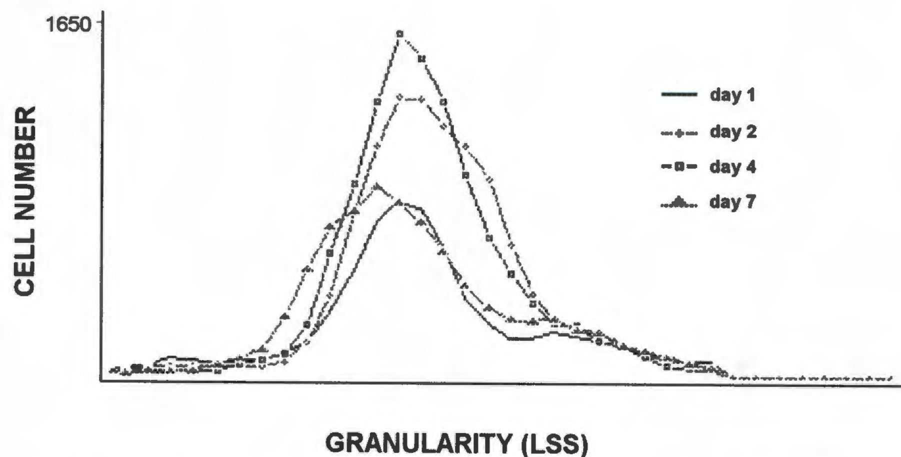
Figure 4.5a

*Modulation in cell size of clone AP2.5e following antigenic stimulation**



*Changes is cell morphology following activation were determined by flow cytometry, as described in materials and methods. Data is displayed as a single parameter projection from the dual parameter histogram of forward scatter (FS) vs log side scatter (LSS).

FIGURE 4.5b Granularity of clone AP2.5e following antigenic stimulation*



*Changes is cell morphology following activation were determined by flow cytometry, as described in materials and methods. Data is displayed as a single parameter projection from the dual parameter histogram of forward scatter (FS) vs log side scatter (LSS).

As the concentration of cytokine in the cell supernatant reflects an equilibrium between secretion and utilization or breakdown, it is possible that the rapid decline in IL4 levels in the supernatants of the Th0 clone reflects autocrine utilization. The maintenance of near peak levels of IFN-γ concentrations in the supernatants of the Th1 clones may indicate a steady state between production and utilization, or the lack of uptake or breakdown of the cytokine once secretion had ceased. To distinguish between these latter two possibilities, the kinetics of intracellular expression of IFN-γ were investigated. As shown in table 4.V, the peak percentage of cells with detectable intracellular expression of IFN-γ occurred at 18h after stimulation and was considerably lower at the subsequent time point (42h). These findings thus indicate that IFN-γ secretion diminishes after an initial peak and the maintenance of high concentrations of this cytokine in the cell supernatants described above may be the result of low levels of utilization or breakdown.

Table 4.IV

*Concentrations of IFN-γ and IL4 in cell supernatants of 3 CD4+ T cell clones following antigenic stimulation**

	4 h#		8 h		D 1		D 2		D 4	
	IFN-γ (iu/ml)	IL4 (pg/ml)	IFN-γ (iu/ml)	IL4 (pg/ml)	IFN-γ (iu/ml)	IL4 (pg/ml)	IFN-γ (iu/ml)	IL4 (pg/ml)	IFN-γ (iu/ml)	IL4 (pg/ml)
PL7.2E (exp 1)	38	< 16	214	< 16	588	< 16	576	< 16	574	< 16
PL7.2E (exp 2)	58	nd	179	nd	177	nd	151	nd	143	nd
PL18.1B (exp 1)	< 0.5	< 16	2.0	191	27	625	32	107	18	< 16
PL18.1B (exp 2)	2	nd	5	nd	32	nd	43	nd	29	nd
PL2.2B (exp 1)	nd	nd	nd	nd	587	< 16	576	< 16	535	< 16
PL2.2B (exp 2)	nd	nd	nd	nd	167	nd	165	nd	165	nd

*T cell clones were stimulated with irradiated autologous PBMNC, antigen and rIL2 and supernatants harvested after the time intervals indicated. Cytokine concentrations in supernatants were measured using commercial kits.
#Incubation period; nd - not done

Table 4.V

*Percentage of T cell clones with detectable intracellular IFN-γ following antigenic stimulation**.

CLONE	8h#	18h	42h
PL18.1B	2.3	6.0	1.3
PL2.2B	nd	18.9	2.3
PL7.2E	2.0	3.6	2.7

*Detection of intracellular IFN-γ was performed by flow cytometry following permeabilisation and fixation of the clones, as described in materials and methods. The peak percentage of cells positive for intracellular IFN-γ are comparable with those previously reported using this technique[14].

#Incubation interval after stimulation; nd - not done

5. The effects of exogenous IL2 on kinetics of activation, proliferation and cytotoxicity.

To determine whether exogenous IL2 added at the time of initial stimulation modulated the kinetics of the functions studied, clone PL2.2b was stimulated with irradiated autologous PBMNC and PPD, in the presence or absence of recombinant IL2 (100 iu/ml). Cytotoxic function, surface immunophenotyping and proliferation were then assayed at the same time points as described above. The kinetics and levels of cytotoxicity at each of three effector to target ratios were unaltered by the absence of IL2 at the time of initial stimulation (Fig 4.6). However, the presence of IL2 at the time of initial stimulation caused markedly elevated proliferative responses and expression of IL2-R. It is interesting to note that peak proliferation, as determined by ^{3}H thymidine incorporation, consistently occurred earlier when cells were stimulated in the absence of exogenous IL2 (day 1), although the magnitude of the proliferative response was considerably lower than for cells stimulated in the presence of IL2 (Fig 4.7). Comparable kinetics of IL2-R expression were found irrespective of the presence or absence of exogenous IL2; however, exogenous IL2 stimulated greater levels of IL2-R expression (Fig 4.8). Peak HLA-DR expression was unaffected by exogenous IL2 (81,9% day 2 without exogenous IL2; 79.4% day 2 with IL2).

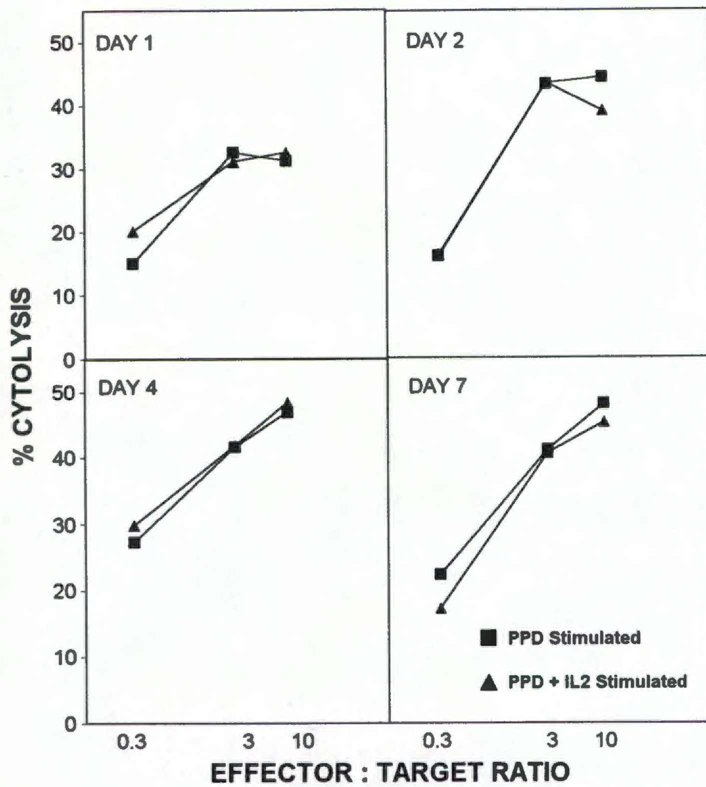


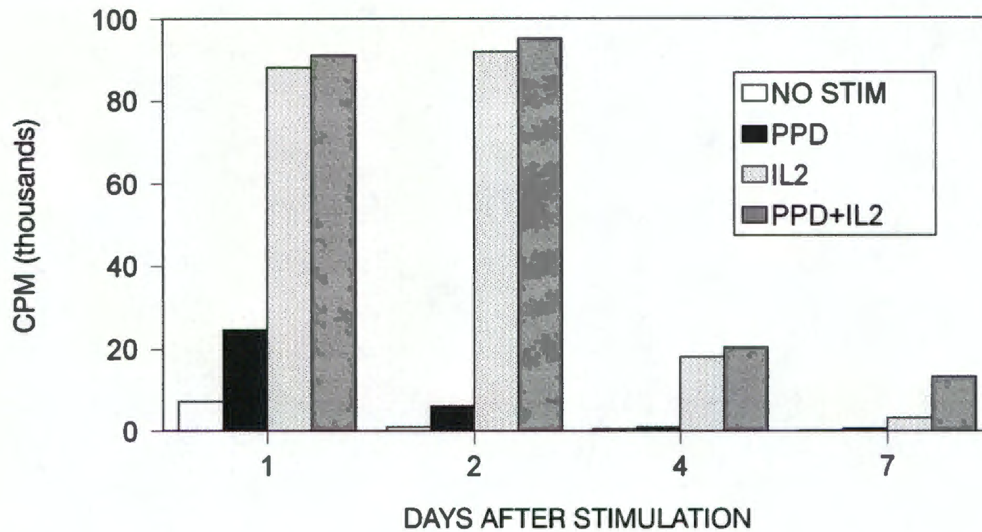
Figure 4.6

The effects of recombinant IL2 on the kinetics of antigen-specific cytotoxicity of clone PL2.2b

Clone PL2.2b was restimulated using autologous irradiated PBMNC and antigen, in the presence or absence of rIL2 (100 iu/ml). Antigen-specific cytotoxicity was measured at each time point in a standard 15h ^{51}Cr release assay. Data points represent the mean of triplicate determinations.

Figure 4.7

*The effects of exogenous interleukin-2 on the kinetics of antigen-induced proliferation of clone PL2.2b**

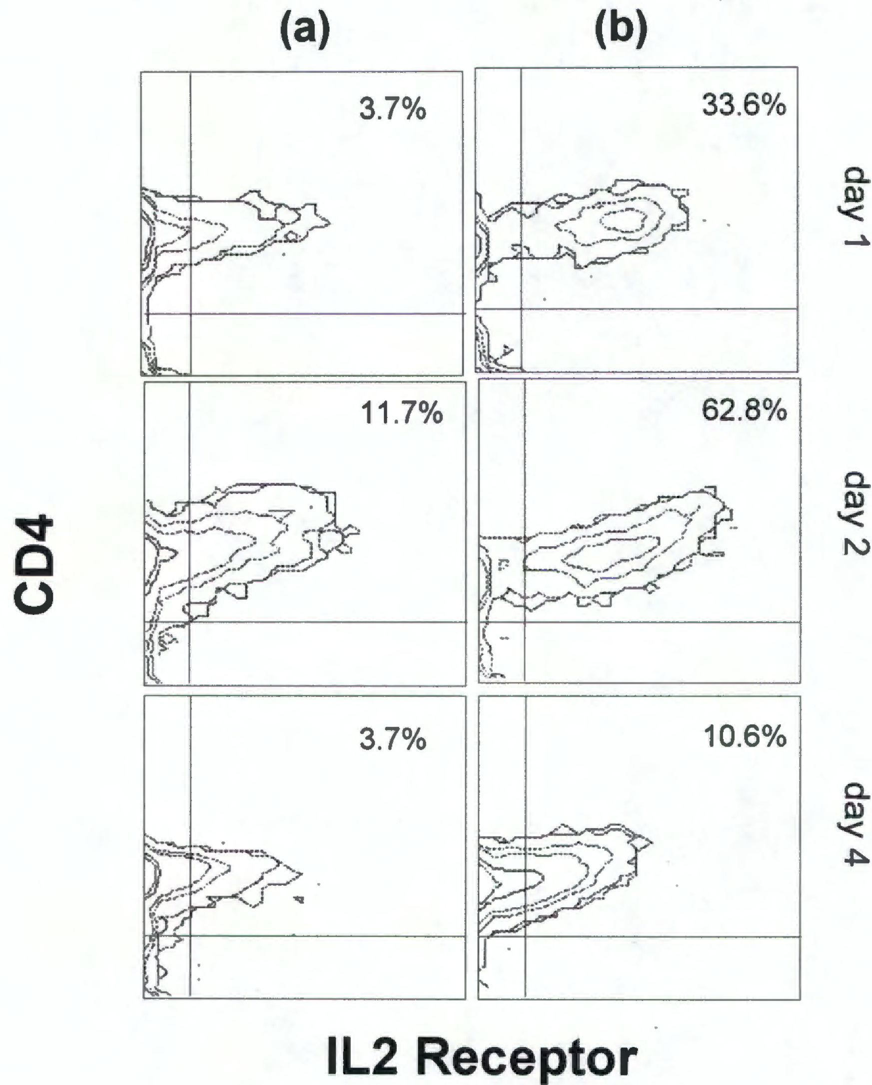


* Clone PL2.2b was restimulated using autologous irradiated PBMNC and PPD, in the presence or absence of rIL2 (100 iu/ml). Proliferation was measured by ^{3}H thymidine incorporation for 15h at the termination of the incubation period. Data shown represent the mean of triplicate determinations.

No stim - unstimulated (background) proliferation

Figure 4.8

The effects of exogenous recombinant IL2 on the kinetics of antigen-induced IL2 receptor expression of clone PL2.2b, (a) cells stimulated without recombinant IL2 and (b) cells stimulated in the presence of recombinant IL2 (100 iu/ml)



Immunophenotyping of effectors used in fig 6 was performed by flow cytometry. Data is displayed as contour plots of dual parameter histograms of CD4 (vertical axis) vs IL2-receptor (horizontal axis). Figures indicate the percentage of CD4+ cells positive for IL2 receptor (upper right quadrant).

DISCUSSION

The retention of cytolytic function by post-activated CD4+ T cell clones observed in the current study has not previously been described and provides novel evidence suggesting a marked difference of kinetics of effector functions in these cells. Thus cytolysis of target cells by cytotoxic CD4+ T cell clones may occur, irrespective of the size, state of activation, proliferation, stage in the cell cycle or cytokine production of the effector cells. These findings may have implications for the pathogenesis of RA; while the rheumatoid synovial CD4+ T cell infiltrate is almost entirely memory cells that display evidence of a state of activation, it has been reported that these cells are generally small, not actively proliferating and not secreting cytokines[19,20,21,22,23,24]. Based on the *in vitro* observations reported in this chapter, it is possible that the rheumatoid synovial CD4+ T cell infiltrate may retain cytolytic potential *in vivo*, despite their morphological appearance and paucity of cytokine production.

The mechanisms of CD4+ T cell mediated cytotoxicity have been discussed previously (Ch1 and Ch3). Delivery of the "lethal hit" is an active process in CD4+ T cells, requires protein synthesis and can be suppressed by metabolic inhibitors and cyclosporin[25,10]. It is probable that interaction of the effector cells with specific antigen-bearing target cells in the course of the 15h incubation of the cytotoxicity assay may have stimulated intracellular pathways within the cytotoxic T cell and in effect constitute a re-prime of the clone; calcium fluxes within CD4+ T cells have previously been demonstrated within similar incubation periods following stimulation[26]. In addition, fas ligand expression occurs rapidly following T cell receptor engagement (ch1 pg 20), although it is difficult to comment on the possible contribution of the fas dependent pathway of cytotoxicity in the current study based on the data in the preceeding chapter. It is also possible that modulation of the other parameters measured in this study may have occurred during the 15h incubation of the cytotoxicity assay, although these changes are likely to have been minor as the assay was performed in the absence of exogenous rIL2. This is supported by evidence from the current study that demonstrates minimal upregulation of IL2 receptor expression of a clone 24h after restimulation by antigen alone (Fig 4.7). IFN-γ secretion, as demonstrated in this study, may also occur within 15h of stimulation of a clone, although the absence of IL2 in the cytotoxicity assay would affect levels of production. Irrespective of potential events occurring during the cytotoxicity assay, the data reported in this chapter demonstrate that considerable differences in activation status, size, cell cycle position, proliferation and cytokine production of CD4+ T cell clones at the commencement of the cytotoxicity assays do not affect the final levels of cytolysis attained. This was shown both in the studies of the kinetics of cytotoxicity and in the comparative study of the effects of rIL2 at the time of antigenic stimulation on the parameters investigated.

The CD4 molecule is a 55kDa cell surface glycoprotein that may be important in the formation of a high affinity complex that stabilizes the T cell receptor/MHC-peptide interaction[27,28]. In addition a role for CD4 in signal transduction has been suggested, with effects on p56lck recently demonstrated[29]. Down regulation of cell surface expression of CD4 following T cell activation has previously been demonstrated and has been suggested to be the result of phosphorylation of the molecule[16]. The current study confirms the previous reported finding that CD4 expression is reduced following T cell activation, but extends the observation to show recovery to preactivation levels with time after stimulation. It has been suggested that down-regulation of CD4 expression following T cell activation may serve as a negative feedback inhibitor of cellular proliferation and activation, based on the experimental evidence of the role of CD4 in these functions[16,27]. In the data reported in this chapter it is interesting to note that reduction in CD4 expression following activation was not associated with modulation of antigen-specific cytotoxic function. Lowest CD4 expression was observed at day 4 following stimulation, the time point at which reduction in proliferation and activation marker expression commenced, while cytotoxicity remained constant. Thus this finding may suggest differential requirements for the CD4 molecule in proliferation and antigen-specific cytotoxicity. Cell surface expression of CD28 slightly increased with time after activation, paralleling antigen-specific cytotoxicity. This observation is compatible with the proposed role of CD28 as an important co-stimulatory factor of T cell mediated cytotoxicity[30].

The potential physiological role for the retention of cytolytic potential by post-activated CD4+ T cells may include the regulation of the extent and duration of an immune response and the prevention of autoimmunity. Thus it has been postulated that fratricidal killing (T cell killing of other T cells) may limit clonal expansion at an inflammatory site and that rapid cytolysis of professional antigen-presenting cells expressing self tissue-specific antigen, before the development of a helper response, may suppress the induction of potentially autoreactive naive T cells[31,9]. The maintenance of cytolytic potential by postactivated CD4+ T cells may contribute to the depletion of CD4+ cells in HIV infection, which may be mediated by cytotoxic CD4+ T cells[32]. The relevance of the findings of the current study to the pathogenesis of RA have been discussed earlier.

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Chapter 5

T-cell proliferative and cytotoxic responses to mycobacterial antigens in RA

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INTRODUCTION

The pursuit of an infective aetiological agent of RA has yielded numerous candidate micro-organisms, including *Mycobacterium tuberculosis*[1,2,3]. Several lines of evidence have been cited in support of a causative association between *Mycobacterium tuberculosis* infection and RA, as discussed previously (Ch1 pg. 11). In particular, the demonstration of *in vitro* proliferation of MNC and T-cell clones from rheumatoid joints in response to mycobacterial antigens has been claimed to have aetiological significance[4,5,6]. Furthermore, a previous report has suggested that mycobacterial antigen-specific cytolytic T-cells present in synovial joints may be relevant to the immunopathogenesis of the disease[7]. Cross-reactivity between mycobacterial HSP65 and host antigens has been proposed as a possible aetiopathogenetic mechanism of RA, based on experimental animal models[8].

Recent evidence has, however, indicated the non-specific nature of the mononuclear cell infiltrate at chronic inflammatory sites; such infiltrates incorporate a diverse array of memory T-lymphocytes recruited to the site by non-specific mechanisms[9]. Standard *in vitro* proliferation assays of cells obtained from these sites may thus reflect responses to a range of antigens for which the individual retains immunological memory. Thus it has been suggested that quantitative (precursor frequency) and qualitative (kinetics of proliferation) differences in MNC antigenic responses may be more specific indicators of aetiological significance[10,11]. While limiting dilution analysis of mycobacterial antigen-reactive T-cells in RA has been reported, there have been no previous reports of the kinetics of *in vitro* MNC proliferation to mycobacterial antigens[12].

In the data reported here, we have studied mycobacterial antigen-reactivity of MNC from the circulation and site of pathology, and CD4+ T-cell clones generated from the site of pathology, of RA patients and controls (non-rheumatoid inflammatory synovitis and PBMNC from healthy laboratory workers) drawn from a population where tuberculosis has been endemic for several decades (estimated prevelance of 700 / 100 000 population[13]). Using these cells, we have investigated (i) the kinetics and specificity of PPD-induced proliferation of circulating and synovial MNC, (ii) the frequency of positive MNC proliferative responses to a recombinant HSP65 of *Mycobacterium bovis* among patients and controls, and (iii) the magnitude and specificity of PPD-specific cytotoxicity. The findings indicate that synovial CD4+ T-cell PPD-specific proliferative and cytotoxic responses are non-specific phenomena, readily demonstrable in patients with inflammatory synovitis of diverse aetiologies. Potential mechanisms for the observed differences in the kinetics of proliferation to PPD between PBMNC and SFMNC are discussed. A lower frequency of MNC reactivity to HSP65 among

PPD-responsive RA patients compared to healthy PPD-responsive controls was found, and may have pathogenetic significance.

MATERIALS AND METHODS

1. Patients and controls

PBMNC and SFMNC obtained from a total of 20 patients with seropositive RA and 6 control patients with synovial effusions due to causes other than RA are included in these studies. Additional clinical details of the RA patients are provided in chapter 2 (pg. 68). The control arthritis group comprised 3 patients with osteoarthritis, 2 patients with acute gout and one patient with psoriatic arthropathy, who were undergoing routine diagnostic or therapeutic arthrocentesis (TABLE 5.I). Diagnoses of these patients were made by characteristic clinical and radiographic findings and the demonstration of birefringent crystals in the SF or SM of the two patients with gout. Furthermore, all patients included in the control arthritis group were seronegative for rheumatoid factor. PBMNC from 9 apparently healthy PPD-reactive individuals were used as normal controls (mean age: 35 ± 4.5; 4 males and 5 females). Synovial membrane from a knee joint was obtained from 3 patients at the time of diagnostic arthroscopy (one 37 yr old male with acute gout) or arthroplasty (one 52 yr old female with seropositive RA and one 37 yr old male with post-traumatic inflammatory OA).

Table 5.I

Control Arthritis Patients (non RA)

PATIENT	AGE (yrs)	SEX	DIAGNOSIS	DURATION (yrs)	TREATMENT
1	63	F	OA	8	Analgaesics
2	62	M	GOUT	7	NSAIDS
3	55	M	PSORIASIS	5.5	NSAIDS
4	37	M	GOUT	2	NSAIDS
5	84	F	OA	15	Analgaesics + NSAIDS
6	54	F	OA	10	Analgaesics + NSAIDS

2. Proliferation and cytotoxicity assays

Tissue culture medium used throughout these studies was RPMI supplemented with 10% pooled AB serum. Proliferation of PBMNC and SFMNC in response to mitogen (PHA) and antigens (PPD, SK-SD and recombinant HSP65 of M bovis) was assayed in microculture by ^{3}H thymidine incorporation, as detailed in chapter 2 (pg. 60). Recombinant HSP65 of *M bovis* was a kind gift of Dr JDA van Embden[14] and was used at final concentration of 5μg/ml. Proliferative responses of CD4+ T-cell clones and T-cell lines obtained from synovial explants were determined as described in chapter 7. Cytotoxic functions of PBMNC, SFMNC and CD4+ T-cell clones were measured in 15h ^{51}Cr release assays, as described in chapter 3 (pg 82).

3. Synovial membrane explants

Fragments of synovial membrane (2 - 3 mm3) were cultured in 1ml complete medium supplemented with recombinant IL2 (Cetus, 100 iu/ml) in 24 well plates (Griner) and passaged weekly, as described in chapter 6 (pg. 155). T-cells growing out of first or second passage explants were cloned or used in functional assays.

4. T-cell clones

T-cells growing out of first passage synovial membrane explants were cloned in limiting dilution using either a non-specific mitogenic stimulus (PHA) or antigens (PPD, SK-SD and recombinant 65kDa heat shock protein of *Mycobacterium bovis* (HSP65)), as described in detail in chapter 7 (pg. 177).

5. Statistical analysis

Statistical analysis was performed using the computer software program Instat (GraphPad Software, San Diego, CA). Analysis of variance (nonparametric), and where appropriate students t test were employed for comparison of proliferation data. Fisher's exact test was used for comparison of frequency data; p values less than 0.05 were considered significant.

RESULTS

1. PBMNC and SFMNC proliferation to bacterial antigens in arthritis

In vitro proliferative responses of PBMNC and SFMNC to mycobacterial antigens (PPD) and, as controls, streptococcal antigens contained within SK-SD, were measured on day 7 following stimulation in a total of 20 seropositive RA patients and 6 patients with non-rheumatoid

synovial effusions. Moderate to high levels of PPD induced proliferation were found in PBMNC and SFMNC from 13 out of 20 RA patients, and in the PBMNC of 4 and SFMNC of 3 out of 6 control patients (FIGURE 5.1) Low or absent proliferative responses (defined as Δ cpm < 20 000) to PPD were associated with significantly longer disease duration in 6 RA patients (see Ch2 pg. 68). While there was a trend to higher PPD-proliferation in PBMNC compared to SFMNC in RA and control arthritis patients, this was not statistically significant.

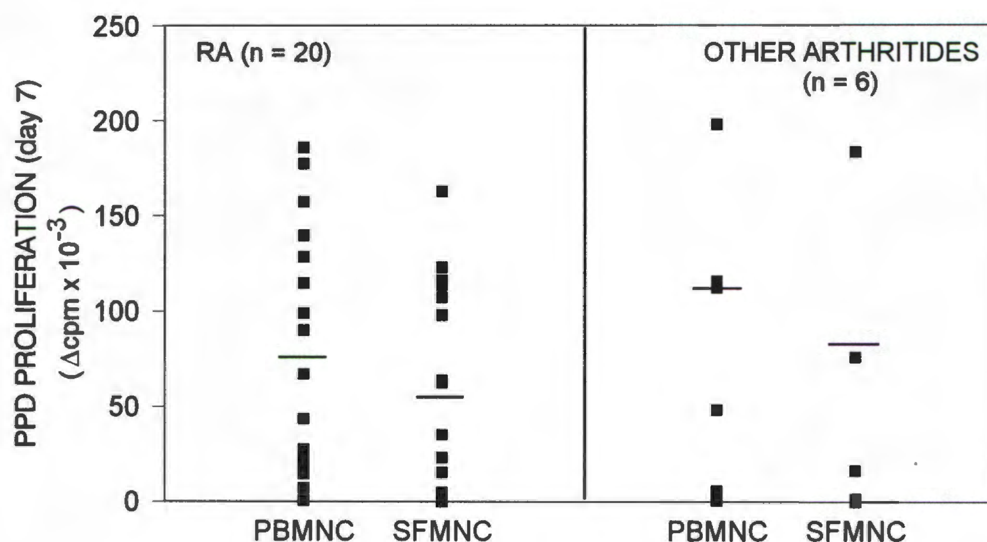


FIGURE 5.1

In vitro PPD-induced proliferation of peripheral blood and synovial fluid mononuclear cells from 20 seropositive RA patients and 6 patients with non-rheumatoid synovial effusions.

Proliferative responses to SK-SD were generally lower than PPD responses in MNC from both compartments in both patient groups (FIGURE 5.2). Among the RA patients, PPD proliferation was significantly higher than SK-SD proliferation in both PBMNC and SFMNC (PBMNC PPD proliferation mean Δcpm = 77820 ± 15965, PBMNC SK-SD proliferation mean Δcpm = 28408 ± 9812: $p < 0.05$; SFMNC PPD proliferation mean Δcpm = 50091 ± 13038, SFMNC SK-SD proliferation mean Δcpm = 6532 ± 3752: $p < 0.001$); in the control patient group this observation was restricted to SFMNC (PPD proliferation mean Δcpm = 91981 ± 39918, SK-SD proliferation mean Δcpm = 33161 ± 15270: $p < 0.01$). In addition, SK-SD induced proliferation was significantly lower in SFMNC compared to PBMNC in both RA and non-rheumatoid control patients (RA $p < 0.005$; non-RA $p < 0.01$) (FIGURE 5.2).

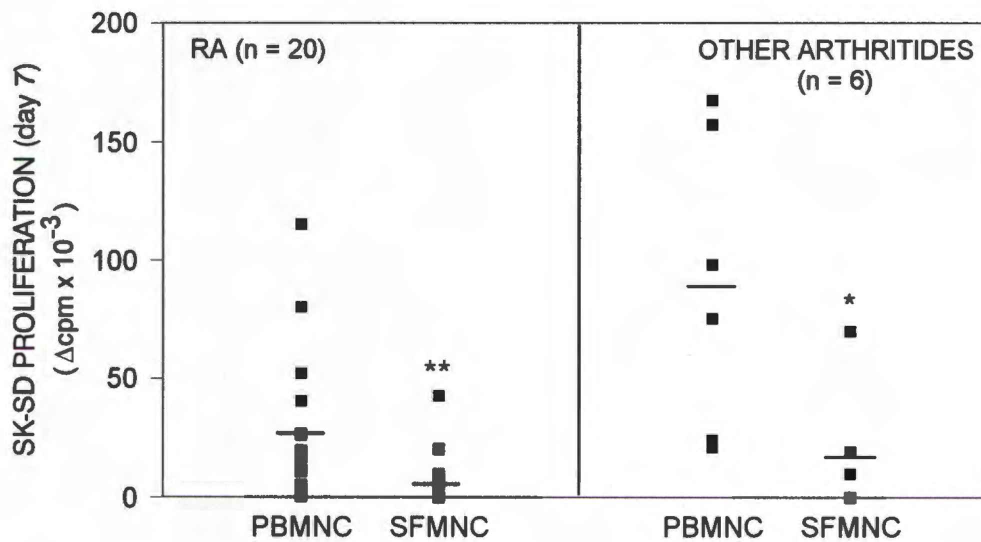


FIGURE 5.2

In vitro SK-SD - induced proliferation of peripheral blood and synovial fluid mononuclear cells from 20 seropositive RA patients and 6 patients with non-rheumatoid synovial effusions.
$*p < 0.01$; $**p < 0.005$

2. Kinetics of PBMNC and SFMNC *in vitro* proliferative responses

The kinetics of PBMNC and SFMNC proliferative responses to PPD and SK-SD were investigated by standard tritiated thymidine incorporation measured over the last 18h of a 4 or 7 day incubation period, in a total of 13 seropositive RA patients and 3 patients with inflammatory synovitis due to other causes. Seven RA patients and 3 OA patients with low or absent proliferation in response to PPD were excluded from this analysis. MNC from the RA patients exhibited peak proliferative responses to both antigens on day 7 following stimulation (Figs. 5.3 and 5.4). However, a difference in the kinetics of PPD responses of PBMNC and SFMNC was observed (Fig. 5.3). RA PBMNC displayed similar kinetics of proliferation to PPD as was found in normal PBMNC (FIGURE 5.5) with a significantly greater response on day 7 than day 4 ($p < 0.001$ RA PBMNC; $p < 0.001$ normal PBMNC). By comparison, RA SFMNC proliferation to PPD acheived near maximal levels by day 4, which was significantly higher than PBMNC proliferation at the same time point ($p < 0.05$). While suggesting a more rapid response to the antigenic stimulus, this finding is distinct from accelerated kinetics of proliferation which has been defined as day 4 proliferation significantly greater than day 7 [11]. The rapid proliferation of SFMNC was antigen-specific, and not a feature of SK-SD responses, which remained uniformly low (FIG. 5.4). Interestingly, proliferation of

unstimulated RA SFMNC was also significantly higher than PBMNC on day 4 (mean Δcpm SFMNC = 2354.8 ± 592, mean Δcpm PBMNC = 721.3 ± 95: $p < 0.01$), but not day 7.

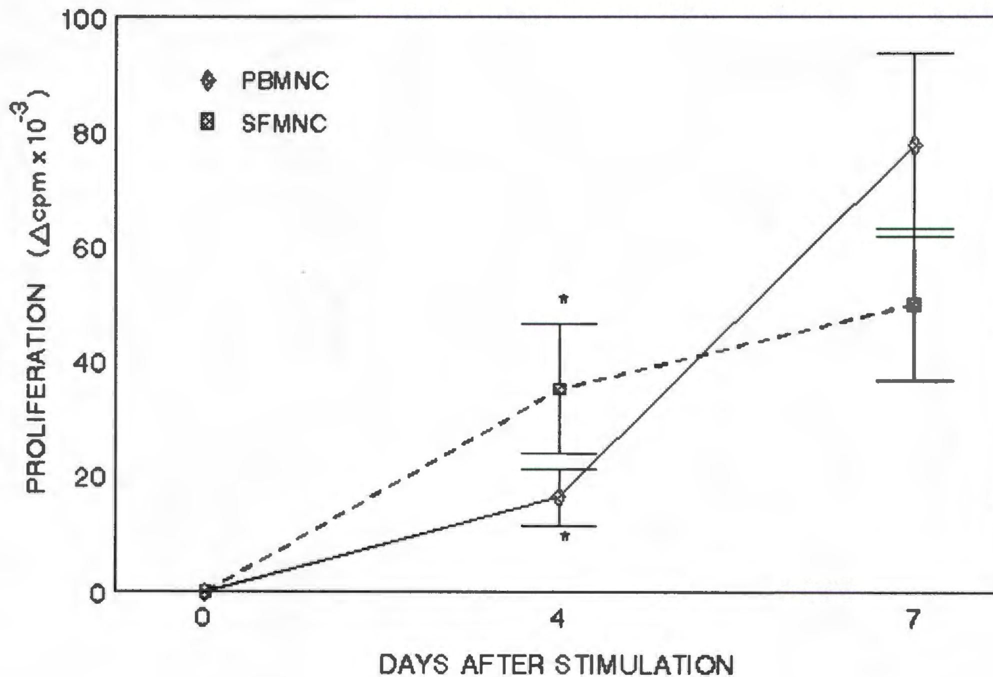


FIGURE 5.3

Kinetics of PPD-proliferation of PBMNC and SFMNC from 13 patients with seropositive RA. PBMNC responses are indicated by the solid line, and SFMNC by the dotted line.

** $p < 0.05$*

Although it has previously been reported that increased *in vitro* proliferation of lymphocytes in patients receiving methotrexate may be due to the high folate concentrations of culture medium[15], this is an unlikely explanation of our findings as (i) only 2 patients were receiving methotrexate at the time of this study and (ii) observations of rapid PPD-induced responses and higher spontaneous MNC proliferation were restricted to cells from the synovial compartment, and were not a feature of PBMNC responses in the same patients.

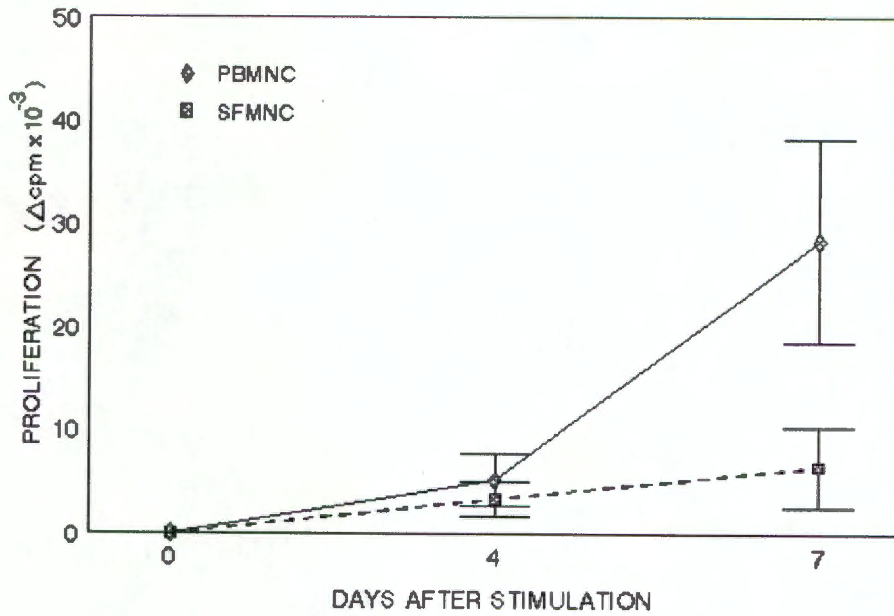


FIGURE 5.4

Kinetics of SK-SD proliferation of PBMNC and SFMNC from 13 patients with seropositive RA.

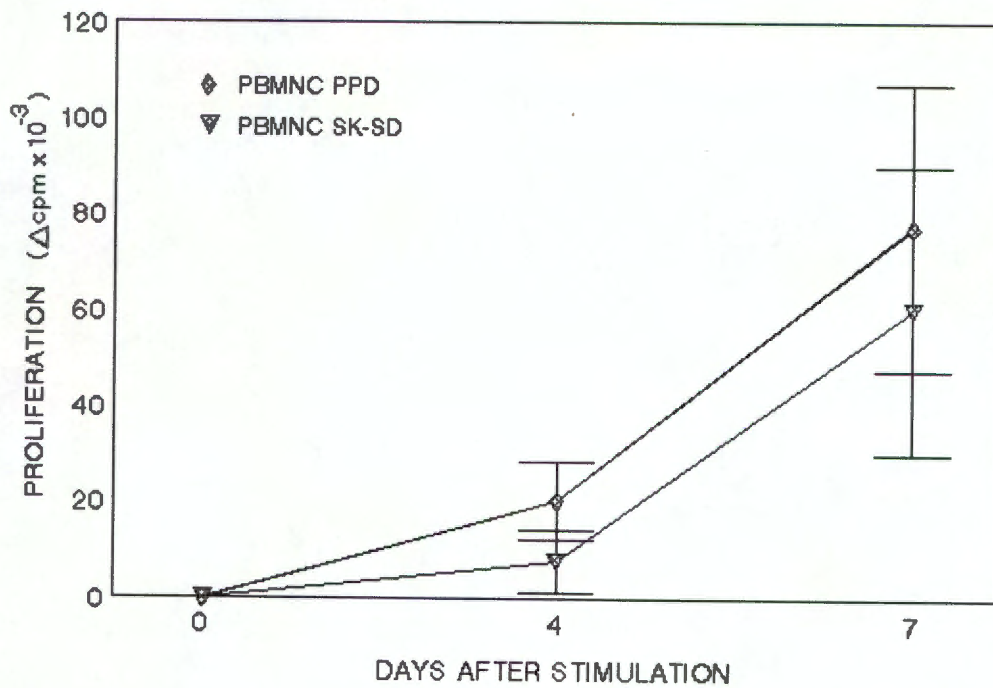


FIGURE 5.5

Kinetics of PPD and SK-SD proliferation of PBMNC from 9 healthy normal PPD-reactive controls.

Kinetics of PPD-induced proliferation of SFMNC from patients with non rheumatoid inflammatory synovitis were similar to those found in RA SFMNC (Figs 5.6 and 5.7). In addition, higher day 4 proliferation to PPD, but not SK-SD, was also found in these patients' SFMNC compared to their PBMNC ($p < 0.05$) (Fig 5.6). Although the numbers of patients included in the control arthritis group are small and thus conclusions remain guarded, the findings indicate that rapid proliferative responses to PPD are not limited to SFMNC from patients with RA.

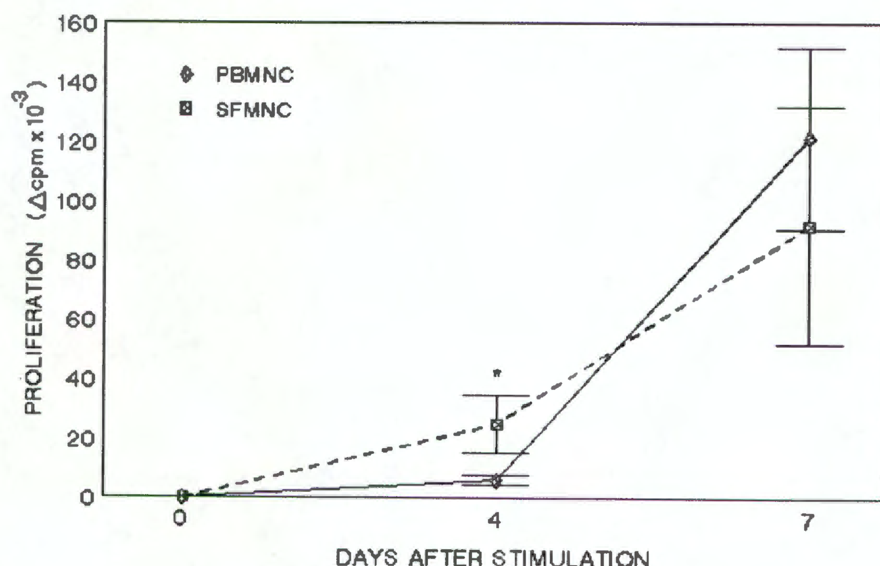


FIGURE 5.6

Kinetics of PPD proliferation of PBMNC and SFMNC from 3 patients with non rheumatoid inflammatory synovitis

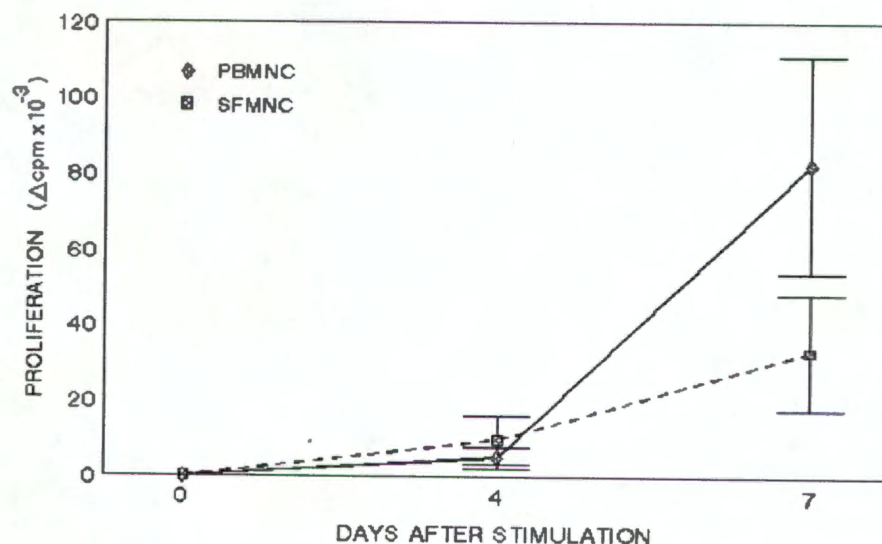


FIGURE 5.7

Kinetics of SK-SD proliferation of PBMNC and SFMNC from 3 patients with non rheumatoid inflammatory synovitis

3. Proliferative responses to HSP65 of *Mycobacterium bovis*

In vitro proliferation of PBMNC and SFMNC to a recombinant HSP65 protein of *M bovis* were investigated. Of the 13 RA patients who demonstrated moderate to high levels of PPD-reactivity in their PBMNC and SFMNC, significant HSP65 proliferation (defined in this setting as Δcpm > 2000 and SI > 3.0) was confined to the SFMNC of 2 patients and the PBMNC of one of these patients (Table 5.II). By comparison, PBMNC from 6 out of 9 healthy PPD-reactive controls demonstrated significant HSP65 reactivity, indicating a lower incidence of HSP65 reactivity among PPD-responsive RA patients than healthy controls ($p < 0.005$ Fisher's exact test). Of the three PPD-reactive patients in the control (non-RA) arthritis group, significant HSP65 proliferation was found in MNC from the site of pathology in both patients with gout but was not found in synovial MNC from the patient with psoriatic arthritis.

4. Restricted localisation of HSP65-reactive T-cells to acutely inflamed synovium in a patient with gout

Multiple sight-directed synovial biopsy samples were obtained from a single knee joint of a patient with acute gout, and classified according to macroscopic and microscopic appearance (Fig. 5.7). Tissue explants of acutely inflamed and macroscopically normal synovium were cultured separately in 24 well plates in standard tissue culture medium supplemented with recombinant IL2 (100 iu/ml). MNC from acutely inflamed synovium (biopsy 2) demonstrated comparatively high levels of HSP65 reactivity and a relative lack of SK-SD response compared to MNC from tissue that was macroscopically normal but had evidence of chronic inflammation on histology (biopsy 1) (TABLE 5.III). MNC from both sites had similar levels of PPD-reactivity, although this was lower than found in PBMNC and SFMNC from the same patient. PBMNC from this patient did not demonstrate significant HSP65 reactivity.

TABLE 5.II

*Proliferation in response to HSP65 of Mycobacterium bovis of mononuclear cells from PPD-reactive patients and controls**

		Peripheral blood MNC		Synovial fluid MNC	
Patient / Control	Diagnosis	Δ cpm	SI	Δ cpm	SI
1	RA	3728	1.1	431.6	2.1
2	RA	619.3	1.3	0	0.6
3	RA	223.5	1.5	1979	2.2
4	RA	1651	1.0	1635	1.6
5	**RA**	**32199**	**15**	**2698**	**2.8**
6	RA	0	0.9	2706	1.8
7	**RA**	787	1.2	**2501**	**11.9**
8	RA	2180	1.3	0	0.4
9	RA	0	0.5	191	1.3
10	RA	455	1.0	0	0.8
11	RA	0	0.8	819.7	1.5
12	RA	552	1.5	1928	1.9
13	RA	1560	1.7	0	0.7
14	**Gout**	**11307**	**12.7**	**9772**	**13.1**
15	**Gout#**	763	1.3	**15483**	**11.7**
16	Psoriasis	0	0.9	836	1.3
1	**Normal control**	**12785**	**14.9**	-	-
2	**Normal control**	**5687**	**5.2**	-	-
3	Normal control	978	1.2	-	-
4	**Normal control**	**40587**	**21.2**	-	-
5	**Normal control**	**13745**	**11.7**	-	-
6	Normal control	744	0.8	-	-
7	Normal control	478	0.9	-	-
8	**Normal control**	**21744**	**15.6**	-	-
9	**Normal control**	**4479**	**2.9**	-	-

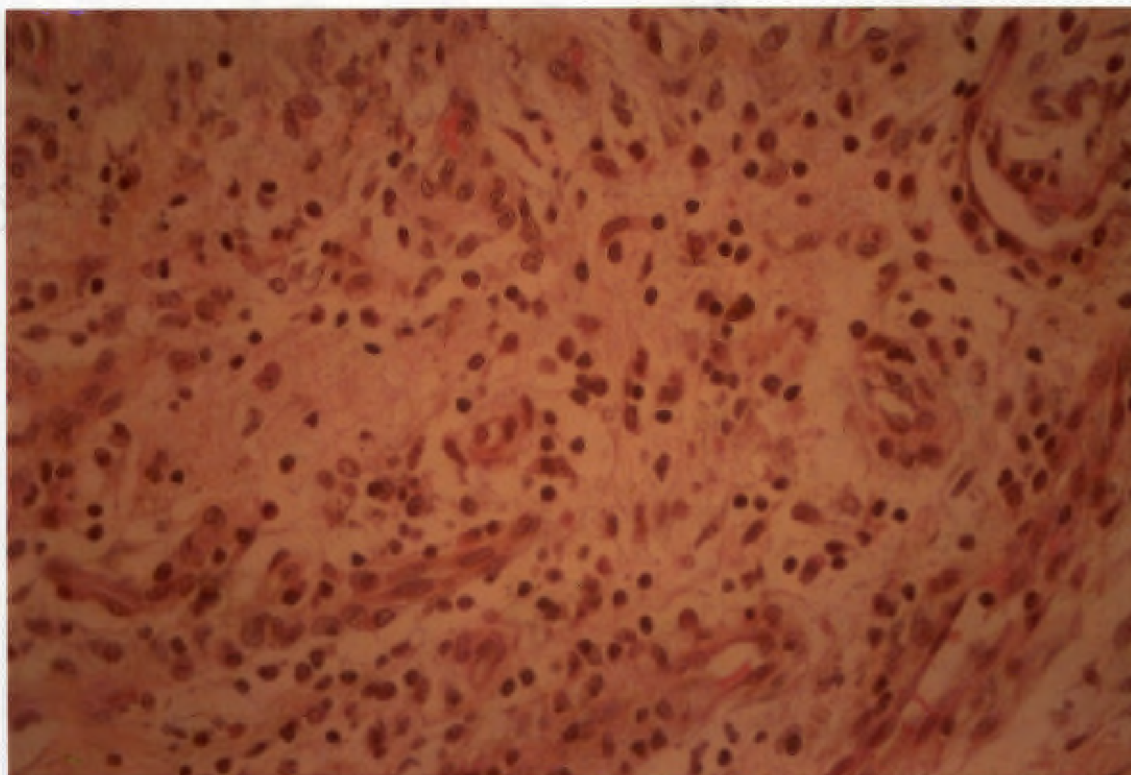
* All patients and controls had significant in vitro MNC proliferative responses to PPD (Δ cpm > 20 000). Proliferation in response to a recombinant 65kDa heat shock protein of mycobacterium bovis was assayed by tritiated thymidine incorporation after a 7 days incubation. Data shown represent the mean of triplicate determinations.

Mononuclear cells from synovial membrane, not synovial fluid.

FIGURE 5.7

*Histological appearance of gout synovium**

(a) Biopsy 1: Chronic synovitis



(b) Biopsy 2: Fibrinous exudate suggestive of acute inflammation

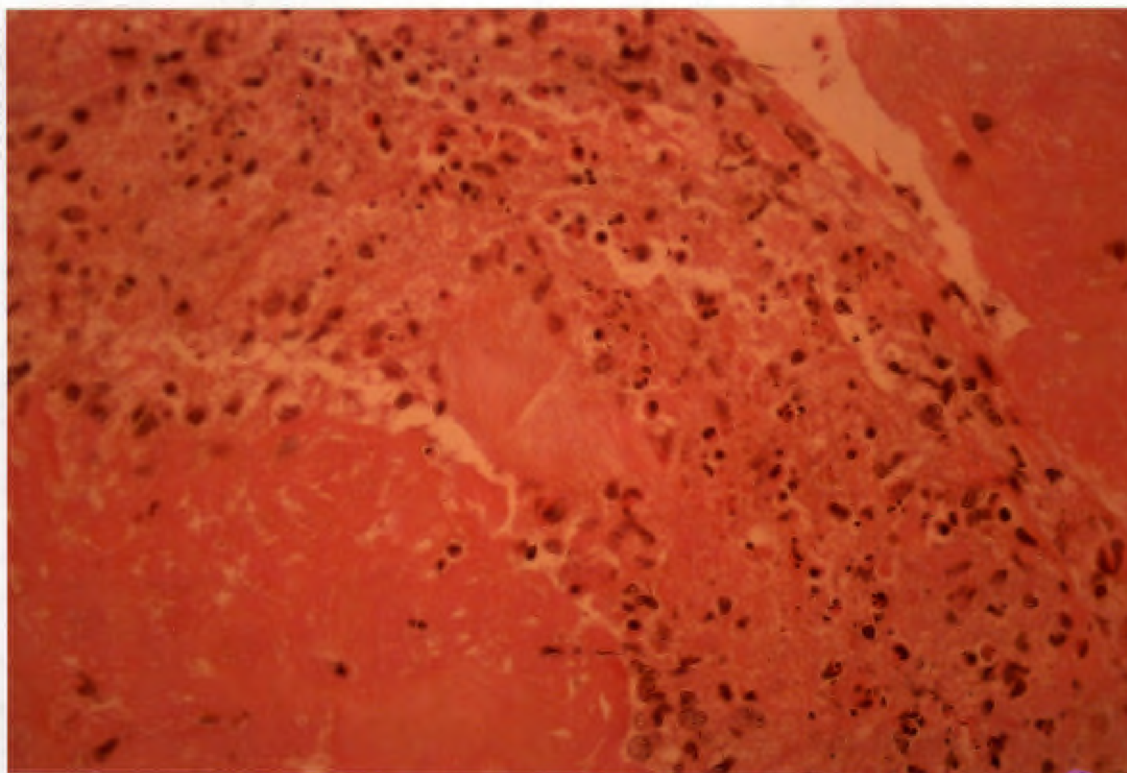
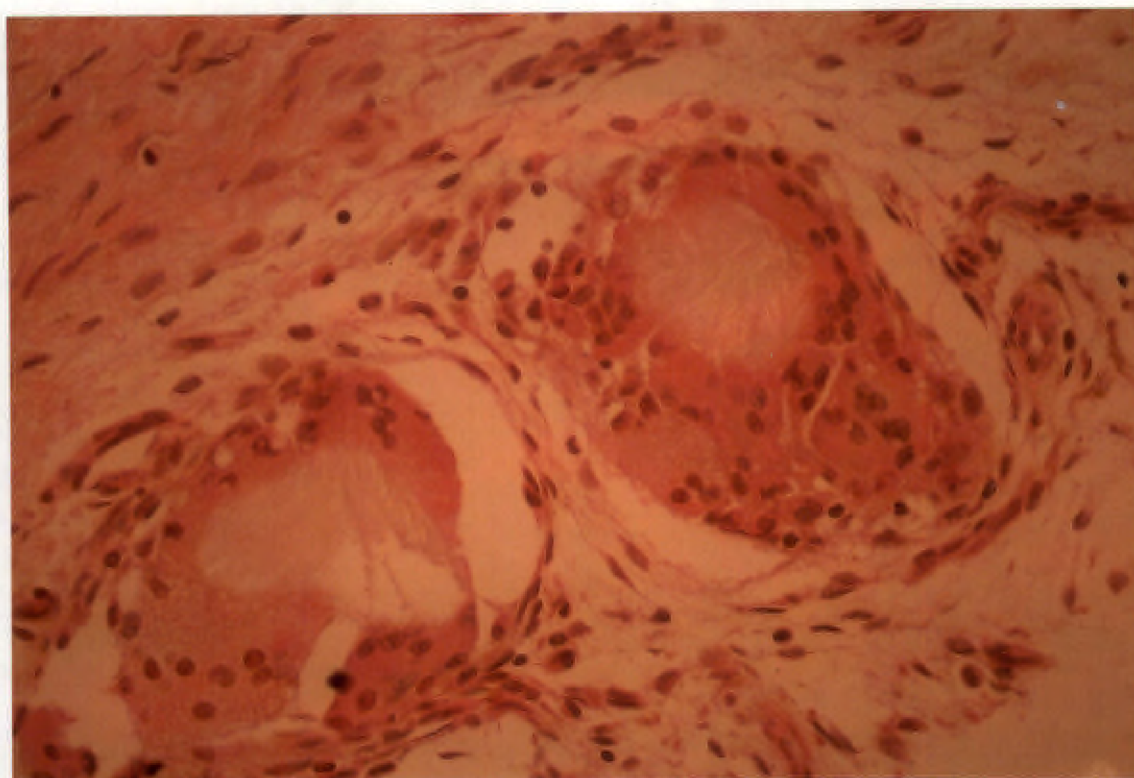


FIGURE 5.7 (cont.)

(c) Multinucleate giant cells containing urate crystals



* Biopsy fragments were prepared using standard histological techniques. Samples in photomicrographs were stained with haematoxylin and eosin.

(a) histological appearance of macroscopically normal biopsy fragment (200x magnification). Predominant mononuclear cell infiltrate and mild non-specific chronic synovitis.

(b) histological appearance of macroscopically inflamed synovial biopsy (200x magnification). Evidence of acute inflammation includes fibrinous exudate, necrosis and the presence of polymorphonuclear neutrophils.

(c) diagnosis of gout was confirmed by the presence of birefringent urate crystals within mutlinucleate giant cells (400x magnification) (section from biopsy 2).

TABLE 5.III

Antigen-induced proliferation of MNC from multiple sites in a patient with gout#

SITE	DESCRIPTION	PHA	PPD	SK-SD	HSP65	rIL2
Biopsy 1	synovium: chronic inflammation	126059 (39.1)	9656 (8.4)	19294 (15.9)	2097 (2.6)	68845 (54)
Biopsy 2	synovium: acute inflammation	33450 (7.9)	13007 (10)	3514 (3.4)	**15483 (11.7)**	38395 (28)
Blood	PBMNC	-	176881 (69)	19479 (8.5)	763 (1.3)	-
Synovial Fluid	SFMNC	-	183410 (11.7)	-	-	-

Values shown are mean Δcpm (Stimulation Index) for triplicate determinations

5. PPD-specific cytotoxicity

The ability of *in vitro* PPD-stimulated PBMNC and SFMNC to cytolyse autologous macrophages in the presence or absence of PPD was investigated in a 15 h ^{51}Cr release assay. Moderate to high levels of PPD-specific cytotoxicity were found in PBMNC of all RA patients with significant PPD-induced proliferation and in the SFMNC of a subset of 7 of these patients receiving NSAID monotherapy at the time of study (see Ch 2). Representative data from a RA patient with similar levels of PBMNC and SFMNC cytotoxicity and a patient with compartmentalized impairment of SFMNC cytotoxicity are shown in figures 5.8 (i) and 5.8 (ii) respectively. As was observed for PPD proliferation, PPD-specific cytotoxicity was also found in PBMNC and SFMNC from a patient with acute gout (Fig. 5.8 (iii)) and psoriatic arthritis (Fig. 5.8 (iv)). In both cases, similar levels of cytolytic function were observed in SFMNC and PBMNC.

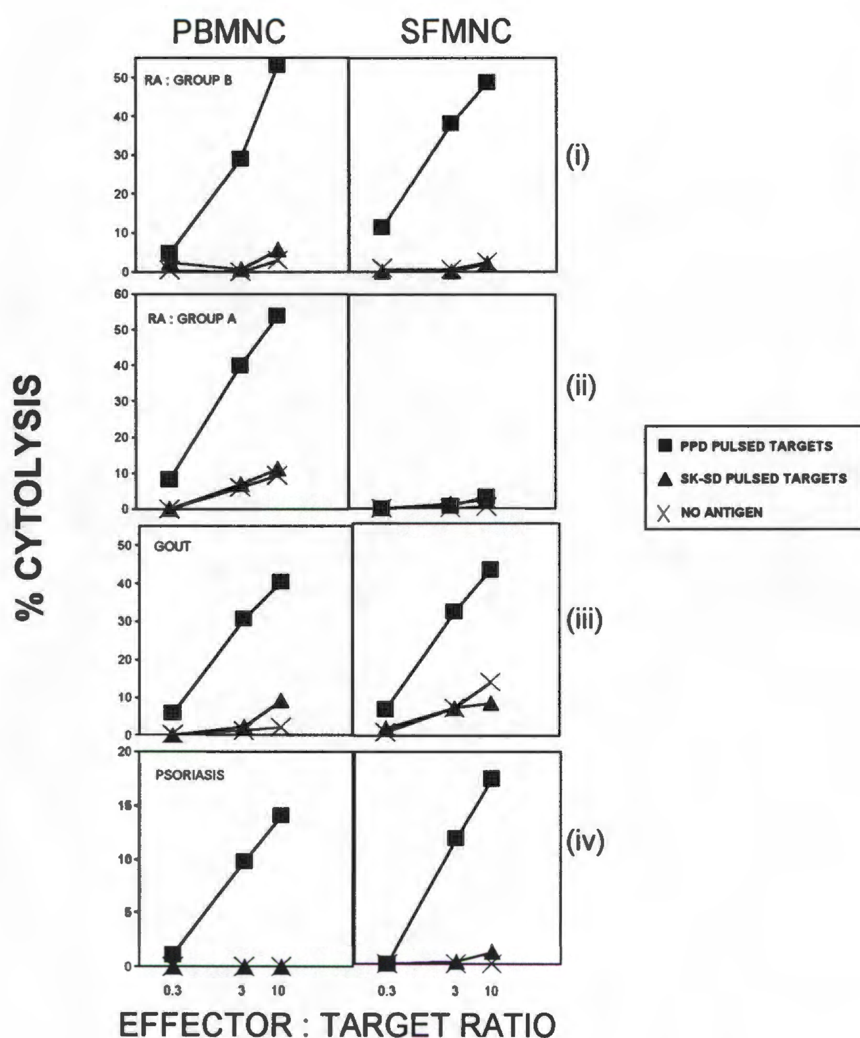


FIGURE 5.8

PPD - specific cytotoxicity of PBMNC and SFMNC from patients with arthritis: (i) RA receiving NSAID monotherapy, (ii) RA receiving DMARD, (iii) Gout and (iv) Psoriatic arthritis

6. Antigen reactivity of CD4+ T-cell clones generated from the synovial membrane of patients with RA, OA and gout.

Mycobacterial antigen reactivity in RA was further investigated by examining proliferative and cytotoxic responses of a panel of CD4+ T-cell clones generated from the synovial membrane of a patient with seropositive RA. CD4+ T-cell clones from the synovial membrane of patients with OA and gout were used as controls. Details of the cloning techniques are provided in chapter 7. Briefly, clones from the patients with OA and RA were generated from synovial explant-derived T-cell lines by limiting dilution using a mitogenic stimulus (essentially non-selective), whereas clones from the patient with gout were generated in the presence of the antigens PPD, HSP65 and SK-SD(selective technique). Data obtained with the CD4+ T-cell clones from the patient with RA are shown in table 5.IV. In contrast to the high proliferative response to mitogen and recombinant IL2, antigenic responses were generally low in these clones. Significant PPD responses (defined as Δcpm > 2000 and SI > 3.0) were observed in 9 out of 27 clones tested. There were no significant proliferative responses to SK-SD or recombinant HSP65 of *M bovis*.

TABLE 5.IV

Proliferation of a panel of CD4+ T-cell clones from rheumatoid synovium in response to microbial antigens, mitogen and recombinant interleukin-2. Values shown are Δcpm (Stimulation Index).

CLONE	PPD	SK-SD	HSP65	PHA	IL2
AP1.8C	**2690 (12.3)**	608 (3.5)	95 (1.4)	76855 (324)	32434 (137)
AP7.2B	1004 (5.7)	243 (2.1)	0 (1.0)	141211 (665)	26226 (124)
AP4.3E	1557 (6.8)	371 (2.4)	34.7 (1.1)	128765 (477)	22510 (84.3)
AP8.2A	**2683 (11.2)**	919 (4.5)	0 (1.0)	160654 (611)	45139 (172.6)
AP6.7E	818 (3.0)	219 (2.1)	0 (0.9)	136290 (676)	28557 (142)
AP2.6F	247 (1.8)	159 (1.5)	0 (0.9)	133877 (414)	31814 (99.2)
AP7.4C	1468 (5.8)	14 (1.0)	34 (1.1)	139076 (458)	31410 (104)
AP3.10C	1014 (5.4)	32 (1.1)	58 (1.2)	104610 (450)	36999 (160)
AP7.9A	1778 (8.9)	710 (4.1)	155 (1.7)	141072 (628)	56185 (250)
AP2.1D	**2012 (7.6)**	222 (1.7)	50 (1.2)	127688 (420)	28965 (96.2)
AP2.5E	**2346 (8.8)**	132 (1.4)	127 (1.4)	140583 (471)	53856 (181.1)
AP3.5B	1124 (4.6)	99 (1.3)	25 (1.1)	118112 (378)	52270 (168.1)
AP8.3C	**2818 (8.4)**	114 (1.3)	0 (0.7)	105646 (278)	62291 (164)
AP3.3A	1324 (4.2)	134 (1.3)	0 (0.9)	129859 (318)	65894 (162)
AP6.6E	1074 (6.5)	1081 (6.4)	20 (1.1)	164678 (837)	41532 (211)
AP8.10B	1240 (6.8)	275 (2.3)	40 (1.2)	133614 (631)	50312 (238)
AP6.3D	**3413 (17.4)**	1002 (5.8)	12 (1.1)	129269 (622)	50541 (240)
AP2.5A	999 (5.2)	116 (1.5)	18 (1.1)	148088 (628)	31769 (135.1)
AP2.9A	1668 (6.9)	207 (1.7)	0 (0.8)	111996 (394)	32521 (132.8)
AP2.4F	1493 (8.7)	136 (1.7)	11 (1.1)	139379 (723)	35729 (186)
AP6.8E	1105 (5.7)	551 (3.3)	93.5 (1.4)	86543 (367)	22226 (95)
AP2.3C	**2045 (9.3)**	946 (5.0)	25 (1.1)	142919 (607)	50949 (217)
AP3.8F	1162 (9.8)	296 (3.2)	2 (1.0)	151138 (1145)	40037 (304)
AP3.10F	198 (2.5)	52 (1.4)	14 (1.1)	19506 (157)	4556 (37)
AP3.7F	**2169 (12.1)**	153 (1.6)	0 (0.9)	134044 (685)	38768 (199.8)
AP4.7E	983 (6.3)	196 (2.1)	24 (1.1)	144397 (785)	49079 (267.7)
AP3.7C	**2565 (14.9)**	613 (4.3)	27 (1.1)	126924 (687)	43382 (235.5)

Proliferative responses of a panel of 7 CD4+ T-cell clones from the synovium of a patient with post-traumatic osteoarthritis of the knee are shown in table 5.V. Five of these clones had significant responses to SK-SD, while no responses to PPD or HSP65 were observed. It is notable that this patient had no clinical or laboratory evidence of streptococcal arthritis or local streptococcal infection during the course of his arthritis. Using an alternative cloning strategy (synovial MNC cloned in limiting dilution in the presence of antigen rather than non-specific mitogenic stimulus), 2 CD4+ T-cell clones with PPD-specific reactivity and 1 clone with SK-SD-specific proliferation were generated from the synovial membrane of a patient with gout (Table 5.VI). In addition, two clones from gout synovium proliferated to both PPD and SK-SD, suggesting a degree of cross-reactivity between these antigens or that the population of cells was not monoclonal. One of these clones (TS8.5A) mediated PPD and not SK-SD specific cytolysis (see Table 5.VII), as did an additional clone with a degree of SK-SD induced proliferation (TS7.5E). The finding of PPD-specific cytotoxicity provides a more reliable indicator of the antigenic specificity of the clones. Two clones generated in the presence of recombinant HSP65 did not have significant proliferative responses to this antigen on subsequent testing.

TABLE 5.V

Proliferation of a panel of CD4+ T-cell clones from osteoarthritic synovium in response to microbial antigens, mitogen and recombinant interleukin-2. Values shown are Δcpm (Stimulation Index).

CLONE	PPD	SK-SD	HSP65	PHA	IL2
VS1.2C	582.4 (2.5)	**3614 (10.3)**	164 (1.4)	191760 (491)	62415 (160.8)
VS1.3D	656.3 (2.3)	**5641 (12.6)**	0 (0.9)	70739 (146)	97229 (200.4)
VS1.5C	259 (2.0)	1141 (5.6)	561 (3.3)	106448 (433)	39524 (161.7)
VS1.6A	605 (1.9)	**6422 (11.1)**	329 (1.5)	180601 (284)	95826 (151.2)
VS2.5A	1285 (2.5)	**11544 (31.6)**	0 (0.9)	165250 (438)	114152 (303.5)
VS2.9D	235.7 (1.8)	**4548 (17.2)**	62.3 (1.2)	179631 (641)	97590 (348.7)
VS2.4D	191.3 (1.5)	273.6 (1.8)	69.3 (1.2)	105899 (303)	22143 (64.3)

TABLE 5.VI

Proliferation of a panel of CD4+ T-cell clones from gout synovium in response to microbial antigens, mitogen and recombinant interleukin-2. Values shown are Δcpm (Stimulation Index).

CLONE	CLONING ANTIGEN	PPD	SK-SD	HSP65	PHA	IL2
TS1.6F	**SK-SD**	**11623 (4.7)**	**6708 (3.1)**	0 (0.7)	184854 (60)	57049 (19.1)
TS7.1C	PPD	5949 (2.7)	**8009 (3.2)**	1018 (1.3)	56627 (17.1)	46610 (14.3)
TS7.2E	**PPD**	**27620 (19.3)**	2835 (2.5)	226 (1.1)	99451 (66.9)	95904 (64.5)
TS7.5E	**PPD**	**51277 (5.5)**	16831 (2.5)	770 (1.1)	175605 (16.3)	145757 (13.7)
TS 8.5A	**PPD**	**14583 (4.7)**	**22709 (6.7)**	815 (1.2)	51534 (13.9)	64616 (17.2)
TS14.3C	HSP65	942 (1.1)	2579 (1.2)	0 (0.6)	115764 (9.8)	85931 (7.6)
TS14.5D	HSP65	0 (0.8)	0 (0.9)	0 (0.5)	71228 (4.1)	35498 (2.5)

Antigen-specific and lectin-dependent cytotoxic functions of a selection of the CD4+ T-cell clones described above were measured in 15h ^{51}Cr release assays. All clones tested had moderate levels of cytolytic potential, as demonstrated in the lectin-dependent assay (Table 5.VII). In addition, antigen-specific cytotoxicity was observed in the clones from OA and gout synovium. Interestingly, although the three PPD-reactive clones from the RA synovium that were tested had moderate lytic potential, as demonstrated in the lectin-dependent cytotoxicity assay, none of these clones demonstrated PPD-specific cytotoxicity. This finding indicates that these clones are very unlikely to be PPD-specific.

TABLE 5.VII

Cytotoxic function of synovial CD4+ T-cell clones from patients with arthtitis.

CLONE	DESCRIPTION	LECTIN-DEPENDENT CYTOLYSIS (%)#	ANTIGEN-SPECIFIC CYTOLYSIS (%)#†
AP2.3C	Cloned from RA	36.3	0 (PPD)
AP2.5E	synovium with	31.3	0 (PPD)
AP3.7F	PHA	27.5	0 (PPD)
VS1.3D	Cloned from OA	38.8	29.8(SK-SD)
VS2.5A	synovium with PHA	18.1	9.3(SK-SD)
TS7.2E	Cloned from gout	35.1	33.9(PPD)
TS7.5E	synovium with	34.4	20.2(PPD)
TS8.5A	PPD	38.2	29.1(PPD)

#Effector:target ratio 10:1

†Levels of cytotoxicity against non antigen-pulsed targets and targets pulsed with antigen other than indicated were less than 2%.

DISCUSSION

Several different strategies have been adopted to identify the putative infectious agent(s) thought to be responsible for the initiation, and perhaps perpetuation, of the chronic inflammatory and immune response in RA[3]. These approaches could be divided into two broad categories: (i) the identification of microrganisms or their components in tissue/fluid samples of patients with RA[16,17,18], and (ii) the elucidation of potentially relevant immunological responses, humoral and cellular, to antigens of candidate pathogens[18,19,20]. Based on observations in reactive arthritis, where specific causative organisms and immune responses to these organisms have been identified, it has been suggested that proliferative responses of synovial lymphocytes to microbial antigens may indicate the microbiological causes of RA[21].

The data reported in this chapter support earlier published observations that proliferation to mycobacterial antigens is frequently observed in RA synovial MNC and T-cell clones[4,5,6,20] but, in common with additional evidence, demonstrates that this phenomenon is neither restricted to cells from the synovial compartment, nor confined to SFMNC from patients with RA[9,12]. These observations thus indicate that mycobacterial antigen-reactivity in RA is a non-

specific occurrence and do not support an aetiological role for M tb infection in RA, and were confirmed by data obtained in studies of the kinetics of PPD-induced MNC proliferation.

Recently it has been reported that MNC from tuberculous effusions respond with accelerated kinetics of proliferation when stimulated *in vitro* with PPD[11]. This phenomenon was specific to the disease-relevant antigen, restricted to MNC from the site of pathology, and closely resembles a secondary / *in vitro* re-prime immune response with peak proliferation occurring on day 4 rather than day 7 following stimulation. In the current study, PPD-induced (but not SK-SD induced) proliferative responses of SFMNC were indeed more rapid than PBMNC, as indicated by greater day 4 proliferation, but this observation was not confined to RA SFMNC, and does not constitute accelerated kinetics of proliferation as previously defined in the tuberculosis model[11]. Nevertheless, rapid kinetics of proliferation to PPD in SFMNC is an interesting finding that may indicate specific properties of the stimulus and the mononuclear cell population localised to an inflammatory site. The precise mechanism responsible for the more rapid PPD-induced proliferative response of synovial MNC compared to circulating MNC is unclear; it is unlikely to be due to an increased precursor frequency of PPD-reactive MNC in the synovial compartment as (i) previous reports have been unable to demonstrate an enrichment of mycobacterial antigen-reactive T-cells in the synovial compartment in patients with RA[12,22] and (ii) kinetics of proliferation appear to be unrelated to the precursor frequency of responding cells in the tuberculosis model [PT Lukey et al, unpublished observations]. While it is theoretically possible that the rapid proliferative response of SFMNC may be due to a superantigenic effect of PPD, especially given the reports of specific accumulation of Vβ T-cell families in RA synovial compartments[23], this mechanism does not explain the finding in non-RA inflammatory synovitis and is not supported by any other evidence of superantigenic properties of PPD. An alternative potential mechanism may involve the superior antigen processing capabilities of synovial antigen presenting cells, as has previously been demonstrated in RA[24], although this does not account for the restriction of the phenomenon to PPD responses.

Nine out of 27 clones generated from rheumatoid synovium displayed low levels of proliferation to PPD (table 5.IV). While it is possible that this finding represents antigen-specific responses, the low levels of PPD-induced proliferation may be due to the phenomenon of "back stimulation". This phenomenon occurs where a major recall antigen such as PPD induces IL2 production by the irradiated antigen-presenting cells, in turn stimulating low levels of proliferation of the clone, and may also account for low levels of proliferation observed in other clones. Proliferative responses of the clones using T cell depleted irradiated autologous PBMNC would assist in distinguishing between "back stimulation" and true antigenic responses, but was not performed in the current study.

Mycobacterial HSP65 is a member of the HSP60 family of heat shock proteins which function as molecular chaperones and are highly conserved throughout evolution, with approximately 48% shared homology between mycobacterial HSP65 and its mammalian homologue, HSP60 [25]. Heat shock proteins are considered to be immunodominant constituents of many microorganisms, possibly due to their increased production by the microbe following host invasion and the suggestion that the immune system is pre-set for their recognition[26]. Upregulation of mammalian tissue expression of HSP60 as a result of a variety of stressful stimuli is a physiological response, and has been demonstrated in synovium of patients with RA and juvenile chronic arthritis[27,28]. Initial evidence from mycobacterial-induced adjuvant arthritis indicated that T-cell responses to amino acids 180-188 of mycobacterial HSP65 were central to the disease process[29]. However, attempts to induce AA by vaccination of susceptible animals with HSP65 (rather than killed mycobacteria) proved unsuccessful and instead conferred protection against the subsequent induction of several forms of experimental arthritis, and other experimental autoimmune diseases[30,31,32]. Recently it has been shown that the protective effect of HSP65 preimmunisation is not due to the induction of tolerance, either to the parent protein or the arthritogenic epitope (amino acids 180-188), but may be the result of regulatory T-cells activated by alternative protective epitopes of HSP65 not present in sufficient quantity in killed organism preparations[33]. As HSP65 preimmunisation is effective in protecting against the development of chemical as well as bacterial-induced forms of experimental arthritis, it has been suggested that the common protective mechanism involves a T-cell population that is cross-reactive between mycobacterial HSP65 and host HSP60, and that regulates subsequent immune responses[33].

Reports of T-cell proliferative responses to HSP65 in humans with inflammatory arthritis have been somewhat varied and controversial and may have, in part, been due to proliferative responses to E coli contaminants contained in preparations of the recombinant protein[34]. Increased synovial lymphocyte proliferative responses to HSP65 have been demonstrated in HLA B27 +ve patients with pauciarticular juvenile chronic arthritis and in patients with reactive arthritis[34,35]. In common with observations in patients with RA[36,37], T-cell responses to HSP65 in JCA appear to be directed against mycobacterial and not human epitopes[34]. In patients with RA, evidence of significant lymphocyte proliferative responses to HSP65 has perhaps been the most controversial; while some studies have found increased proliferative responses in early disease[4,38], our data indicate that significant proliferation to HSP65 occurs less frequently in RA patients with early disease than in healthy normals, despite vigorous lymphocyte proliferation in response to other mycobacterial antigens. Our findings are supported by similar observations in patients with RA from an area with comparatively low tuberculosis prevalence [JSH Gaston - personal communication], and the evidence from

limiting dilution analysis that the rheumatoid synovial compartment does not contain a specific enrichment of HSP65-specific T-cells[22].

Interpretation of our findings in the light of current theories emanating from animal models would suggest that the observed relative lack of MNC proliferation to HSP65, in patients with RA, may be indicative of a failed regulatory T-cell response in these individuals that may have contributed to the persistent synovial immuno-inflammatory process. In this context, our observation of significant HSP65 proliferation restricted to T-cells from an acutely inflamed area of synovium in a patient with gout is of interest. The localisation of this HSP65 T-cell proliferative response to an area of acute inflammation does not discriminate between a potentially pathogenic or immunoregulatory role for these cells, but may indicate their specific involvement in the local acute immune response.

Cytotoxic CD4+ T-cells may be important in the immune response to infections, particularly against facultative intracellular pathogens[39], and host responses to tumours and transplanted organs[40,41]. These cells mediate their cytotoxic function, following specific binding of their T-cell receptor and antigenic epitope in association with class II MHC, by fas-dependent or granule-mediated mechanisms resulting in target cell apoptosis and/or cytolysis, as discussed previously (Ch1, pg. 20). While target cell disruption in the immune response to an intracellular infection has the potential beneficial effect of exposing the pathogen to alternate defence mechanisms[42], it is likely that this process may be pro-inflammatory and contribute to joint destruction in RA. An isolated previous report of cytolytic function of T-cells from RA synovial fluid and synovial membrane indicated high levels of antigen-specific cytotoxicity in all mycobacterial antigen-specific T-cell lines and clones studied[7]. However, although it was suggested that T-cell mediated cytolysis of mycobacterial antigen-expressing target cells in the synovium may be relevant to the pathogenesis of RA, this phenomenon was not investigated in control patients with non-rheumatoid inflammatory arthritis. The data in this chapter indicate that mycobacterial antigen-specific cytotoxicity can also be demonstrated in MNC from the circulation and synovial fluid of patients with gout and psoriatic arthritis. These data, together with the finding of PPD and SK-SD - specific cytotoxicity among CD4+ T-cell clones generated from the synovium of patients with gout and OA, further emphasize the non-specific nature of the T-cell infiltrate in inflammatory synovitis. Although the demonstration of T-cell mediated microbial antigen-specific cytotoxicity thus has limited aetiological significance in RA, cytotoxic CD4+ T-cells directed against the as yet unidentified "rheumatoid antigen" may have relevance to the immunopathogenesis of the disease. In addition, the apparently quiescent, non-specific cytotoxic CD4+ T-cell infiltrate also has immunopathogenic potential as discussed in chapter 4 (pg. 105).

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Chapter 6

A high molecular weight component of synovium elicits a compartmentalised proliferative T-cell response in rheumatoid arthritis

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INTRODUCTION

As the rheumatoid synovium is characterised by an accumulation of T cells that display evidence of a state of activation, identification of the T cell stimulus is of considerable importance in the understanding of the disease and for the development of specific immunomodulatory agents[1,2,3,4,5,6,7]. Based on current understanding of T cell activation, it is possible that T cell stimuli in RA synovium may include specific MHC-restricted T cell epitopes (foreign or self), or non-specific factors[8,9,10,11,12,13]. To date, the approach to the identification of the synovial T cell stimulus in RA has focussed on the investigation of proliferative responses to microbial or selected purified or synthetic self antigens or specific cells[14,9,15,11]. However, as discussed in chapter 5, identification of synovial lymphocyte proliferation to microbes or their products in RA may have limited aetiological significance. In addition, synovial T cell proliferation to selected self antigens has yielded controversial results[15,16].

An alternate strategy used in the examination of T cell stimuli in organ-specific autoimmune disease has been the investigation of T cell responses to a wide range of target organ-derived constituents[17,18,19]. T cell proliferation identified with this method may be in response to self constituents or locally-sequestered microbial antigens present in the target organ. Using this approach, it has previously been reported that a high molecular weight component of synovial fluid (not restricted to rheumatoid synovial fluid) exerted a costimulatory effect on the IL2-induced proliferation of rheumatoid synovial T cell lines and clones[20]. A recent report has demonstrated T-cell reactivity to synovial fluid and plasma antigens in the > 100kDa and 40 - 60kDa range in patients with RA[21]. However, as the pathogenetic processes in RA would appear to be centred largely in the synovial membrane, identification of T cell stimuli in this tissue may also have relevance, and has not previously been reported.

The studies reported in this chapter have been based on the hypothesis that the rheumatoid synovium may be the source of both the pathogenetic T cell population and the on-going stimulus of these cells. With the use of synovial explant culture techniques, the phenotypic and functional characteristics of T cells growing out of synovial membrane under conditions that may select for in vivo activated T cells were investigated. Samples of synovium obtained from patients with RA and a control arthritis group were included in these investigations. The in vitro proliferative response of synovial explant-derived T cell lines to a broad range of constituents of autologous synovial membrane were examined using a T cell immunoblot assay. Prolonged outgrowth of T cells from rheumatoid synovial explants was observed, with a clustering of synovial T cell proliferative responses to a high molecular weight constituent of autologous synovium in patients with RA but not other arthritides. Additional investigations

indicated that the stimulatory fraction of synovium includes components of the extracellular matrix proteins, collagen and fibronectin. The approach to further characterisation of the synovial T cell stimulus and the potential role of extracellular matrix proteins in T cell activation within the rheumatoid joint are discussed.

METHODS

1. Patients and controls.

Samples of synovial membrane and venous blood from a total of 12 patients with arthritis undergoing arthroplasty are included in studies reported in this chapter. Clinical details of the patients are shown in Table 6.I. In all cases, diagnoses were made according to the revised ARA criteria and typical clinical, radiographic and laboratory findings[22]. In addition, venous blood from 3 healthy laboratory workers (1 male and 2 female, mean age 42 ± 8.8 years) was used as a source of normal PBMNC.

TABLE 6.I

Patient information

PATIENT	DIAGNOSIS	AGE	SEX	DISEASE DURATION (YRS)
1	RA	32	F	2.5
2	RA	64	F	6
3	RA	63	F	8
4	RA	72	F	15
5	RA	64	F	8
6	RA	52	F	7
7	JRA	27	F	13
8	SLE	43	F	2
9	OA	71	M	12
10	OA	68	F	14
11	OA	64	F	8
12	OA	58	M	7

2. Synovial membrane culture and antigen-reactivity of T cell lines

Fragments of freshly-harvested synovial membrane (2 - 3 mm^3) were cultured in 1ml complete medium (RPMI 1640 containing 100U/ml of penicillin, 100μg/ml of streptomycin and 10% pooled AB human serum) supplemented with recombinant IL2 (100 iu/ml, Cetus) in 24 well plates (Greiner). Growing cultures were fed with fresh medium supplemented with recombinant

IL2 every 3 - 4 days. Tissue explants were passaged weekly to new culture plates and mononuclear cells harvested by vigorous re-suspension, washed in PBS and either used in experiments or cryopreserved for later investigations.

Autologous and heterologous PBMNC were obtained by ficoll-hypaque density centrifugation of buffy layer cells, as described previously[23]. Following the final wash cells were resuspended at 10^6/ml in complete medium. Proliferative responses of T cell lines to soluble antigens and mitogen were performed in triplicate at least 3 days after last feeding with rIL2. Details of the methods are as described for the T cell clones in chapter 7. Briefly, T cell lines (5×10^4/well) were stimulated by antigen or mitogen at optimal concentration in the presence of 2.5×10^4 irradiated autologous PBMNC, but in the absence of rIL2. Incorporation of ^{3}H thymidine was measured over the last 8h of the 48h incubation. Wells with feeders and T cell line but no antigen/mitogen served as background controls.

3. Separation of synovial and collagen constituent proteins by polyacrylamide gel electrophoresis

Fragments of synovial membrane (1cm^3) from each patient were immersed in liquid nitrogen and ground into a fine powder using a pestle and mortar[24]. Tissue particles were solubilised using the following buffer: 20mM Tris-HCL (pH 8.4), 7M urea, 5% 2-mercaptoethanol (2-ME) containing 1% sodium dodecyl sulphate (SDS) and then diluted 1:2 with sample buffer (125mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 10% 2-ME and 0.01% bromophenol blue) and boiled for 3 minutes. Aliquots of samples prepared in this manner (40 - 50µl) were electrophoresed using standard Laemmli SDS-polyacrylamide gel electrophoresis (SDS-PAGE) techniques with a 3% stacking gel and 10-12,5% running gel in a vertical slab electrophoresis unit (SE 600, Hoeffer Scientific Instruments, San Francisco, CA)[25]. High and low molecular weight standards (Biorad) were boiled in sample buffer and added to relevant vacant lanes. Separation was performed at 4°C under constant current conditions for approximately 4 h in tank buffer consisting of 0.025M Tris-HCL (pH 8.3), 0.192 M glycine and 0.1% SDS. On completion of the electrophoresis, gels were stained with coomassie blue (0.025% coomassie blue R-250, 40% methanol, 7% acetic acid) for at least 8 h with constant shaking, and then de-stained with 50% methanol and 10% acetic acid solution. Gels were not stained when transfer of separated proteins to nitrocellulose was to be performed. Human type I and bovine type II collagen were purchased from Sigma and prepared for electrophoresis as described for the synovial membrane samples; 40-50µl of the 1mg/ml collagen preparation were loaded per lane.

4. Transfer of separated proteins to nitrocellulose.

Electrophoretic transfer of separated synovial and collagen constituents to nitrocellulose membrane (0.2µm and 0.45µm, Hoeffer) was performed using a transphor electrophoresis unit

(TE 52, Hoeffer Scientific Instruments) with the same tank buffer as described above. Transfer was conducted over 18h at 4°C, using a current of 0.5mA. Successful transfer of separated proteins and location of lanes was determined by temporary staining of the nitrocellulose with amido black (0.01% in 0.5% acetic acid solution) followed by washing in distilled water[17]. Residual dye and SDS were removed from the nitrocellulose by washing in PBS for 2 h with repeated changes of washing solution. Nitrocellulose with bound proteins was then either stored in cling film at 4°C, or used in assays as described below.

5. Preparation of nitrocellulose membranes for use in proliferation assays.

Nitrocellulose was cut into individual lanes, as demarcated by prior staining with amido black, and each lane fractionated into 2mm horizontal bands, as previously described[26]. As each lane was approximately 12cm in length, this procedure yielded 60 fractions of nitrocellulose with bound protein of known molecular weight. Individual nitrocellulose fragments were dissolved and sterilized in 250 μl of DMSO in glass tubes (1hr at room temperature). Recovery of the protein was obtained by precipitating the mixture with the slow addition of an equal volume of carbonate/bicarbonate buffer (0.05M, pH 9.6) during continuous vigorous vortexing. This method results in the production of small protein-bound nitrocellulose particles. DMSO was removed from the nitrocellulose particles by washing three times in PBS. Following the final wash, RPMI 1640 (0.5ml) was added to each sample, which were then stored at -20°C until use in the proliferation assays. A particle suspension of nitrocellulose prepared as above but without bound protein was used as a background control in all proliferation assays.

For detection of proliferative responses to nitrocellulose-bound proteins, 20 μl of 5 pooled samples (recently thawed and vortexed) were added to triplicate wells containing 10^5 mononuclear cells in a final volume of 200 μl of complete medium in 96 well flat bottomed microtitre plates (Greiner). Where the proliferative responses of T cell lines were investigated, 5×10^4 irradiated (3000 rads) autologous PBMNC were added as a source of phagocytic antigen-presenting cells. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days (T-cell lines) and 7 days (PBMNC). Tritiated thymidine (Amersham International, Buckinghamshire, England; specific activity 5 Ci/mmol) was added (2 μCi/well) for the final 18 h of incubation. Means of triplicates were calculated and results expressed as a stimulation index (SI), where SI = mean counts per minute in stimulated wells/mean counts per minute in background (unstimulated) wells.

6. Standardisation of T cell immunoblot technique

The above techniques have been used extensively in the study of T cell protein responses[17,27,28]. Prior to use in these investigations of synovial T cell responses to synovial proteins, the T-cell immunoblot method was standardised using recombinant HSP65 protein of

Mycobacterium bovis as antigen (kind gift of Dr J.D.A van Embden[29]), and PBMNC and a T cell line from HSP65-reactive normal individuals. The HSP65-reactive T cell line was generated from the PBMNC of a normal PPD-reactive individual by stimulating freshly isolated PBMNC (10^6/ml) in vitro with recombinant HSP65 (5μg/ml). After 7 days recombinant IL2 (rIL2) was added (100 iu/ml), and the cells fed with fresh medium and rIL2 every 3 - 4 days and passaged weekly with additional antigen, rIL2 and autologous irradiated PBMNC (5 x 10^4/ml) as feeder cells. Antigen-reactivity of the T-cell line was tested at least 3 days after the last addition of rIL2 and 7 days after passaging with fresh feeders and antigen. Recombinant HSP65 (1mg/ml) was subject to electrophoretic separation as described above and transferred to nitrocellulose. Fractions of nitrocellulose were prepared as described and used in proliferation assays of PBMNC from HSP65-reactive donors and the HSP65-reactive T cell line.

7. Detection of collagen and fibronectin in rheumatoid synovium by western blotting

Monoclonal antibodies used included a mouse monoclonal antibody specific for human type III collagen (Sigma), a mouse monoclonal antibody specific for human fibronectin (CJ2) and a rabbit polyclonal antibody to human fibronectin (the latter two antibodies both kind gifts of C.A. de Jager[30]). Following electrophoretic transfer of separated synovial proteins to nitrocellulose, individual lanes of membrane were incubated for 1 h at room temperature in a blocking solution of 2% casein in tris buffered saline (TBS - pH 7.4), with continuous rocking. Antibodies were then added in fresh blocking solution (1:500 dilution for monoclonal antibodies and 1:1000 dilution for the polyclonal antibody) and incubated for 1 h at room temperature with continuous rocking. Lanes of nitrocellulose were then washed three times in TBS with 0.05% tween-20, and immersed in peroxidase conjugated goat anti-mouse (or goat anti-rabbit where appropriate) immunoglobulin solution (Organon Technika, Cappel, West Chester, PA) (1:500 dilution in blocking solution) for 1 h at room temperature. Following a further three washes in TBS and 0.05% tween-20, detection of bound immunoglobulin was performed by the addition of substrate for 15 minutes, and then rinsing in distilled water[24]. Detection substrate was constituted as follows: 24mg 4 chloronapthol (Merck) dissolved in 8 ml methanol, and diltued with 40ml TBS; hydrogen peroxide (10μl of 30%) was added immediately prior to use.

8. Immunophenotyping of synovial mononuclear cell lines

Phenotypic analysis of cell lines harvested from synovial explants was performed by dual parameter flow cytometry, using an Epics Profile II (Coulter Electronics). Monoclonal antibodies used were CD3-RD1, CD4-FITC, CD8-FITC, all from Coulter Immunology. Mononuclear cells were analysed at 10^6/ml in complete medium, using 100μl per assay. Histograms generated were (i) forward scatter (FS) vs log side scatter (LSS), (ii) log

fluorescence 1 (LF1 - green fluorescence) vs log fluorescence 2 (LF2 - red fluorescence, (iii) and (iv) single parameter histograms of LF1 and LF2.

RESULTS

1. Standardisation of the T-cell immunoblot technique using a recombinant protein

The T-cell immunoblot technique was standardised using a recombinant HSP65 protein and PBMNC from HSP65 reactive individuals. In order to establish the technique for use in assays of T-cell lines, a T-cell line was generated in the presence of HSP65 from the PBMNC of a normal individual. This T-cell line demonstrated proliferative responses to PPD and recombinant HSP65, but not SK-SD, when assayed by tritiated thymidine incorporation over the last 8 h of a 48 h culture in the presence of the antigens and irradiated autologous feeders (Table 6.IIa). HSP65 was subject to electrophoretic separation and transferred to nitrocellulose. Specific proliferation to nitrocellulose-bound protein in the molecular weight range of 60-75kDa was found with PBMNC from the HSP65 reactive individuals and confirmed with assays of the HSP65-specific T cell line (Table 6.IIb). Levels of proliferation were 60-70% of that observed with the soluble recombinant protein at optimal concentration. No significant proliferative response to other nitrocellulose fractions (higher or lower molecular weight range) prepared from the HSP65 sample were found.

Table 6.IIa.

Reactivity of a T cell line generated in the presence of recombinant HSP65.*

STIMULUS	Δ cpm	SI
PHA	12612	5.8
PPD	17298	7.6
SK-SD	0	0.4
HSP65	12687	5.8

*Data shown are means of triplicate determinations. Similar results were obtained on an additional occasion.

Table 6.IIb.

Standardisation of T cell immunoblot technique: Proliferation of PBMNC and a HSP65-reactive T cell line to nitrocellulose-bound recombinant HSP65 of appropriate molecular weight range.

Fractions*	Mol wt range (kDa)	PBMNC 1#	PBMNC 2	T Cell line†
1 - 5	175 - 217	1.0	1.0	1.1
6 - 10	142 - 175	2.1	1.8	1.0
11 - 15	114 - 142	1.3	1.7	1.3
16 - 20	93 - 114	1.1	2.1	1.1
21 - 25	75 - 93	0.9	1.6	1.7
26 - 30	**60 - 75**	**9.7**	**17.9**	**3.6**
31 -35	49 - 60	2.1	1.9	0.8
36 - 40	39 - 49	1.9	0.8	1.2
41 - 45	32 - 39	2.0	0.7	1.4
46 - 50	26 - 32	0.7	2.0	1.1
51 - 55	21 - 27	1.0	1.2	0.8
56 - 60	17 - 21	1.4	1.1	1.4

* Pooled 2mm horizontal bands of nitrocellulose membrane

\# Cells from normal healthy PPD and HSP-reactive individuals; data shown are mean stimulation indices obtained from triplicate determinations. Δcpm for positive results (SI > 2.5) were in the range 6130 - 25 670.

† Additional details of this T-cell line are provided in Table 2a

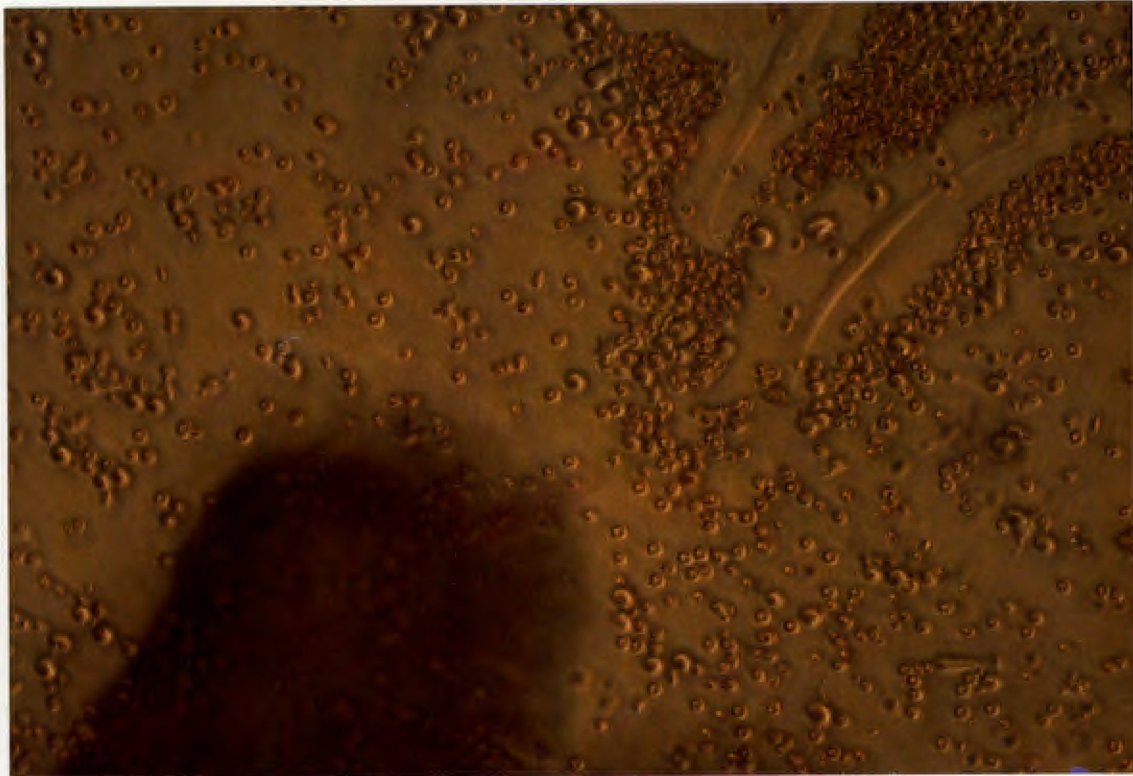
2. Sustained out-growth of T cells from rheumatoid synovial explants.

Fragments of synovium from a total of 12 patients with arthritis were cultured in complete medium supplemented with recombinant IL2 (100 iu/ml), and passaged weekly. Out-growth of mononuclear cells was observed, typically clustered around dendritiform cells (Figure 6.1a and 1b). A wide interpatient variation in the number of mononuclear cells from the synovial explants with each passage was found (2 - 20 x 10^6 cells per passage); maximum yields were obtained with rheumatoid synovium. Flow cytometric analysis was performed with cells harvested from explant cultures. In all cases, irrespective of the type of arthritis, the cells were predominantly CD3+ (> 84%) and CD4+ (table 2), but variation in the percentage of CD4+ and CD8+ cells were observed with long-term passaging of rheumatoid synovium (Fig 2). Synovial explants could be passaged for up to 13 weeks with sustained out-growth of T cells in 3 patients with RA; out-growth of T cells from OA synovial membrane ceased by 3 weeks in culture. In addition, explants of synovium from a further 5 patients with OA did not yield sufficient T-cells for investigation; these patients were excluded from this study.

FIGURE 6.1

Rheumatoid synovial explant culture

(a) T cells and dendritiform cells growing out of a fragment of rheumatoid synovial membrane after 3 days in culture (100x magnification)



(b) High power view with typical appearance of T cells clustered around dendritiform cells following 7 days of *in vitro* culture of rheumatoid synovial explant. Tissue fragment is not included in field of photomicrograph. (200x magnification)

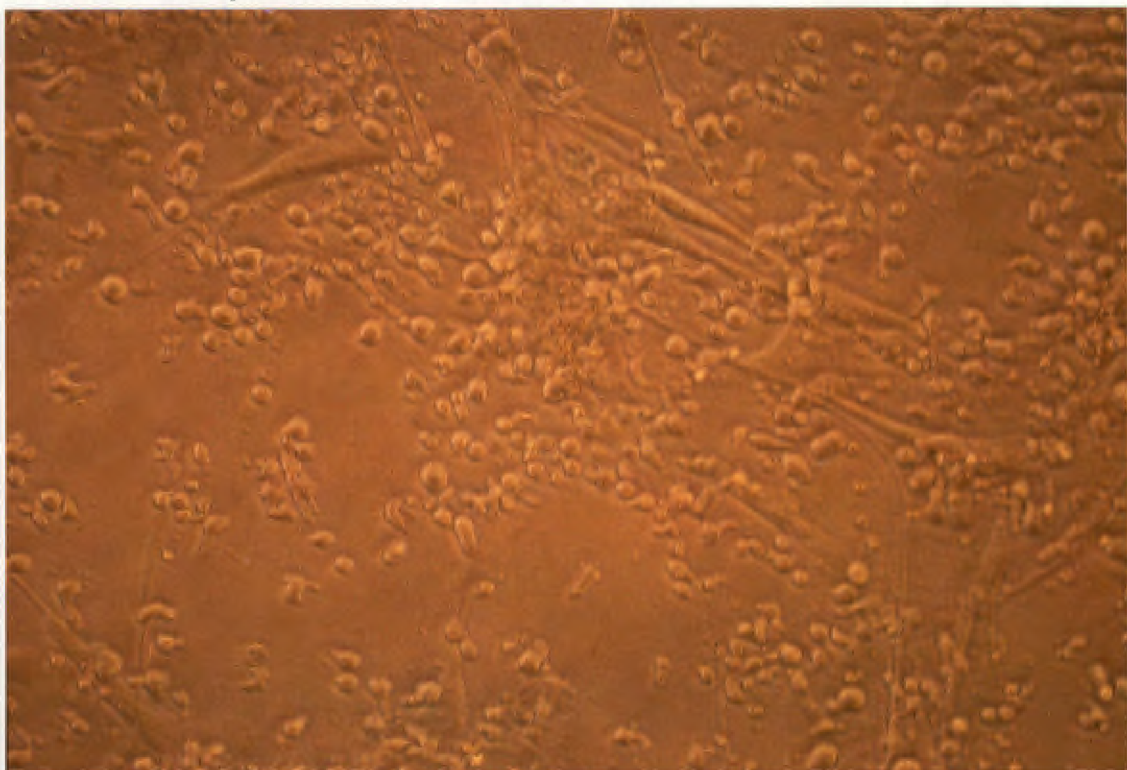


TABLE 6.III

Phenotypic analysis of mononuclear cells obtained from early passage synovial explants.*

PATIENT	DIAGNOSIS	CD3 (%)	CD4 (%)	CD8 (%)	CD4:CD8 ratio
1	RA	96.3	69.2	21.7	3.2
2	RA	84.1	72.6	10.8	6.7
3	RA	97.3	88.7	7.7	11.5
4	RA	98.1	96.2	1.6	60.1
5	RA	92.7	88.6	2.9	30.6
6	RA	88.7	78.4	6.9	11.4
9	OA	91.8	65.0	25.9	2.5
10	OA	98.9	91.3	1.0	91.3
11	OA	91.5	57.9	26.9	2.2

*Phenotypic analysis was not performed on synovial explant-derived cells from individual patients with JRA, SLE and OA included in this study.

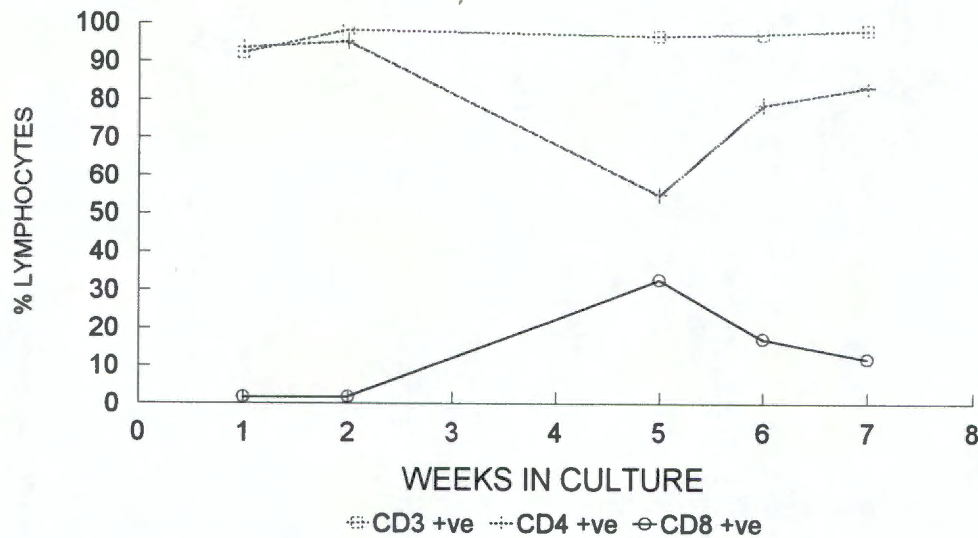
3. Proliferative responses of synovial membrane T cell lines

Functional analysis of early passage synovial membrane T cell lines described above included investigation of proliferative responses to mitogen (PHA), soluble antigens (PPD, SK-SD and recombinant HSP65), and autologous synovial proteins separated by gradient SDS-PAGE. Significant response to soluble antigens was found in T cell lines from an individual patient with RA, JRA and OA (Table 6.IV). In these cell lines, similar responses to PPD and SK-SD were found, while no significant proliferation to HSP65 was observed.

Proliferative responses of synovial T cell lines to autologous synovium was investigated using the T cell immunoblot technique[26]. Separation of synovial constituents was performed by 10-12.5% gradient SDS-PAGE (Figure 6.3). Significant proliferation (in this system defined as stimulation index > 2.5 and Δcpm > 2000) in response to nitrocellulose particles containing a high molecular weight component of synovium (114-142 kDa) was confined to synovial T cell lines of 5 out of the 6 patients with RA (Figure 6.4). Three prominent protein bands were present in this molecular weight range (rf: 0.209, 0.218 and 0.242 respectively) (see Fig 6.3). In addition, responses to synovial constituents of lower molecular weight were noted in a single patient with RA (75-93 kDa, 49-60 kDa and 39-49 kDa). No significant proliferation of synovial T cell lines from patients with OA, SLE or JRA in response to autologous synovium was found.

FIGURE 6.2

Changes in T cell subsets obtained from a rheumatoid synovial explant with prolonged in vitro culture.*



*Synovial explants were passaged weekly as described in materials and methods and cells harvested at the end of each passage and cryopreserved. Phenotypic analysis was performed by flow cytometry

TABLE 6.IV

Proliferation of synovial membrane T cell lines and autologous PBMNC in response to mitogen and soluble antigens.*

Patient	Diagnosis	P H A		P P D		SK- SD		HSP 65		IL 2	
		PBL#	SML†	PBL	SML	PBL	SML	PBL	SML	PBL	SML
1	RA	**212**	**13.5**	**7.9**	1.3	**5.3**	0.4	0.7	0.8	**51.3**	**263**
2	RA	nt‡	nt	1.1	**6.0**	0.9	**4.2**	0.6	2.5	nt	nt
3	RA	**45.5**	**11.2**	**3.5**	0.9	1.1	0.6	1.0	0.7	nt	nt
4	RA	**12.5**	**9.6**	nt	0.7	nt	0.6	nt	0.9	nt	**21.5**
5	RA	nt	nt	**3.6**	0.9	1.1	0.7	2.0	0.8	nt	**22.1**
7	JRA	nt	nt	**26**	**7.3**	**160**	**4.5**	**3.1**	1.2	nt	nt
9	OA	nt	**15.3**	nt	0.43	nt	0.2	nt	0.3	nt	**50.3**
11	OA	nt	nt	**38.0**	**5.0**	**32.0**	**19.5**	2.7	1.5	**30.0**	**41.7**

*Values indicated are stimulation indices obtained from experiments performed in triplicate

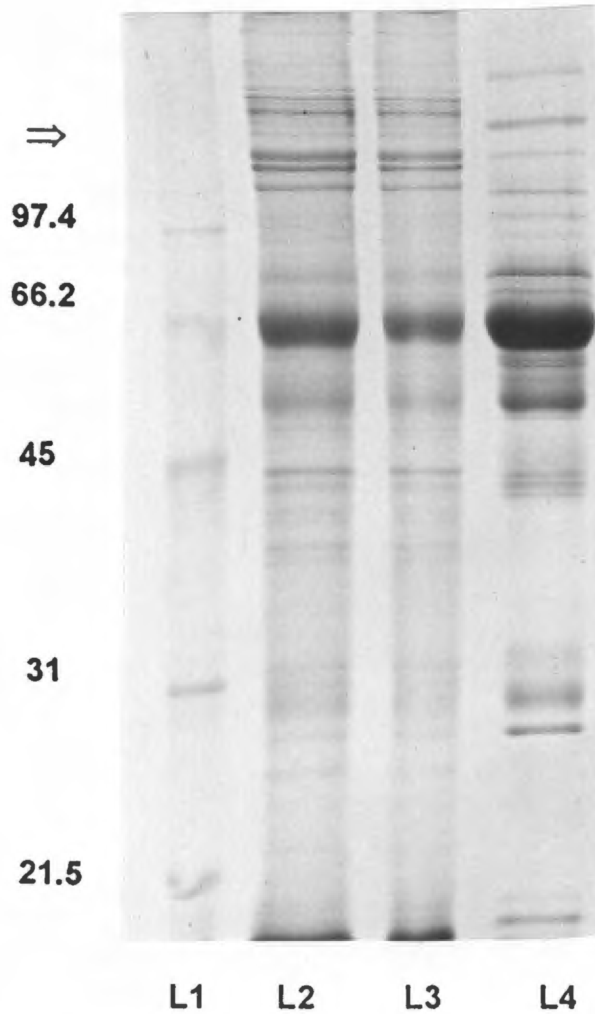
autologous peripheral blood mononuclear cells

† synovial membrane T cell line

‡ not tested

FIGURE 6.3

*Separation of synovial membrane and synovial fluid by gradient SDS-PAGE**



Separation was performed using a 10-12.5% gradient gel, under reducing conditions - as described in materials and methods.

Lane 1: molecular weight standards

Lane 2: rheumatoid synovial membrane

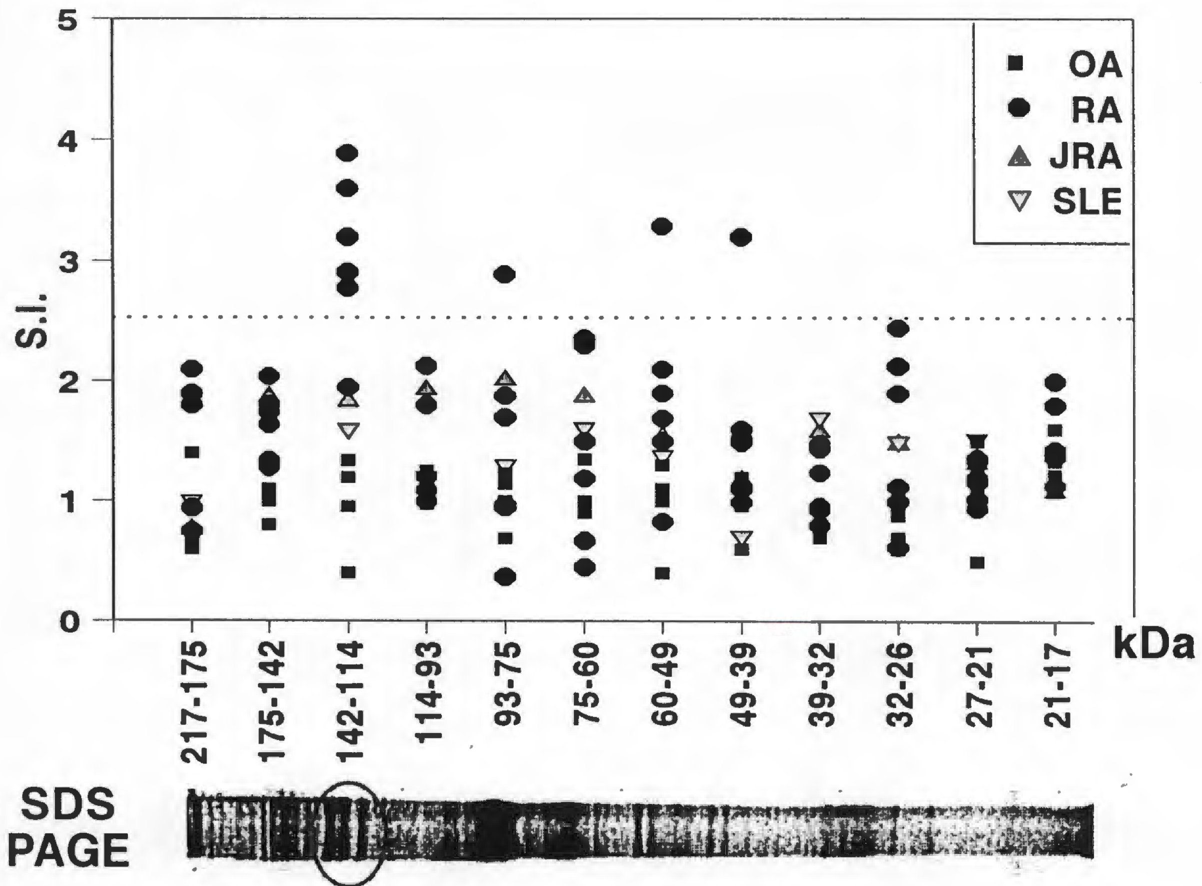
Lane 3: rheumatoid synovial membrane

Lane 4: rheumatoid synovial fluid

Three prominent bands contained within the stimulatory fraction of synovium are indicated by the arrow.

FIGURE 6.4

*Synovial T cell responses to autologous synovial constituents**.

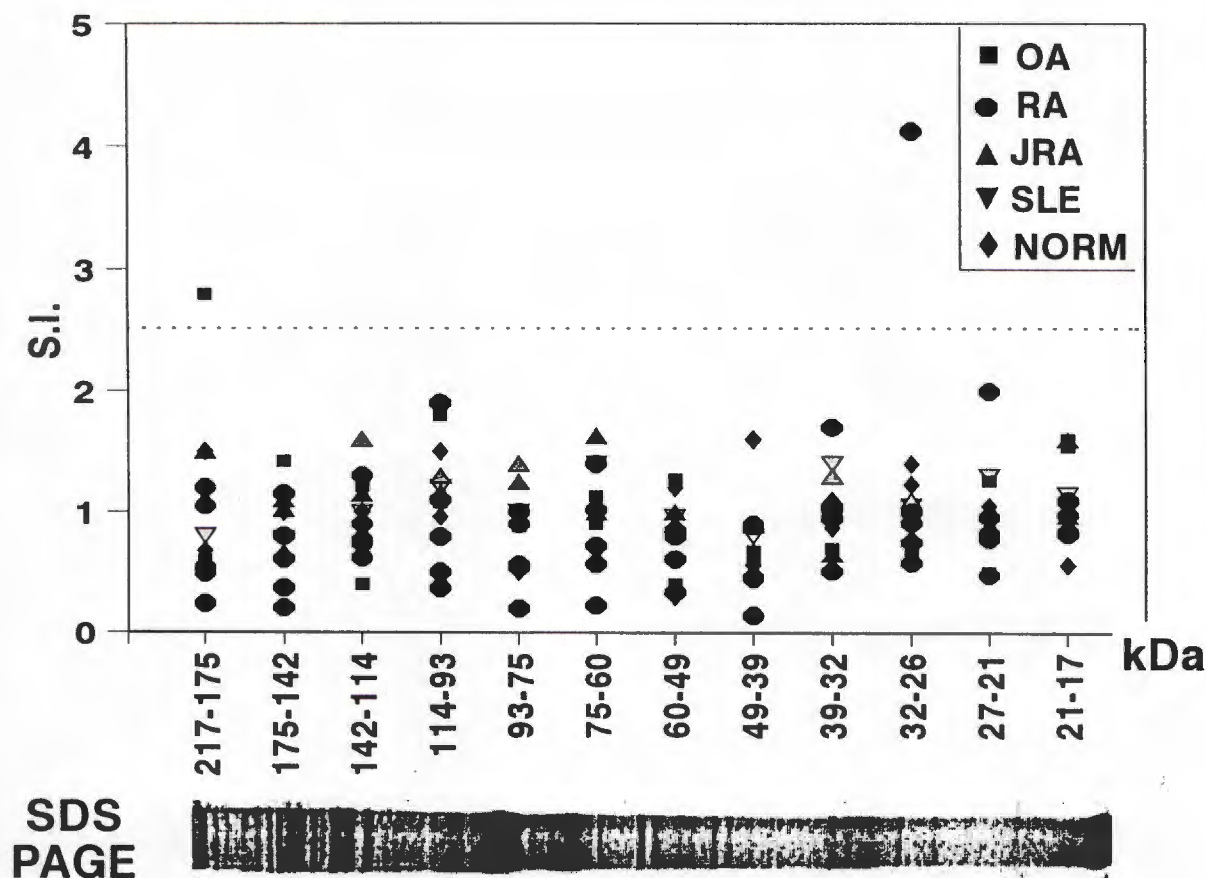


*Proliferative responses of synovial explant-derived T cell lines to components of autologous synvoium were measured using a T cell immunoblot technique, as described under material and methods. Data shown are mean stimulation indices of triplicate determinations. OA - osteoarthritis (n = 4); RA - rheumatoid arthritis (n = 6); JRA - juvenile rheumatoid arthritis (n = 1); SLE - systemic lupus erythematosus (n = 1).

The proliferative response to the high molecular weight component of synovium was restricted to cells from the synovial compartment of patients with RA, and was not found with PBMNC from the same patients or 3 normal donors (Fig 6.5). Similarly, PBMNC from patients with other arthritides did not proliferate in response to the synovial constituents. A single RA patient displayed a significant PBMNC proliferative response to a low molecular weight synovial component (26-32 kDa). In addition, there was no detectable response of a synovial T cell line from a patient with osteoarthritis to the stimulatory fraction of synovium from 2 patients with RA (SI < 1.2; Δcpm < 1100 for each fraction tested).

FIGURE 5

*Peripheral blood mononuclear cell proliferative responses to synovium**



*Peripheral blood mononuclear cell (PBMNC) proliferation in response to autologous synovial constituents was determined as for synovial T cell lines indicated in Fig. 4. NORM - 4 healthy normal individuals tested against rheumatoid synovial membrane.

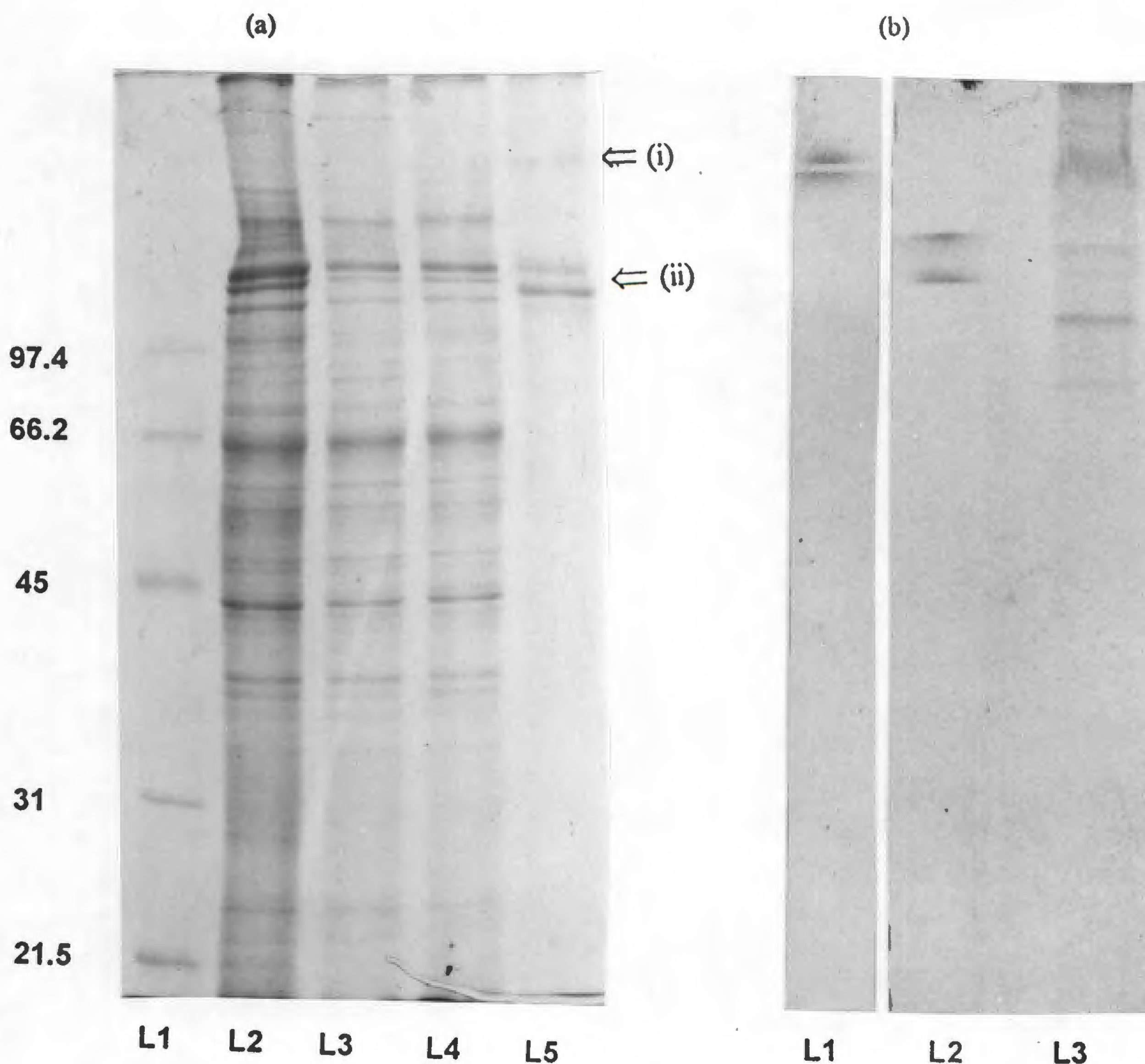
4. **The stimulatory fraction of synovium includes extracellular matrix proteins**

Fibronectin and collagen (types I, II and III) are extracellular matrix proteins present in significant quantities in the synovial compartment[30,31]. Both proteins are large complex molecules (fibronectin 440 kDa, collagens approx 300 kDa) that may be degraded within the joint by the action of a variety of enzymes or dissociated by reducing SDS-PAGE[32]. Type I and type II collagen were separated by SDS-PAGE under reducing conditions and in parallel with samples of RA and OA synovium. Collagen (both type I and II) separated into three bands (rf: 0.1, 0.218 and 0.233); two of these bands corresponded with protein bands present in OA and RA synovium under the same experimental conditions (rf 0.1 and 0.218), one of which was found in the stimulatory fraction of synovium (rf 0.218) (Figure 6.6a).

FIGURE 6.6

(a) *Separation of RA and OA synovial membrane and collagen by SDS-PAGE*

(b) *Detection of collagen and fibronectin in rheumatoid synovium by western blotting.*



(a) Lane 1: molecular weight standards; Lane 2: OA synovium; Lane 3: RA synovium; Lane 4: RA synovium; Lane 5: Type I human collagen

Arrows in lane 5 indicate: (i) faint band approximately 300kDa (rf 0.1) [collagen]; (ii) 2 prominent bands (rf 0.218 and 0.233) [collagen]; the prominent band of collagen with an rf of 0.218 corresponds with a band present in the stimulatory fraction of synovium (lane 4).

(b) Western blotting of synovium was performed using the tissue shown in (a) lane 4

Lane 1: Mouse monoclonal antibody (anti- human type III collagen); non-reducing conditions;

Lane 2: Mouse monoclonal antibody (CJ2 - anti-human fibronectin); non-reducing conditions;

Lane 3: Rabbit polyclonal antibody (anti-human fibronectin); non-reducing conditions.

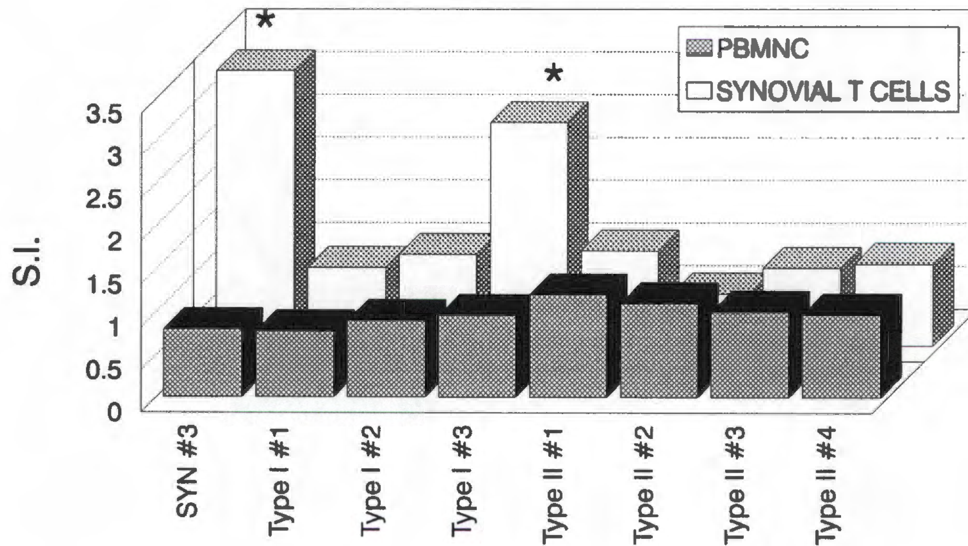
The presence of collagen in synovial membrane was confirmed by western blotting using a mouse monoclonal antibody specific for human type III collagen (Figure 6.6b, lane 1). According to the manufacturer's specifications, this antibody may cross-react with type I or type II collagen, but this was not confirmed in the current study. Under non-reducing conditions, two bands in the high molecular weight range (300kDa) were detected in a sample of rheumatoid synovium, and correspond to the high molecular weight band obtained when type I collagen was subject to SDS-PAGE under reducing conditions (Figure 6.6a, lane 5). When electrophoresis of rheumatoid synovium was performed under reducing conditions, western blotting with the mouse anti-human collagen type III antibody did not detect the presence of collagen. Using monoclonal and polyclonal antibodies to fibronectin, degradation products of fibronectin were also detected in rheumatoid synovium (Fig 6.6b, lanes 2 and 3), under the same experimental conditions as for the detection of collagen. Two bands detected by the anti-fibronectin monoclonal antibody and one detected by the polyclonal antibody were in the same molecular weight range as the stimulatory synovial fragment (114-142 kDa) (Fig 6.6b, lanes 2 and 3).

5. Cross-reactivity between the synovial stimulatory fraction and nitrocellulose-bound type I human collagen of similar molecular weight.

Human type I and bovine type II collagen were separated by gradient SDS-PAGE and transferred to nitrocellulose as described above. Lanes of nitrocellulose were fractionated into 2mm bands and dissolved and precipitated as for the preparation of synovial membrane. Fractions were pooled (5 per test) and their effects on the proliferation of a T cell line from rheumatoid synovium examined as detailed above. Pooled fractions of nitrocellulose-bound type I collagen corresponding to the molecular weight range of the stimulatory synovial fraction caused a low but definite proliferative response of the T cell line but not autologous PBMNC (Δ cpm 6520)(Figure 7). There were no proliferative responses to fractions of bovine type II collagen prepared in a similar manner. This finding was confirmed on repeat testing with the same T cell line, but was not observed using T cell lines from an additional two RA patients who demonstrated proliferative responses to autologous synovium, and in two OA patients.

FIGURE 6.7

Cross-reactivity between nitrocellulose-bound synovial stimulatory fraction and nitrocellulose-bound type I collagen of similar molecular weight range#



Human type I and bovine type II collagens were subject to electrophoretic separation and transferred to nitrocellulose, as described for the synovium. Proliferative response of a T cell line from rheumatoid synovium to fractions of collagen and autologous synovium were assayed. Data shown are mean stimulation indices of triplicate determinations. SYN #3 - third pooled synovial fraction 114-142 kDa; Type (n) #(n) - collagen type(n) pooled nitrocellulose fraction (n). Fraction #1 - 175-217kDa, fraction #2 - 142-175kDa, fraction #3 - 114-142kDa.

* Stimulation index > 2.5

DISCUSSION

It has previously been suggested that the synovial compartment in RA represents a special environment that either induces or maintains a degree of T cell activation[20]. In the current study, the sustained outgrowth of T cells from rheumatoid synovial explants, cultured in the presence of exogenous recombinant IL2 but without the addition of other T cell stimuli, provides additional evidence that the rheumatoid synovial membrane may contain a co-stimulatory factor for T cell proliferation.

With the use of the T cell immunoblot technique, a compartmentalised T cell proliferative response to a component of autologous synovium in the molecular weight range 114-142kDa was found in 5 out of 6 patients with RA but not in patients with other arthritides. The comparatively low levels of proliferation in response to this synovial constituent may be a result of the technique used, suboptimal concentration of the stimulatory fraction in the nitrocellulose preparation, or the due to the nature of the stimulatory constituent[27]. Nevertheless, the observation of a proliferative response to a component of autologous synovium in the absence of costimulatory factors may provide insight into the source of the T cell stimulus in rheumatoid synovial membrane. The limitation of the proliferative response to autologous T cells from the site of pathology in RA indicates a degree of specificity of the finding. Thus the observed proliferation may have been an antigen-specific response with compartmentalisation of the specific antigen-reactive T cells to the site of pathology, as has been demonstrated in a T cell dependent disease of known aetiology[34]. Furthermore, the restriction of the finding to rheumatoid synovium may imply that the antigenic stimulus or the specific antigen-reactive T cells are not present in the synovium of patients with other arthritides; the observation that a T cell line from osteoarthritic synovium did not proliferate to the stimulatory fraction of rheumatoid synovium supports the latter hypothesis.

It is, however, also possible that the proliferation of rheumatoid synovial T cell lines in response to a fraction of autologous synovium may be due to the interaction of a certain subset of CD4 T cells enriched in the rheumatoid joint and a non-specific stimulus. In particular, upregulated cell suface expression of integrins in synovial lymphocytes of patients with arthritis has previously been described, and may increase non MHC-restricted proliferative responses to extracellular matrix proteins[35,36]. Evidence in support of this hypothesis in the current study includes the demonstration that the stimulatory fraction of synovium incorporates components of collagen and fibronectin. Both these extracellular matrix proteins contain RGD peptide sequences that bind integrins on the CD4 T cell surface, independent of MHC interactions, and augment several T cell functions including proliferation, cytokine production, release of proteases by cytotoxic T cells, IL2 receptor expression and cell migration[37,38,36,39,40,41,42]. A

120 kDa fragment of fibronectin that may be cleaved from the parent molecule by the action of several proteases previously identified in the rheumatoid joint, contains the cell attachment site for neutrophils and is a chemoattractant for T cells expressing the $\alpha5\beta1$ integrin that are enriched within the rheumatoid synovial compartment[43,41,35].

The potential role of collagen as the T cell stimulus in rheumatoid joints is somewhat controversial, as discussed earlier (Ch1 pg. 16). In the current study, low levels of proliferation of a rheumatoid synvoial T cell line in response to human type I but not bovine type II collagen in the same molecular weight range as the stimulatory fraction of synovium was observed. With the use of the T cell immunoblot technique, it is unlikely that this proliferative response was due to contaminants. The lack of proliferation to bovine type II collagen may be due to species-specific differences, although the collagens are generally conserved proteins[32]. Alternatively the finding may reflect differences in tissue expression of collagen subtypes; type I collagen being predominantly a product of synovium and type II collagen essentially chondrocyte-derived[32]. The lack of proliferation to nitrocellulose-bound collagen (either type I or II) of the additional 2 rheumatoid synovial T cells lines tested indicates that collagen reactivity in this model is not universal in RA and that the observed proliferation to a constituent of synovium of similar molecular weight range cannot generally be attributed to a collagen-induced response.

Synovial T cell reactivity to foreign antigens in RA has been discussed earlier (Ch5 pg. 126). Significant proliferative responses of the synovial explant-derived T cell lines to PPD and SK-SD were limited to 3 patients with arthritis of diverse aetiology in the current study and emphasize the non-specific nature of these responses. In addition there was no evidence of compartmentalisation of the responses and none of the patients demonstrated significant reactivity to recombinant HSP65 of *Mycobacterium bovis*. The finding of proliferation to recall antigens in the T cell lines from three patients does, however, indicate that cell lines generated using the synovial explant culture technique are capable of antigen-specific responses and are not anergic[44].

As indicated in the preceeding discussion, further characterisation of the T cell stimulus contained within the stimulatory fraction of synovium is required. The experimental approach to this further characterisation may include the isolation of a soluble product of narrower molecular weight range from rheumatoid synovium and the use of synovial T cell clones to improve specificity. Use of a soluble form of synovial stimulatory factor will overcome several of the limitations of the nitrocellulose-bound product, including the need for a phagocytic feeder cell population and the inherent difficulties associated with particle suspensions in lymphocyte proliferation assays[27]. Determining the mode of T cell stimulus will require MHC blockade or substitution experiments to investigate whether the effect is class II restricted, and RGD

peptide inhibition assays to demonstrate integrin-mediated effects[28,45]. Precise characterisation of the stimulatory protein may be performed by sequence analysis techniques[46]. This study thus provides the rationale for the further investigation of synovial constituents and their effects on T cell function in RA, with implications for the understanding of the pathogenesis of the disease and the development of new immunotherapeutic agents.

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Chapter 7

Isolation, expansion and characterisation of T- cell clones from the synovial membrane of patients with inflammatory synovitis.

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INTRODUCTION

A T cell clone is defined as a population of T cells arising from a single precursor cell.[1] Techniques for the *in vitro* generation of human T cell clones have been established for more than ten years. In addition to having potential clinical application, human T cell cloning permits the investigation of monoclonal human T cell populations *in vitro*[2,3]. Such *in vitro* studies of cloned human T cells have several advantages over studies of 'bulk' mononuclear cell populations or polyclonal T cell lines, including (i) the precise definition of antigen reactivity, specific epitope mapping and HLA restriction, (ii) the determination of cytokine profiles, (iii) modulation of specific cell functions with minimal confounding variables introduced by other cell populations, (iv) investigation of phenotypic expression in response to antigenic stimuli, (v) specific delineation of intracellular signalling pathways and cellular regulation, and (vi) defining mechanisms of effector functions, including cytotoxicity[4,5,6,7,8]. As a T cell clone constitutes a uniform population of cells, these studies can be performed repeatedly, permitting greater reproducibility of results.

A variety of methods have been used to generate T cell clones, including growth of colonies from cells plated in soft agar, micro-manipulation or limiting dilution techniques[9,10]. The latter method is probably most widely applied at present and permits the estimation of clonality by application of Poisson statistics[1]. When required, precise definition of clonality of a T cell population may be achieved by analysis of T cell receptor Vβ gene expression[11]. All cloning techniques have limitations and will, to an extent, bias the selection of cells obtained. Cloning strategies using the limiting dilution method may involve stimulation with an antigen to obtain specific antigen-reactive clones, or the use of a non-specific stimulus to obtain a panel of individual clones that reflects, as closely as possible, the constituents of the original 'bulk' population. In the present studies, limiting dilution cloning was undertaken using both selective and non-specific stimuli. Cloning of cells from the synovium of patients with inflammatory synovitis and the circulation of a normal control was performed. As detailed in previous chapters, investigation using these clones included phenotypic and functional analyses, examination of the effects of eicosanoids and other agents, and a study of the kinetics of activation marker expression and effector functions following antigenic stimulation.

GENERATION OF T CELL CLONES

1. Patients and controls

Limiting dilution cloning was performed using T cell lines generated from synovial explants of 3 patients with inflammatory synovitis of diverse aetiologies, and from the peripheral blood of a normal healthy control. Details of the patients and control have been provided previously, and are included below in table 7.I for completeness.

Table 7.I

Patients and control used as sources of CD4+ T cells for cloning.

PATIENT	DIAGNOSIS	AGE	SEX	CELL SOURCE
AP	RA (sero positive)	52	F	Synovium: arthroplasty left knee
VS	Post traumatic inflammatory OA	37	M	Synovium: arthroplasty right knee
TS	Gout	37	M	Synovium: arthroscopic biopsy right knee
PL	Normal control	38	F	Venous blood

2. Generation of T cell lines

Synovial explant-derived T cell lines were obtained as described in chapter 6. Briefly, fragments of recently-obtained tissue (2-3mm^3) were cultured in complete tissue culture medium supplemented with rIL2 (100iu/ml)(Cetus), in 24 well tissue culture plates (Greiner). Early passage mononuclear cells growing out of the explants were harvested by vigorous resuspension and used either for cloning immediately (patients VS and AP) or to generate antigen-specific T cell lines which were subsequently cloned (patient TS - gouty synovitis). These procedures are summarised in table 7.II. Mononuclear cells were harvested from synovial explants of patient TS after 48h growth in the presence of rIL2 (100iu/ml), and stimulated in 24 well plates (2.5 x 10^5 cells per well) with irradiated (4000rads) autologous PBMNC (1.25 x 10^5 per well) and one of the following antigens: PPD (Central Veterinary Laboratory - final concentration 3µg/ml), streptokinase-streptodornase (SK-SD, Lederle - 1:40 dilution), or recombinant HSP65 of *Mycobacterium bovis* (provided by Dr JDA van Embden - final concentration 5µg/ml). Cultures were fed with fresh medium containing rIL2 (100iu/ml) after 3-4 days and cells harvested at day 5 for limiting dilution cloning.

PBMNC were separated from buffy layer cells of a venous blood sample from the normal control by density centrifugation. Antigen-stimulated T cell lines were generated prior to cloning by culturing PBMNC (5 x 10^6 cells at 10^6/ml in complete medium) with either PPD (Central Veterinary Laboratory - final concentration 3μg/ml) or tetanus toxoid (Lederle Laborotories, American Cyanamid Co, Pearl River, NJ; 1:40 dilution) in 25cm^2 tissue culture flasks (Becton Dickinson Labware, Lincon Park, NJ) for 7 days. Cells were then fed with fresh medium and rIL2 (final concentration 100iu/ml) and cloned in limiting dilution after 4 days.

Table 7.II

Summary of cloning strategies

Patient	Cell Source	IL2-dependent T-cell lines	Antigen-stimulated T-cell lines	Stimulus used in limiting dilution cloning
AP	synovial explant	yes	no	PHA
VS	synovial explant	yes	no	PHA
TS	synovial explant	yes	PPD, SK-SD, HSP65	PPD, SK-SD, HSP65
PL	venous blood	no	PPD, Tettox	PPD, Tettox

PHA - phytohaemagglutinin; PPD - purified protein derivative of Myocbacterium tuberculosis; SK-SD - streptokinase-streptodornase; HSP65 - recombinant 65kDa heat shock protein of Mycobacterium bovis; Tettox - tetanus toxoid.

3. Limiting dilution cloning

Limiting dilution cloning of T cell lines detailed above was performed as previously described[12]. Cells to be cloned were washed in PBS and resuspended at 10^6/ml in fresh culture medium containing RPMI 1640 supplemented with 10% autologous serum, 2-mercapto-ethanol (BDH Chemicals, Poole, England; final concentration 5 x 10^{-5}M), non-essential amino acids (Highveld Biologicals, Johannesburg, South Africa; final concentration 10mM), l-glutamine (Highveld Biologicals; final concentration 2mM), sodium pyruvate (Highveld Biologicals; final concentration 1mM) and antibiotics (100U/ml of penicillin, 100μg/ml of streptomycin) (cloning medium). Cells were re-counted at this concentration and serially diluted as required. Cloning was performed in Terasaki plates and responder cells seeded in the range 100, 50, 10, 1, 0.5 and 0.3 cells per well. Feeder cells used were autologous irradiated (4000 rads) PBMNC (10^4/Terasaki well). Cells were stimulated with rIL2 and either mitogen (PHA) or antigens

(PPD, SK-SD, HSP65 or tetanus toxoid) at optimal concentrations, as indicated above, in a final volume of 20µl. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere; given the small volumes of the Terasaki wells, care was taken to ensure accurate temperature and humidity control to avoid evaporation. A single row of feeder cells without responders was used to provide a negative control for screening of cell growth. Cell growth was determined by visual inspection using an inverted microscope after 7-10 days. Wells were scored as positive if greater than one third of the well surface was occupied by cells.

Cloning efficiency has been defined as the proportion of seeded cells that give rise to a growing clone[13]. Several factors contribute to the final cloning efficiency, including culture conditions (plating efficiency) and the precursor frequency of cells in the 'bulk' population capable of responding to the particular stimulus. As indicated in table 7.III, cloning efficiency was generally low in the current study; in the case of mitogen-stimulated cultures, this may reflect suboptimal culture conditions or inadequacies of the responder cells[13]. The low proportion of positive wells obtained when antigenic stimuli were used in the cloning of cells from gout synovium may be an indication of the low precursor frequency of these cells in the parent T cell lines, in addition to other factors.

Limiting dilution cloning permits estimation of clonality according to the zero term of the Poisson distribution, $F_0 = e^{-u}$, where F_0 is the fraction of negative cultures per total number of cultures, e the base of the natural logarithm and u the average number of precursor cells per culture[6]. As clonality is defined as the growth of cells from a single precursor, u = 1; for u =1, $F_0 = e^{-1} = 0.37$. Thus clonality is likely (95% probability) when > 37% negative wells are obtained (see Table 7.III), although this assumption may be compromised in settings of low plating efficiency[1].

Table 7.III

Limiting dilution cloning of T cells from synovium and circulation

CELL SOURCE	CLONING: Cells/well	STIMULUS	Positive wells/ total wells	% Negative wells	Cloning Efficiency‡
AP	100	PHA	108/108	0*	100%
(RA synovium)	50	PHA	108/108	0*	100%
	10	PHA	300/354	15.3*	85%
	0.5	PHA	49/474	89.7†	20.7%
VS	10	PHA	70/108	35*	64.8%
(OA synovium)	1	PHA	8/108	92.5†	7.4%
TS	10	PPD	7/108	93.5†	6.5%
(Gout synovium)	1	PPD	0/216	100	0%
	0.3	PPD	0/324	100	0%
	10	SK-SD	3/108	97.2†	2.8%
	1	SK-SD	0/216	100	0%
	0.3	SK-SD	0/324	100	0%
	10	HSP65	9/108	91.7†	8.3%
	1	HSP65	0/216	100	0%
	0.3	HSP65	0/324	100	0%
PL	10	PPD	45/600	92.5†	7.5%
(Normal PBMNC)	10	Tettox	63/600	89.5†	10.5%

* As the probability of clonality is extremely low in these cultures, the term culture efficiency may be more appropriate than cloning efficiency

† Positive wells are likely to be monoclonal according to Poisson statistics

‡ May be affected by minor errors in cell counting, due to the amplification effect of repeated dilutions; assumes 'single hit' model even when > 1 cell per well was plated

PHA - phytohaemagglutinin; PPD - purified protein derivative of *Mycobacterium tuberculosis*; SK-SD - streptokinase-streptodornase; HSP65 - recombinant 65kDa heat shock protein of *Mycobacterium bovis*; Tettox - tetanus toxoid.

EXPANSION AND MAINTENANCE OF CLONES

Growing cells were 'picked' when greater than 50% of the well surface was occupied by cells (usually between 7-12 days after cloning) using a 20μl microtitre pipette and transferred to u-bottomed 96-well microtitre plates. Re-stimulation was performed using irradiated autologous PBMNC as feeder cells (5 x 10^4 /well), rIL2 (100iu/ml) and antigen or mitogen at optimal

concentrations, in a final volume of 200μl of cloning medium. Autologous PBMNC were employed as the optimal source of feeder cells throughout these studies as preliminary experience with Epstein-Barr virus transformed lymphoblastoid cell lines (LCL) indicated inadequate function of these cells in soluble antigen-induced proliferation of T cell lines and clones (data not shown). Growing cultures were fed by replacing 100μl of medium with fresh cloning medium containing rIL2 (100iu/ml) at day 3-4 following transfer and cells were resuspended regularly to avoid the formation of large clumps. At day 7 following transfer, cells were counted, seeded into new 96 well microtitre plates (10^5/well) and re-stimulated with feeders and antigen or mitogen as indicated above.

As the objective at this stage of culture was to obtain maximal cell numbers within relatively few passages, cultures were divided when confluent or near confluent, fed every 3-4 days as detailed above, and transferred to 24 well tissue culture plates, usually by the end of the second passage in microtitre wells. Approximately 2.5×10^5 cells were seeded in each well of the 24 well plate and stimulated with irradiated autologous PBMNC (10^5 per well), antigen or mitogen and rIL2, at concentrations indicated previously, in a final volume of 1ml. Cultures were resuspended regularly, fed every 3-4 days and passaged weekly. Once sufficient cells had been obtained, aliquots of clones were cryopreserved and the remainder of cells analysed as indicated below, or expanded further. Using these techniques, sufficient quantities of each clone ($20 - 30 \times 10^6$) were usually obtained within 4-6 weeks of cloning; additionally, it was possible to perform repeated functional studies on individual clones, previously cryopreserved at early passage, for over 2 years after initial cloning.

CHARACTERISATION OF CLONES

1. Phenotypic analysis

Phenotypic analysis of the clones obtained was performed by flow cytometry, as described earlier (chapter 3 pg. 83). As the objective of these studies was the investigation of CD4+ T cells, clones were initially screened for CD4 expression, and CD4 negative cultures, with one exception, excluded from further analyses. Only four CD4 negative cultures in total were identified (3 from patient AP (RA) and one from patient TS (gout)).

2. Proliferation

T cell clones were screened for proliferative responses to mitogen (PHA), a range of antigens (PHA, PPD, SK-SD, HSP65 and tetanus toxoid) and rIL2. Assays were performed at least 3 days after the clone had been fed with fresh rIL2 and 7 days after re-stimulation with feeders and antigen. All proliferation assays were performed in triplicate in u-bottomed microtitre wells

in cloning medium. Clones ($5x10^4$/well) were stimulated by antigen or mitogen at optimal concentration in the presence of 2.5 x 10^4 irradiated autologous PBMNC but in the absence of rIL2. Incorporation of ^{3}H thymidine (2μCi/well) (Amersham International) was measured over the last 8 hours of the 48h incubation. Wells with feeders and clone but no antigen/mitogen served as background controls; ^{3}H thymidine incorporation of feeders in the presence of antigen/mitogen but in the absence of the clones, was also determined to ensure effectiveness of the irradiating procedure. Results were expressed as either stimulation index (SI) (where SI = mean counts per minute in stimulated wells/mean counts per minute in background wells) or Δcpm (mean counts per minute in stimulated wells - mean counts per minute in background wells). Data obtained from these assays are provided in chapter 5.

3. Cytotoxicity

Cytotoxic function of a selection of the clones was determined in a standard 15h ^{51}CR release assay, as detailed in previous chapters. Both antigen-specific and lectin-dependent cytotoxic functions were investigated. Assays were performed in triplicate and usually at multiple effector to target ratios. Clones were used as effectors at day 2 or 3 following re-stimulation with feeders, antigen and rIL2, although similar levels of cytotoxocity at other time intervals after restimulation were demonstrated (chapter 4 pg. 105). Cytotoxicity assays were performed in complete medium, without the addition of rIL2. Data obtained from these assays are provided in chapters 3,4 and 5.

4. Cytokine production

Cytokine profiles of a selection of the clones were determined as described in chapter 3. As for the proliferation assays, cytokine production of a clone was assayed at least 3 days following feeding the clone with fresh rIL2 and 7 days after re-stimulation with feeders and antigen/mitogen. Cytokine production in response to specific (antigen and autologous irradiated PBMNC) and non-specific stimuli (mitogen and autologous irradiated PBMNC or plastic adherent anti-CD3 and rIL2) were investigated. Where irradiated PBMNC were employed, background cytokine production by these cells alone, in the presence of the mitogenic or antigenic stimulus, was also determined. Cell supernatants were harvested after 48h incubation, confirmed in the kinetics studies (chapter 4) as the optimal incubation period. Cytokine concentrations in the cell supernatants was assayed using commercial kits, as described in chapter 3. Data obtained from these assays are provided in chapters 3 and 4.

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Chapter 8

Conclusions

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1. Introduction

The studies described in the preceeding chapters were undertaken to address several aspects of the role of CD4+ T-cells in the immunopathogenesis of RA and identify potential novel therapeutic targets. In meeting the objectives of the study (as tabulated in chapter 1), the data obtained provide insight into potential pathogenetic mechanisms of RA that may have therapeutic relevance.

2. Novelty of the approach

The approach adopted in the studies was innovative in the following respects: (i) antigen-induced proliferation and antigen-specific cytotoxicity of mononuclear cells from the synovial fluid and circulation of patients with RA and other arthritides were examined in parallel, permitting comparison of functions between cells from either compartment, and correlation with clinical parameters, (ii) antigen-specific cytotoxicity of mononuclear cells in the patient-based studies was determined using short-term cell lines stimulated with antigen alone; an isolated previous report of antigen-specific T-cell mediated cytotoxicity in RA was based on IL-2 dependent lines and clones from the synovial compartment and did not include clinical correlations[1], (iii) the *in vitro* effects of leukotrienes and prostanoids on CD4+ T-cell mediated cytotoxicity (either murine or human) have not previously been reported and neither have there been reports of the effects of leukotrienes on cytokine secretion by human CD4+ T-cell clones, (iv) the dissociation between proliferation/cytokine production and cytotoxicity of human CD4+ T-cell clones has not previously been demonstrated; in addition, the approach to defining the cellular regulation of these functions following a single inciting stimulus has not previously been adopted, (v) the investigation of mycobacterial antigen-reactivity of mononuclear cells from the site of pathology in RA and other arthritides was performed with patients drawn from a population where tuberculosis is endemic, and included a novel investigation of the kinetics of responses, and (vi) the studies of synovial T-cell reactivity to autologous synovial constituents used a novel application of the T-cell immunoblot technique and tissue explant culture techniques.

3. Salient findings

In the patient-based study reported in chapter 2, a positive linear relationship between PPD-induced proliferation and PPD-specific cytotoxicity was confirmed in peripheral blood mononuclear cells (PBMNC) and synovial fluid mononuclear cells (SFMNC) of 20 patients with seropositive RA. Three subsets of RA patients were classified according to their SFMNC proliferative and cytotoxic responses to PPD. This distinction, based on SFMNC function, correlated with disease duration and therapy. Low or absent SFMNC proliferative and cytotoxic responses were associated with significantly longer disease duration. In patients with

shorter disease duration, moderate to high levels of both proliferation and cytotoxicity were associated with NSAID monotherapy. Intact proliferation but impaired PPD specific cytotoxicity was a feature of SFMNC from 5 out of 6 patients receiving DMARDs and supplemental NSAID therapy. The potential mechanisms for these findings were explored. Dissociation of proliferation and cytotoxicity in normal PBMNC was not an *in vitro* effect of the DMARDs tested, but was suggested to be an effect of PGE2. Thus it was postulated that the specific impairment of SFMNC cytotoxicity despite intact proliferative responses may have been due to higher production of PGE2 by cells from the synovial compartment in patients receiving DMARDs and supplemental NSAID therapy, compared to patients receiving NSAID monotherapy. Support for this hypothesis was provided by (i) confirmation of higher PGE2 production by CD14+ synoviocytes from patients receiving DMARDs, and (ii) demonstration that the suppressive effects of synovial CD14+ cell supernatants on the cytotoxic function of a CD4+ T-cell clone may be abrogated by the generation of the supernatants in the presence of the cycloxygenase inhibitor, indomethacin. Given the relatively small numbers of patients, conclusions about these data remain guarded and may require confirmation in a larger series.

The mechanism of PGE2 inhibition of CD4+ T-cell cytotoxicity and the effects of this agent and other eicosanoids on CD4+ T-cell effector functions and phenotypic expression were examined (chapter 3). Studies were performed *in vitro* using PBMNC from normals and panels of clones generated from the synovium of patients with inflammatory synovitis and the circulation of a healthy control. PGE2 inhibition of antigen-specific cytotoxicity was demonstrated in bulk PBMNC effectors and in the clones. Misoprostol (PGE1 analogue) and forskolin (cAMP agonist) displayed similar inhibitory effects on the clones, although the suppression mediated by misoprostol was directly dose-dependent. Inhibition of both binding and post-binding events by PGE2 was indicated by data from lectin-dependent and antigen-specific cytotoxicity assays. Long-term culture of a clone in the presence of PGE2 did not inhibit the maintenance of cytolytic potential, as demonstrated in the lectin-dependent cytotoxicity assay, but suppressed antigen-specific cytotoxicity. Moderate but significant augmentation of cytotoxicity by LTB4 and LTC4 was demonstrated in the clones, although the effects of LTB4 were not titratable. Modulation of CD4+ T-cell cytotoxicity by the eicosanoids tested was mainly due to effects on calcium-dependent pathways of cytotoxicity. Reciprocal effects of PGE2 and leukotrienes on IFNγ production by the clones was also found.

In chapter 2, a positive linear relationship between antigen-induced proliferation and antigen-specific cytotoxicity was demonstrated in bulk PBMNC and SFMNC. However, dissociation in these functions (proliferation in the absence of cytotoxicity) was observed in the SFMNC of a subset of patients and also shown to be an effect of PGE2 on normal PBMNC responses. These findings suggested that proliferation and cytotoxicity of CD4+ T-cells may be independently

regulated. This hypothesis was tested by measuring a range of morphologic, phenotypic and functional parameters of a panel of CD4+ T-cell clones as a function of time following a single initiating stimulus (chapter 4). Dissociation of functions was noted, with maintenance of cytolytic potential independently of other parameters. Thus these findings provide evidence, at a clonal level, of the separate regulation and different kinetics of cytotoxicity in CD4+ T cells and that post activated CD4+ T-cells retain cytolytic potential irrespective of their size, activation status, cell cycle position or cytokine production.

Previous reports have suggested that mycobacterial antigen reactivity of SFMNC in RA may have aetiological significance. In the current study, PBMNC and SFMNC proliferative and cytotoxic responses to PPD were indeed found in a subset of patients with RA, but also demonstrated in SFMNC and CD4+ T-cell clones from patients with non-rheumatoid inflammatory synovitis (chapter 5). Studies of the kinetics of PPD induced proliferation in SFMNC and PBMNC from RA and control patients yielded similar results, supporting the contention that PPD reactivity in RA reflects the non-specific nature of the synovial T-cell infiltrate. In addition, the frequency of PBMNC reactivity to HSP65 of *Mycobacterium bovis* in PPD-responsive RA patients was significantly lower than that found in PPD-responsive healthy controls. Specific localisation of HSP65 reactive T-cells at the site of acute synovial inflammation was found in a patient with gout.

Proliferation of synovial T-cell lines in response to a high molecular-weight component of autologous synovial membrane was found in patients with RA but not other arthritides (chapter 6). The stimulatory fraction of synovium was shown to contain components of extracellular matrix proteins, including collagen and fibronectin.

4. Immunopathogenetic relevance of this study

The present paradigm of CD4+ T-cells in the immunopathogenesis of RA has focussed on the 'helper' function of these cells, mediated by their release of cytokines[2,3,4]. However, the role of CD4+ T cells in the pathogenesis of the disease has been disputed, due to the paucity of T-cell derived cytokines in the rheumatoid synovial compartment and evidence suggesting that these cells are not activated, not progressing through the cell cycle, and small in size[5]. Data from this study have highlighted the cytotoxic potential of rheumatoid synovial CD4+ T cells. In addition, investigation of the *in vitro* regulation of these cells indicated that small, post activated CD4+ T-cells retain maximal cytotoxic potential, independently of cytokine production and cell cycle progression. Taken together, these findings suggest that CD4+ T-cell mediated cytotoxicity (including target cell lysis and apoptosis) may be involved in the pathogenesis of RA.

The reciprocal modulation of CD4+ T-cell effector functions by prostanoids and leukotrienes B4 and C4 demonstrated in this study highlights a potential regulatory network between macrophage-lineage cells and CD4+ T-cells in the rheumatoid synovial compartment, and indeed between CD4+ T-cells and other cellular sources of eicosanoids. Although prostanoids are generally viewed as deleterious in RA, data from this study indicates the need for reappraisal of their function in the pathogenesis of RA. PGE2 may play an important role in the inhibition of cytotoxic effector function of CD4+ memory T-cells sequestered at a chronic inflammatory site. In addition, prostanoid inhibition of IFNγ production by CD4+ T-cells, as shown in this study, may also be of benefit in the rheumatoid synovial compartment. Conversely, the immune stimulatory properties of LTB4 and LTC4 demonstrated in the current study support a role for these products of lipoxygenase metabolism in promoting the immuno-inflammatory cascade in RA.

Data from this study do not support a role for mycobacterial infection in the aetiopathogenesis of RA. The finding of a lower frequency of HSP65 reactivity among PPD-reactive RA patients compared to PPD-reactive healthy controls may be indicative of a failed regulatory T-cell response in the patients that may have contributed to the persistent local immune response. The sustained outgrowth of CD4+ T-cells from rheumatoid synovial explants and the demonstration of T-cell reactivity to a component of autologous synvoium supports the previous suggestion that the synovial compartment in RA represents a special environment that either induces or maintains a degree of T cell activation.

5. Prospects for immunotherapy

Given the potential role of CD4+ T-cells in the pathogenesis of RA, modulation of CD4+ T cell function has been a major objective of immunotherapeutic regimens to date[6,7]. This study indicates the potential for modulation of CD4+ T-cell functions (including cytokine production and cytotoxicity) at the site of pathology in RA by therapeutic manipulation of local eicosanoid concentrations. Thus specific inhibition of 5 lipoxygenase metabolism, or LTB4/LTC4 antagonism, in the rheumatoid synovial compartment may reduce the stimulatory effects of these eicosanoids on CD4+ T-cell functions. As reviewed in chapter 1, reduction in the leukotriene stimulus of cells of other lineage in the synovial compartment (including macrophage production of TNFα) may also be beneficial in RA. The *in vitro* immunosuppressive properties of prostanoids and the association of NSAID monotherapy with high levels of SFMNC cytotoxicity in patients with RA demonstrated in this study both suggest that cycloxygenase inhibition as the sole form of therapy in RA may remove the potentially protective effects of local prostanoids in the synovial compartment. In addition, the

immunosuppressive properties of the PGE1 analogue misoprostol demonstrated in this study indicate its therapeutic potential in RA.

The dissociation of CD4+ T-cell functions demonstrated in both bulk and clonal populations in the current study indicate the potential for selective modulation of effector mechanisms in these cells. Thus it may be possible to inhibit CD4+ T-cell mediated cytotoxicity independently of effects on cytokine production and thereby increase the specificity of the immunosuppression.

Further elucidation of the nature of the high molecular-weight T-cell stimulus identified in the synovium of patients with RA may have therapeutic potential. Identification of a specific antigen within this synovial fraction may permit the therapeutic targeting of individual T-cell populations in RA. Alternatively, as suggested by the current study, confirmation that the stimulus is due to T-cell binding to components of extracellular matrix proteins may indicate the therapeutic potential of inhibiting such processes in RA.

6. Future research

The findings of the current study provide the rationale for further research incorporating *in vitro* and *in vivo* models, and perhaps early phase clinical trials. These studies include:

(i) investigation of the potential contribution of CD4+ T-cell mediated cytotoxicity to joint destruction in animal models of inflammatory arthritis; these studies may include analysis of cytolytic and apoptotic cell death mechanisms,

(ii) further definition of the *in vitro* effects of eicosanoids on CD4+ T-cell mediated cytotoxicity, including the modulation of fas/fas ligand expression and intracellular granzyme/perforin contained within cytotoxic granules,

(iii) characterisation of the high molecular weight T-cell stimulatory factor in rheumatoid synovium, as detailed in chapter 6,

(iv) phase I clinical trials of 5 lipoxygenase inhibitors and specific LTB4 antagonists in the treatment of patients with RA, and

(v) early phase clinical trial to investigate the potential disease-modifying properties of the PGE1 analogue, misoprostol.

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