LYMPHOCYTE-SYNOVIAL MICROVASCULAR ENDOTHELIAL CELL INTERACTIONS IN EXPERIMENTAL POLYARTHRITIS

: A microassay for screening monoclonal antibodies that block adhesion

by
Elizabeth-Anne Louise Farmer B.Sc.(Hons)
(nee: Vinall)

Department of Molecular Biosciences
The University of Adelaide

and

Arthritis Research Laboratory
Hanson Centre for Cancer Research

A dissertation submitted to the University of Adelaide
in candidature for the degree of
Doctor of Philosophy
in the Faculty of Science

DECLARATION

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Elizabeth-Anne L. Farmer B.Sc.(Hons)

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Now I can paint the kitchen ceiling and have the plumbing fixed!

ABSTRACT

Although the causes of rheumatoid arthritis (RA) are still not well defined, an improved understanding of the pathogenesis of this disease has the potential to allow the development of new and innovative therapies. An understanding of each facet of the adhesion cascade leading to T cell migration into synovium should help to identify molecules that are involved directly with propagation of synovial inflammation. By targeting these molecules, treatments might be developed that are more efficacious, specific and less toxic systemically than current therapies. Furthermore, if adhesion molecules can be identified that have expression limited to synovium, novel therapeutic approaches aimed at blocking the function of adhesion molecules offer advantages in the management of arthritis. Thus, offering an immunotherapy for arthritis that is limited in its systemic effects on the immune system

With this in mind, an experimental system was developed to allow direct investigation of the adhesion of activated lymphocytes to microvascular endothelial cells (EC) from the inflamed synovium of rats with adjuvant arthritis (AA). The experimental system that was developed is novel, in that the EC were prepared from the microvasculature as primary cultures. Previous studies, utilising various models, have highlighted a role for a number of adhesion molecules in the recruitment of inflammatory cells during the pathogenesis of immunologically mediated polyarthritis. Earlier *in vitro* studies used either EC derived from large vessels, such as the rat aorta or the human umbilical cord, or from the microvasculature of non-articular tissues such as the normal rat heart. EC populations from normal tissues have required *in vitro* stimulation with pro-inflammatory mediators prior to use in an adhesion assay, in order to upregulate expression of adhesion molecules. In contrast, the EC used in the experimental system described herein are derived from synovium and they have been activated *in vivo* during the pathogenesis of polyarthritis. It was possible, therefore, to investigate the adhesive interactions between freshly isolated populations of synovial microvascular ECs that were activated *in vivo* and activated T cells. Two different sources of activated T cells were used. They were T cells from lymph nodes activated *in vitro* with Con A and *in vivo* activated CD4+ T cells obtained from the thoracic

duct (TD) lymph of donor rats during the late prodromal period of adjuvant induced arthritis. Cells in thoracic duct lymph during this phase of AA are known to enter inflamed synovium and they can transfer the disease adoptively.

This project investigated lymphocyte-EC adhesive interactions in a novel assay system. The molecular basis for lymphocyte-EC adhesion was investigated using an existing panel of monoclonal antibodies against rat adhesion molecules. The study has demonstrated a role of adhesion molecules CD54, CD11a/CD18, CD49d, CD62L and CD62P in the adhesion lymphocytes from both sources, to synovial microvascular EC. In addition, hybridomas were generated from the spleen of mice immunised with synovial cells produced from rats with established AA. Two hybridoma supernatants have been identified that contain antibodies that significantly inhibit lymphocyte adhesion to synovial microvascular EC monolayers. The results indicate that the microassay of lymphocyte–EC adhesion that has been developed in this work has wider application in characterising new monoclonal antibodies against adhesion molecules and could also be used to explore interactions between immune cells and EC from other sites of inflammation.

Development of a technique for preparation and purification of EC from synovium has allowed studies of other cells in synovial tissues. Lymphocyte subsets found in the synovim have been defined and expression of activation markers and adhesion molecules expressed by T cells have been examined by flow cytometry. Most of the T cells recovered from this tissue belong to the CD4+ subset and they express activation markers and elevated levels of the adhesion molecules: CD44, CD11a, CD54, CD49d, MHC class II, CD71 and CD25 accompanied by low levels of CD45RC and CD62L. This phenotype is consistent with that of effector T cells. The method for producing a mixed cell suspension from rat synovial tissue has shown great utility in studies on the pathogenesis of polyarthritis.

ABSTRACTS, CONFERENCE PROCEEDINGS

& SCIENTIFIC PAPERS

Publications and Abstracts

Zhang, X., Fitzsimmons, R.L., **Farmer, E.A.**, Cleland, L.G., Ey, P.L., Zannettino A.C., Mayrhofer, G. CD36/fatty acid translocase in rats: distribution, isolation from hepatocytes, and comparison with the scavenger receptor SR-B1, Lab. Invest., 2003 Mar; 83 (3):317-332

Farmer E.L., Cleland L.G. and Mayrhofer G. Endothelial Cell Monolayers in Adjuvant Arthritis, Australasian Society for Immunology, 1997, W2.3, pp 56 (abstract)

Farmer E.L., Cleland L.G. and Mayrhofer G. Endothelial Cell Monolayers in Experimental Polyarthritis, The Federation of Immunological Societies of Asia-Oceania, 1996, P12 (abstract)

Zhang X., **Farmer E.**, James M., Cleland L., Ey P.I., Zannettino A., Sincock P., Murphy C., Hutchinson M and Mayrhofer G. The Distribution of CD36 In The Rat, Demonstrated By Monoclonal Antibodies UA009, UA011 and OX43, 10th International Congress of Immunology, 1998 (abstract)

Zhang X., **Farmer E.**, James M., Cleland L. and Mayrhofer G. Characterisation of a surface Membrane Antigen Expressed On Synovial Microvascular Endothelial Cells And At Other Sites In The Rat, Australian Rheumatology Association, 1997 (abstract)

Zhang X., **Farmer E.**, James M., Cleland L. and Mayrhofer G. Characterisation Of A Rat Cell Surface Membrane Antigen Related To CD36 And Its Function On Macrophages, Australasian Society for Immunology, 1997, W3.15, pp 66 (abstract)

Papers in Preparation-

Farmer E.L., Cleland L.G. and Mayrhofer G. Flow Cytometric Analysis of Lymphocyte Subsets Recovered From Inflamed Synovium In Adjuvant Arthritis. Paper in preparation

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ABBREVIATIONS

AA	adjuvant arthritis
Ab	antibody
Ac-LDL	acetylated low density lipoprotein
APC	antigen presenting cells
APC	antigen presenting cell
Asc	ascites
Az	azide
BSA	bovin serum albumin
CAMs	cell adhesion molecules
CD	cluster of differentation
CFA	complete freunds adjuvant
CFDA,SE	5- and 6-carboxyfluorescein diacetate, succinimidyl ester
CFSE	5- and 6-carboxyfluoroscein diacetate succinimdyl ester
CIA	collagen induced arthritis
CLA	cutaneous leucocyte antigens
CMI	cell-mediated immunity
Con A	concanavalin a
CT	connective tissue
DA	dark agouti
DAB	3,3'-diaminobenzidine peroxidase
DC	DC
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,',3'-tetramethylindo-carbocyanine conjugated ac-ldl
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell(s)
EDTA	ethylenediaminetetraacetic acid
EIU	experimental immune uveoretinitis
ELAM-1	endothelial-leucocyte adhesion molecule-1
FACS	fluorescence activated cell sorting
FB	fibroblast
FCS	fetal calf serum
FSC	forward scatter
G	gauge
GIT	gastrointestinal tract
GlcNAc	n-acetylglucosamine
GlyCam	glycosylation-dependent cell adhesion molecule
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP-140	granule membrane protein-140
H&E	haematoxylin & eosin
HEPES	n-2-hydroxyethylpiperazine n'-2-etanesulphonic acid

HEV	high endothelial venules
HRP	horseradish peroxidase
HSP	heat shock protein
HUVECs	human umbilical vein endothelial cells
Hybr.	hybridoma
i.v.	intravenous
ICAM-1	intracellular adcsion molecule
IDDM	insulin-dependent diabetes mellitis
IFA	incomplete freunds adjuvant
IFN-γ	interferon-gamma
Ig	immunoglobulin
IgG_1	immunoglobulin gamma-1 isotype
IgG_{2a}	immunoglobulin gamma-2a isotype
IL	interleukin
kDa	kilodaulton
LAD	leucocyte adhesion deficiency
LFA-1	leucocyte function-associated antigen-1
LN	lymph node
L-VAP-1	lymphocyte-vascular adhesion protein
mAb	monoclonal antibody
MAdcam	mucosal addressin cell adhesion molecule
MALT	mucosal associated lymphoid tissue
MHC	major histocompatability complex
MPC	magnetic particle concentrator
mϕ	macrophage
NHS	normal human serum
NK	natural killer cells
NK T cell	natural killer t cell
NMS	normal mouse serum
NRS	normal rat serum
NSS	normal sheep serum
OIA	oil-induced arthritis
PADGEM	platelet activation-dependent granule external membrane protein
PAF	platelet activating factor
PBS	phosphate buffered saline
PCV	post capillary venule
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule
PLN	peripheral lymph node
PMN	polymorphonuclear
PNA	peripheral lymph node addressin
PNAd	peripheral node addressin
PSGL-1	p-selectin glycoprotein ligand-1
QTL	quantitative trait locus
R	receptor

RA	rheumatoid arthritis
RF	rheumatoid factor
RPMI	roswell park memorial institute
SEM	standard error of the mean
SGNL lipids	sulphated glycosphingolipids
Sgp200	sulphated glycoprotein 200
sLe	sialyl-lewis
SLE	systemic lupus erythematosus
SN	supernatant
SSC	side scatter
SSEA-1	sialyl stage-specific embryonic antigen
TCR	t cell receptor
TD	thoracic duct
TLR	Toll-like receptors
TNF	tumour necrosis factor
TNP	trinitrophenol
VAP-1	vascular adhesion protein
VCAM	vascular adhesion molecule
VLA	very late antigen
w/o	week old
w/v	weight per volume
v/v	volume per volume

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Erratum

Abstract , page iv, paragraph 3 line 2
Change 'synovim' to 'synovium'

Section 1.4.7, page 16, line 2
Change 'antigenic' to antigen

Section 1.9.4, page 44 line 2
delete 'the', should read ' arthritogens in autoimmunity...'

Section 1.10.4.1, page 50 paragraph 3, line 5
Delete 'only'

Section 1.10.4.5, page 53, paragraph 3 line 1
should read - 'It is significant that successful knockouts tend to exhibit a less compromised phenotype than predicted.'

Section 2.11.2, page 82, paragraph 3 line 2,
insert- Cells to be labeled with mAb Ki67 against intracellular antigen, were permeabilised prior to incubation by exposure to 80% cold AR grade ethanol for 10 minutes. They were then washed twice in FACs wash buffer.

Section 2.15.2, page 92, paragraph 1, line 12
insert-To set a baseline, control wells containing fibronectin coating only (without EC), were analysed for adhesion of lymphocytes through fibronectin - VLA-4 interaction. This controlled for lymphocyte adhesion to the fibronectin substrate.

Section 4.7.6.4, page 155, paragraph 1
insert-Several researchers have used anti-PECAM-1 mAb with immunomagnetic beads to purify EC. However, a disadvantage of using this mAb this is that PECAM-1 is present on EC of both small and large vessels. A better choice of mAb is one that detects an antigen that is present on only EC from the microvasculature (refer Section 4.9.3, page 163 paragraph 2).

Section 5.1, page 167, line 13
insert-These are likely to be CD25+ activated T cells, rather than CD25+ T regulatory cells, as in this model the former have been shown to adoptively transfer disease to naïve rats (Spargo et al., 2001).

Section 5.4, page 181 last word on page
replace 'artefactural' with 'artifactual'

Section 6.3.1, line 4
insert- The flow cytometric analysis after 0, 24, 48 and 72 hours ConA stimulation was repeated twice for each antibody listed in Table 6.2. The results for each experiment were similar and comparison between replicated experiments detected inter-experiment variation of less than 5%. The data presented in Table 6.2 are presented as representative.

Section 6.4.3.2, Page 202, paragraph 4 line 2 (end of sentence)
insert- (see section 2.11.2)

Section 7.3.2.1, page 218 paragraph 4 line 4
insert- Cells positive for antigen were identified by localisation of bound antibody, visualised by light microscopy, as visible brown coloured (immunoperoxidase) staining, as described in Section 2.4.3. Individual cells were identified by morphology and by anatomical / histological location.

Section 8.1.8.3, page 247, paragraph 3
insert- Because this is a static assay, it is not possible to comment on the strength of lymphocyte adhesion (ie., differentiate between loose tethering and whether a chemokine-induced transition to firm adhesion has occurred).

Section 7.3.1, page 215, line 8
insert- No statistical difference was detected between lymphocyte adherence in the presence of RPMI compared with isotype negative antibody controls prepared in PBS.

TABLE OF FIGURES

Chapter 1

Review of the literature

This literature review encompasses relevant literature until February 2004.

1.1 Introduction

1.1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a debilitating, chronic inflammatory disorder with many systemic manifestations and a primary focus on the synovial joints. Inflammation of the synovium, bursae and tendon sheaths, with synovial cell proliferation and infiltration by inflammatory cells leads to progressive erosion of articular cartilage and bone, with resultant loss of joint function. Clinical manifestations of RA are heterogeneous in nature and can range from a mild arthritis, requiring little treatment, through to a crippling, destructive joint disease with life-threatening involvement of major organs.

Destruction of the bone and cartilage of the joints tends to occur mainly in the early stages of the disease process. In RA, multiple joints are commonly affected in any one individual and the affected joints are typically swollen and painful, with loss of normal function. The pain and loss of function that occurs in early arthritis is caused by inflammation.

RA affects 1-2% of people in Western populations, of all ages and with a higher incidence in females. The earliest evidence of this disease in Europe is documented in early 17^{th} century art. The first case report was documented in 1676, but, the disease was not recognised until 1859. From examination of skeletal remains from Native American tribes of North America, RA has been present in the population for thousands of years. In this group, the incidence of RA is high affecting up 5% of individuals (Firestein, 2003).

Because auto-antibodies have been observed in patients with RA, it has been considered an

autoimmune disease. The first evidence of this putative "self-reactivity' was the early discovery of rheumatoid factor (RF), which is present in about 80% of individuals with the disease. The first autoantibody associated with RA was described by Waaler in 1940. Almost 20 years after its initial discovery, this antibody was designated as 'RF' and found later to bind to the Fc portion of immunoglobulin G (Migrom, 1988; Firestein, 2003). Since this time, a number of other autoantibodies for RA have been detected, including anti-perinuclear factor, anti-Epstein-Barr nuclear antigen, RA33, anti-Sa, anti-p68 anti-keratin antibodies and anti-collagen antibodies (Smolen and Steiner, 2001).

Genetic factors have been implicated in the development and severity of RA. The presence of a unique amino acid sequence in some alleles of the MHC Class II antigens, HLA-DRB1*0404 and HLA–DRB1*0401 (MacGregor *et al.*, 1995; Lanchbury, 1992) confers a predisposition to the disease. An individual diagnosed with early RA, who has an elevated rheumatoid factor auto-antibody titre, accompanied by a genetically defined predisposition, is likely to suffer a more severe form of RA (MacGregor *et al.*, 1995). However, even in the genetically susceptible individual, a specific trigger (either an exogenous or endogenous) appears to be necessary to initiate the onset of autoreactivity. As with most autoimmune diseases, the initiating trigger is unknown (Davidson and Diamond, 2001).

The widespread inflammation, characteristic of RA, is the result of infiltrating leucocytes, recruited from peripheral blood. These cells migrate through endothelial intercellular junctions. The process of recruitment and migration involves many steps. This multi-step cascade of events is mediated by the cell adhesion molecules (CAMs) and their ligands, which are expressed by activated ECs and leucocytes. The following discussion will explore what is known about the events that precede and orchestrate inflammation in arthritis.

1.2 Synovium

1.2.1 Normal synovium

Synovium can be defined as a nonadherent, soft connective tissue that lines the closed spaces of diarthrodial or synovial joints, tendon sheaths and bursae (Henderson and Edwards, 1987). It is composed of two layers, the intima and subintima. The most superficial cellular layer is the synovial intima. The deeper subintima is composed of relatively compact loose connective tissue with an associated vascular plexus (Edwards, 1994). Below the subintima, the subsynovium is gradually replaced by a more loosely organised connective tissue (Firestein, 1994).

The appearance of the intimal layer of synovium is variable and in some cases it may be absent. If present, the intima appears as one or more layers of flattened or cuboidal cells. Unlike epithelial linings, both basal lamina and tight junctions are absent. However, like epithelium, some polarisation is evident in cells from normal tissue. For example, nuclei are located basally and cytoplasmic processes are evident on the apical surface (Barland *et al.*, 1962). The intima is composed of two cell types, known as type A (that constitute 20-30% of the intimal cells) and type B (making up the remaining 70-80% of cells in this layer). Type A cells have been identified as macrophage-like cells of haematopoietic origin, and type B cells are specialised fibroblast-like cells derived from connective tissue (Barland *et al.*, 1962; Roy and Ghadially, 1967). These intimal cells are also referred to as synoviocytes (Edwards, 1994).

The synovial FB-like cells of the intima exhibit several specialisations compared with other fibroblasts (FB), including high levels of the enzyme uridine diphosphoglucose dehydrogenase (UDPGD). This enzyme is not expressed strongly by the FBs of the subintimal layer or by chrondocytes (Wilkinson *et al.*, 1992). The intimal FB cells are believed to produce normal synovial fluid, as UDPGD is involved in hyaluronic acid synthesis, a component of synovial fluid (Edwards, 1994).

Synovial type A cells express many of the markers associated with normal tissue

macrophages. These include markers of non-specific esterase activity, tartrate labile acid phosphatase activity, CD14 (commonly expressed on newly recruited macrophages), CD68 (a lysosome-associated protein), high-affinity immunoglobulin Fc receptors (FcRI) and a number of other macrophage markers (Edwards, 1994). However, they do not express CD36 (personal observation). In the diseased joint, cell populations of the intima undergo complex changes, involving modification of function and increases in both size and number (Broker *et al.*, 1990). For example, UDPGD activity is reduced or absent from synovial cells and chondrocytes from inflamed joints, compared with levels detected in normal cells.

1.2.2 Classification of normal synovium

Synovial tissue has been classified into three main categories, according to the type of tissue that it overlies. These are areolar, adipose and fibrous synovia. Areolar synovium is described as a mobile fibrous surface sheet over a deeper layer of areolar connective tissue (CT), also called loose CT. Adipose synovial surfaces are found where fat pads lie directly adjacent to the synovial cavity, and fibrous synovium overlies the dense connective tissue of underlying ligaments and tendons. These are the three main types of synovium, but in some instances the intima may lie on other tissues such as muscle, bone or cartilage or a combination of tissue types (Edwards, 1995).

1.2.3 Normal synovial vasculature

The synovium and its vasculature provide nutrition, via the synovial fluid, for the avascular cartilage (Edwards, 1994). Beneath the synovial surface is a microvascular network (Davies and Edwards, 1948; Wilkinson and Edwards, 1989). Capillaries are present within and adjacent to the intima and vary in type, consisting of a mixture of fenestrated and continuous capillaries (Suter and Manjo, 1964). The number of capillaries in normal synovium is reported to decrease and the tissue becomes more fibrotic with age. The density and distribution of the vasculature within synovial tissue shows variability between different joints, as well as within each individual joint and this depends on the nature of the synovium present. For example, areolar synovium is highly vascular, with capillaries tending to lie superficially, whereas fibrous synovial surfaces are less vascular (Wilkinson

and Edwards, 1989).

1.3 Endothelium

1.3.1 Historical perspective of endothelium

In the 1800s, von Recklinghausen hypothesised that cells lined the network of blood vessels and that blood vessels were not the simple 'tunnels' described by Malphigi in the 1600s. Initially the lining was thought to be inert, with primary roles in physical separation of blood and tissue, as well as vessel wall permeability. It was not until the 1950s that the ultrastructure of EC was revealed by Palade (1953), and a few years later the details of physiological EC functions were revealed by Gowans (1959). These studies provided the foundations for subsequent insights into the dynamic and heterogeneous nature of endothelium and its important secretory, synthetic, metabolic and immunological functions (Cines *et al.*, 1998).

EC were among the earliest cell types to be cultured *in vitro*. Although not the first to be cultured, an early method for the culture of HUVECs was reported by Maruyama in the early 1960s (Kumar *et al.*, 1987). The earliest microvascular EC cultures were derived from white matter of the CNS, where large vessels are restricted to the grey matter and other cells from within this tissue did not survive *in vitro* (Phillips *et al.*, 1972; Bowman *et al.*, 1979; 1981; Kumar *et al.*, 1987).

1.3.2 Endothelial cell function

EC line the vessels of every organ system and regulate the exchange of nutrients, biological substances and blood cells. EC are multifunctional cells that have a broad range of roles that are fundamental to physiological and pathophysiological processes. The role of endothelium as a simple permeability barrier is overshadowed by its multifunctional paracrine and endocrine capacity (Sumpio *et al.*, 2002).

The cardiovascular system is among the first systems to develop and become functional during embryonic development. EC arise from angioblasts or hemangioblasts and the latter can also differentiate into haematopoietic cells. The two processes responsible for the formation of new blood vessels are vasculogenesis and angiogenesis. Vasculogenesis occurs during the process of embryogenesis. Angiogenesis however, occurs in both embryonic development and postnatally in such process as wound healing, inflammation, ischaemia, reproductive organs (female), and pathologies (Pepper, 1997). EC have multiple roles in vascular homeostasis (Pearson, 2000) as detailed in Table 1.1 and the numerous compounds that are produced by the EC are detailed in Table 1.2 (Vapaatalo and Mervaala, 2001).

The role of endothelium as 'gatekeeper' between the blood and tissues is mediated by the presence of numerous surface receptors that facilitate the activities of molecules such as growth factors, coagulation factors, lipid transporter molecules (lipoproteins), metabolites, hormones and specific junctional proteins involved in EC interactions with cells or extracellular matrices (Kirkpatrick *et al.*, 1997).

Highly reactive in character, ECs play a vital part in maintaining the dynamic state of balance in normal vascular physiology. Disturbance of this balance, which is strictly controlled under normal physiological conditions, may result in vascular pathology. Major areas in which EC have a vital homeostatic role include haemostasis, modulation of vascular tone, growth-regulatory signalling and regulation of leucocyte migration in inflammatory responses (Cines *et al.,* 1998; Kirkpatrick *et al.*, 1997).

The quiescent endothelium is an efficient anti-thrombogenic surface. This state can change rapidly. For example, procoagulant activity can be evoked by pro-inflammatory cytokines and toxins of bacterial origin (Kirkpatrick *et al.*, 1997). Vasoactive substances produced by ECs, blood cells, plasma cascade systems and smooth muscle cells are important regulators of vascular tone. In vessel walls, EC and smooth muscle cells produce

Table 1.1 Summary of EC role in regulation of vascular homeostasis

Control of....
vessel wall growth, development and differentiation
vascular tone, regional blood flow and blood pressure
leucocyte traffic to extravascular tissues
solute flux and fluid permeability across vessel walls
platelet adhesion and aggregation
blood coagulation
fibrinolysis

(from Pearson, 2000).

Table 1.2 Summary of the major endothelial derived factors. These agents have vasoactive, hemostatic, growth modulating or inflammatory actions.

Vasodilators	**Vasoconstrictors**
Nitric oxide	Endothelin
Prostacyclin	Angiotensin II
Endothelium derived hyperpolarising factor	Endoperoxide
C-naturetic peptide	Thromboxane
Antithrombotic	**Prothrombotic**
Tissue type plasminogen activator	Tissue type plasminogen inhibitor
Prostacyclin	Thromboxane A2
Nitric oxide	Tissue factor
	von Willebrand factor
Growth inhibitors	**Growth promotors**
Nitric oxide	Superoxide radicals
Prostacyclin	Endothelin
C-naturetic peptide	Angiotensin II
	β fibroblast growth factor
	Vascular endothelial growth factor receptor
	Epidermal growth factor
	Platelet derived growth factor
Inflammation inhibitors	**Inflammation promotors**
Nitric oxide	Superoxide
	Other free radicals
	TNF-α
	IL-1

Adapted from Vapaatalo and Mervaala (2001); David and Harrison (1992)

growth factors eg. VEGF and growth inhibitory factors. Under normal physiological conditions, synthesis of growth inhibitory factors predominates. However, cytokines such as TNF-α can stimulate the production of mitogenic factors like basic fibroblast growth factor, and platelet derived growth factor (Kirkpatrick *et al.*, 2000).

As discussed above, EC function in a multifactorial manner, producing and interacting with numerous molecules associated with physiological and pathophysiological processes. Only those factors or molecules involved with the process of inflammation will be discussed further.

1.3.3 Endothelium and the inflammatory response.

The endothelium is an important participant in inflammatory responses, through interactions between EC and leucocytes. The endothelium provides a physical interface for interaction with leucocytes and produces its own proinflammatory agents, which include IL-1β; IL-6, IL-8 and platelet activating factor (PAF) (Warner *et al.*, 1987; Sironi *et al.,* 1989; Kirkpatrick *et al.,* 1997). The cascade of the events in the process of leucocyte margination, adhesion and migration into the extravascular space have long been recognised. However, the detail of mechanisms that control this process have been understood only recently. A major breakthrough in this understanding was the discovery of CAMs that are expressed on EC and their ligands on circulating leucocytes. A number of different CAMs are involved in homotypic interactions between EC, while heterotypic interactions mediate interactions between EC and circulating blood cells at the luminal surface and between EC basement membrane components at the subintimal surface.

1.3.4 Endothelial cell culture

Following the development of techniques to culture EC, numerous *in vitro* studies of EC have been undertaken (Jaffe *et al.,* 1973, Gimbrone *et al.*,1974, Lewis *et al.*, 1973). While much has been learnt using cultured EC, several important issues have come to light with respect to the validity of direct comparison between the functions of EC *in vitro* and *in vivo*. The *in vivo* phenotype of EC is plastic and under *in vitro* culture conditions the cells are

also able to undergo phenotypic and functional changes (Cines *et al.*, 1998; Grant *et al.*, 1989). This is important, especially in consideration of microvascular EC, where phenotypic characteristics are linked closely to the tissues from which the cells are isolated.

Much of the *in vitro* work carried out to investigate the functions of EC have utilised cells harvested from human umbilical vein (HUVEC). HUVECs are a useful tool, as human umbilical cords are readily available and provide a source from which EC can be obtained reproducibly without the need for invasive and ethically unacceptable methods. However, extrapolation of experimental data and comparisons between the characteristics of umbilical vein EC and microvascular EC has been questioned. This large vessel is not usually exposed to the pathological processes that occur more commonly in the smaller vessels of the systemic vasculature (Cines *et al.*, 1998).

1.3.5 EC heterogeneity

Morphological and functional differences in endothelium from different vascular beds have long been recognised (Auerbach *et al.*, 1995). For instance, differences in permeability have been correlated with structural differences in the cells and in their intercellular junctions. Such specialisations are apparent in the CNS and retina, where a closed endothelium helps maintain the blood-brain-barrier. By contrast, in the liver discontinuous endothelium in the sinusoids allows percolation of blood macromolecules through the extravascular compartment of this organ. Sinusoidal endothelium also allows blood cells to easily penetrate the interstitial spaces of the spleen and bone marrow. Intermediate examples are the selectively permeable fenestrated endothelium found in endocrine glands and the kidney (Dejana, 1996).

More subtle heterogeneity has been recognised recently with respect to the proteins expressed by EC from various parts of the vasculature. The commonly used EC marker, von Willebrand factor, is not expressed uniformly by EC from all vascular beds or from various parts of individual vascular beds. Importantly, microvascular endothelium exhibits significant heterogeneity, particularly at the level of surface protein expression

(Kumar *et al.*, 1987; Cines *et al.*, 1998; Gumowski *et al.*, 1987). Phenotypically distinct EC populations have been identified within individual organs. For example, in the human liver, sinusoidal EC in the periportal region express PECAM-1 and CD34, whereas those from the intrahepatic region do not express these molecules (Morin *et al.*, 1984).

Comparisons of microvascular EC and HUVECs indicate functional differences in the responsiveness of the cells to pro-inflammatory stimuli. ICAM-1 expression has been compared in monolayers of HUVECs and synovial ECs from rheumatoid tissue samples. Although similar initially, the application of IFNγ brought about a significant increase of ICAM-1 expression in the EC from synovium but not in EC from HUVECS (Gerritsen *et al.*, 1993).

Under *in vitro* culture conditions, the surface protein phenotype of HUVECs changes. In contrast, the surface proteins of microvascular EC appear to maintain more of their tissue specific phenotypic characteristics (Grau *et al.*, 1997). Microvascular and macrovascular EC, also differ in their growth rates, growth requirements, and their ability to form capillary-like tubes *in vitro*. The expression of CAMs varies markedly on EC from different tissue and different vascular beds (Ades *et al.*, 1992). Although diverse surface phenotypes have been identified, few of the factors that control these have been defined. In the future, the elucidation of these as yet unknown controlling factors will be important in allowing experimentation on EC with stable expression of differentiated characteristics and associated consistency in responsiveness to inflammatory stimuli.

Because migration of lymphocytes involves interactions with ECs of the microvasculature rather than EC of the large vessels, this study utlizes EC from small vessels in the synovium of rats with AA.

1.4 The immune/ inflammatory response

1.4.1 Mechanisms of immunity

The immune system involves both cellular and humoral mechanisms. Together, they mediate and amplify responses to invasion by foreign organisms or to tissue injury. In general, this response involves processes of innate and/or acquired immunity. Innate immunity involves physical barriers, physiological components, phagocytic cells of the blood and tissues and the actions of soluble factors in the blood and tissue fluids. Acquired immunity is characterised by antigen specific defence mechanisms that in some cases focus and utilise the machinery of innate immunity to execute antigen specific responses.

The cellular component of immunity comprises the leucocytes of the blood. Bone marrow is the site of haematopoiesis in the adult, where all cells of the blood (or their precursors) are produced, including leucocytes (Erslev and Gabudza, 1979). All leucocytes originate from the pluripotent haematopoietic stem cells (Kennedy *et al.*, 1997), which give rise to myeloid progenitors and the common lymphoid progenitor cell. The myeloid progenitors differentiate to give rise to granulocytes (neutrophils, basophils and eosinophils), and to monocytes (which differentiate into macrophages in the tissues). It is from the common lymphoid progenitor that T and B lymphocytes arise (Roitt, 1993).

The immune system can be divided into 3 separate divisions or compartments. Primary lymphoid organs form the first compartment, where lymphocytes develop from undifferentiated precursors to express antigen specific receptors (Ikuta *et al.,* 1992). Bone marrow and thymus (as well as foetal liver) are regarded as primary lymphoid organs. B cells become functional in the bone marrow and T cells in the thymus. The secondary or peripheral lymphoid organs, including spleen, lymph nodes and mucosa associated lymphoid tissue, make up the second compartment, and are sites where mature lymphocytes interact with foreign antigen. Cells presented with antigen in the secondary lymphatic compartment undergo activation and proliferation. These effector cells will carry out their immune functions in the third and final compartment, the extra-lymphoid tertiary lymphatic tissues (Salmi and Jalkanen, 1997).

1.4.2 T lymphocytes

The thymus is important in the development of a functional immune system because this is where T cell precursors from bone marrow undergo maturation (Miller, 1961; 1962; 1994). This organ provides a suitable microenvironment for selective clonal expansion and deletion (Ritter, 1992). T cells are characterised by the expression of an antigen specific receptor, the cell receptor (TCR). For a functional immune system to develop, positive selection of cells expressing TCR with appropriate MHC affinity and removal of self-reactive cells by negative selection needs to occur (von Boehmer *et al.*, 1989). As a result, less than 5 % of the cells produced each day are ever released. The fate of the rest is apoptosis and phagocytosis by thymic macrophages (Boise and Thompson, 1996; Shortman and Scollay, 1994; Surh and Sprent, 1994).

1.4.3 Autoimmunity

Autoimmunity is the loss of self-tolerance that occurs when an immune response is directed against autologous antigen and causes damage to tissue and /or autoimmune disease (Heath *et al.*, 1997). Most autoreactive lymphocytes are destroyed during lymphocyte development. This occurs in the bone marrow in the case of B cells and thymus in the case of T lymphocytes (Hanahan, 1998). It is likely that some autoreactive T cells will escape deletion, remaining quiescent unless activated by either the loss of suppressive elements (eg. regulatory T lymphocytes), exposure of previously concealed antigen, or expansion of autoreactive T cells due to the presence of infectious agents expressing antigenic epitopes that cross react with the self-antigens (Elson *et al.*, 1995). Autoreactive T lymphocytes that escape deletion are 'potentially' autoreactive and once activated may initiate an immune response causing autoimmune disease.

Autoimmune diseases are influenced by genetic and environmental factors (Theofilopoulis, 1995). Triggers for autoimmune disease may include bacterial or viral infection. For example psoriasis can be triggered by beta-haemolytic streptococcus infection (Valdimarsson *et al.*, 1995). Chronic inflammation can also occur following bacterial or

viral infection, due to inappropriate presentation of self-antigens to T cells by dendritic cells (DC) (Di Rosa and Barnaba, 1998).

1.4.4 Activation of T cells

Upon presentation of antigen, naïve T cells ($CD45RA^+$) undergo activation, proliferation and differentiation. Activated T cells differentiate into $CD45RO^+$ memory cells (Mackay, 1993a) or effector cells. Whereas B lymphocytes possess the ability to recognise epitopes on native antigens via the B cell receptor, T cells express the T cell receptor (TCR) and recognise processed antigens presented by MHC molecules. Processing involves proteolytic cleavage of protein antigen to peptide fragments that are loaded onto major histocompatibility complex molecules (MHC) and presented on the surface of antigen presenting cells (APC). The interaction between MHC and TCR is stabilised by CD4 or CD8, which bind to cognate sites on the MHC (Davis *et al.*, 1998). Signalling via the TCR requires the involvement of the invariant CD3 with its components γ, δ, ε, and ζ:ζ or ζ:η dimmers, CD45 (a transmembrane protein with an intracellular tyrosine phosphatase moiety) and the recruitment of the tyrosine kinase by CD4 or CD8 (Janeway, 1992).

T lymphocytes are divided into subsets, depending whether they express the α/β or γ/δ TCR. Of the latter subset, most express CD8, whereas mature α/β T cells express either CD4 or CD8. As a result of the process of selection in the thymus, CD4+ cells are restricted to recognising antigenic peptides presented by MHC class II molecules, while CD8+ cells are restricted to recognise peptides associated with MHC class I (Swain, 1983). CD4+ cells are referred to as "helper T cells", based on the ability of these cells to assist in the differentiation of B cells into antibody secreting cells (Vidal *et al.*, 1999). CD8+ T cells are also called "cytolytic" or "cytotoxic" T cells. This is in reference to their ability to induce lysis of target cells by the intracytoplasmic deposition of substances such as perforin and granzymes. Some exceptions to these generalisations exist. For example, some CD4+ cells exhibit "cytotoxic" behaviours and CD8+ cells have shown immunoregulatory behaviours (in particular, secretion of IFN-γ) in addition to their cytotoxic functions. T cells can be further divided into functional subsets according to the surface molecules that

they express and their production of cytokines (discussed in Section 1.4.8).

1.4.5 T cell effector cell function

T lymphocytes require three distinct stimuli to become functionally activated. The first is recognition of MHC-associated antigenic peptide via the TCR (Dustin and Springer, 1989). Secondly, they require co-stimulatory signals from the antigen-presenting cell, involving interaction of CD80 and/or CD86 molecules with CD28 on the T cell (Chambers and Allison, 1997). Finally, the activation may require further stimulation by cytokines. Once activated, the cells undergo further differentiation and maturation, to yield either effector T cells or memory function. Engagement by T cells with cognate MHC- associated antigenic peptide alone generally fails to activate T cells and leads to a state of anergy (Janeway and Goldstein, 1992). This has important implications, since self antigens encountered on cells other than APCs will not induce a response to self antigens and may induce anergy (Bretscher and Cohn, 1970).

Upon activation, the T cells enter the G_1 stage of the cell cycle, characterised by an increase in size of up to three times that of the resting cell. These relatively large cells are known as "lymphoblasts". Characteristically they have abundant polyribosomes, high levels of RNA, relatively low levels of endoplasmic reticulum and they are highly mobile (Delorme *et al.*, 1969). Lymphoblasts progress to S phase and then proliferate by cell division.

1.4.6 Antigen presentation in T cell activation

Dendritic cells (DCs) appear to be necessary for the initiation of a primary T cell response. Immature DCs take up antigen and in response to stimulation through pattern recognition receptors, they undergo maturation. Following maturation, they migrate via afferent lymphatics to secondary lymphoid organs (Lane and Broker, 1999; Romani *et al.*, 1989). Chemokines producd constitutively in the tissues, or increased at a site of injury, play a role in the recruitment of immature DCs. Another subset of chemokines is involved in the attraction of mature DCs in to secondary lymphoid organs (Cyster, 1999). In the secondary lymphoid tissues, DC-derived chemokines are important in directing the movement of T

cells and B cells, and also influence immune responses to antigen (Hart, 1997; Lane and Brocker, 1999). In the remainder of this chapter, discussion will focus on the activation of the CD4+ subset.

Naïve T cells interact with DCs in the secondary lymphoid tissues, where proliferation of antigen specific cells occurs and effector cells are produced (Jenkins *et al.*, 2001). Following activation, the CD4+ lymphocytes migrate to the margins of the B cell follicles and play a role in producing a humoral immune response (Garside *et al.*, 1998). Other activated lymphocytes leave the lymph nodes via the efferent lymph (Hall and Morris, 1963). These include effector cells for cell-mediated immunity and memory cells. The latter impart long-lived antigen-specific memory, manifested by more rapid reponse to previously encountered antigen (Mackay, 1993a; Sprent, 1997). Previously activated T cells can be (re)-activated with antigen presented by B cells or macrophages, in contrast to naïve cells that require antigen presentation by DC (Fuchs and Matzinger, 1992).

1.4.7 Recirculation and surveillance by lymphocytes

After release from the thymus and bone marrow, naïve cells migrate between blood and lymphoid tissues to maximise the chance of encounter with antigenic. This lymphocyte recirculation requires the transmigration of lymphocytes from the vascular compartment across the vascular EC lining and into the lymphoid tissue. After movement through lymph node or Peyers patches, lymphocytes re-enter the systemic circulation via the efferent lymphatics. Naïve lymphocytes recirculate continuously between the blood circulation and secondary lymphoid tissues. Following activation, the pattern of recirculation changes considerably (Salmi and Jalkanen, 1997). Effector and memory T cells enter tertiary lymphoid tissues and they exit via lymph nodes and efferent lymphatics to the blood stream. This distribution is not random since extravasation into the tissues is biased, depending on the tissues in which activation has occured. Distinct patterns of circulation appear to exist and these functionally distinct recirculation pathways are peripheral lymph nodes, mucosal associated lymphoid tissue, inflamed skin and synovium (Griscelli *et al.*, 1969; Chin and Hay, 1980; Salmi and Jalkanen, 1997).

The directed traffic of lymphocytes maximises immune surveillance through blood, tissues and lymph by allowing the distribution of previously activated memory and effector cells to sites where they are more likely to encounter cognate antigen (Gowans and Knight, 1964; Pabst and Binns, 1989). Activated T lymphocytes migrate from the blood to sites of inflammation (delivering effector cells to the tissues), while others return to the lymphatic tissues. Ultimately most lymph drains into the thoracic duct (TD) via the efferent lymphatics. Thus cells within the lymph enter the blood via the TD and recirculating cells and newly formed effector cells can be collected by cannulation of the TD and collection of lymph (Bollman *et al.*, 1948; Gowans and Knight, 1964).

Lymphocytes enter lymph nodes and mucosa-associated lymphoid tissues via high endothelial venules (HEVs). HEVs have a lumenal diameter of between 7 and 30μm and are hence classified as post-capillary venules. HEVs are characterised by a taller, cuboidal shaped cells, rather than the flat endothelial lining found in post-capillary venules at other sites (Gowans and Knight, 1964). The lining cells of HEVs are thought to represent a functional adaptation of postcapillary venules that enables a larger influx of lymphocytes into the tissues than can occur through the typical flat endothelial lining (Yednock and Rosen, 1989; Ager, 1987a; Girard and Springer, 1995). They have been observed in some chronically inflamed non-lymphoid tissues, where it is believed that they facilitate lymphocyte migration to inflammatory sites (Girard and Springer, 1995). An example is the synovium in RA (Freemont *et al.*, 1983).

1.4.8 Cytokines

Cytokines are intercellular chemical messengers that are involved with regulation of production, activation and interactions between both non-immune cells and cells of the immune system (Kelso, 1993). At this time there is no international standard for the classification of these molecules. Cytokines can be divided into groups based on their functional or structural characteristics. The families include TNF- related molecules, the interferons (IFN), the chemokines, the interleukins (IL) and haemopoietins (Kelso, 2000).

Helper T cells ($CD4^+$) cells can be further divided into subsets relating to their pattern of secreted cytokines. Their cytokine profiles have led to the division of helper T cells into Th1 or Th2. These patterns of cytokine production were first described in the mouse (Mosmann *et al.*, 1986) and later defined for human T cells (Mosmann and Sad, 1996). T-cells of the Th1 subset produce IFN-δ, IL-2 and lymphotoxin, and those of the Th2 subset produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann and Sad, 1996). Human T cells show similar patterns of Th1 and Th2 cytokines to the mouse, except that the production of IL-2, IL-6, IL-10 and IL-13 differ. In humans, production of these cytokines is not as rigidly restricted to a single T helper subset and additional proteins are produced by both subsets, including IL-3, TNFα, GM-CSF, [met]enkephalin and some chemokines (Mosmann and Sad, 1996).

In immunopathology, Th1 cells are generally involved in the regulation of delayed–type hypersensitivity and Th2 cell are involved with immediate-type allergic responses and antibody isotype switching (Mosmann and Sad, 1996). A Th1 cytokine bias is detected in RA synovium and in some animal models of autoimmunity. These include experimental autoimmune encephalomyelitis (EAE), experimental immune uveoretinitis (EIU) and insulin-dependent diabetes mellitis (IDDM) (Miossec and van den Berg, 1997; Mosmann and Sad, 1996). In these animal models of autoimmunity, Th2 cells have been shown to produce cytokines that have a suppressive action on disease activity, whereas the Th1 subset is implicated in initiation of disease (Miossec and van den Berg, 1997). The role of cytokines in RA is discussed in Section 1.7.5.

1.5 Cell adhesion molecules

Cell adhesion molecules are involved in the regulation of a diverse range of physiological and pathological processes. These processes include leucocyte traffic, cancer metastasis, immune and inflammatory responses, wound healing, thrombosis, fertilisation and embryogenesis (Faull, 1995). Adhesive interactions are central to the functions of the immune system throughout life (Springer, 1990).

The components and steps involved in the process of adhesion and leucocyte migration in inflammation have been reviewed comprehensively by a number of authors. Those surveyed for the purposes of this study include Yednock and Rosen (1989), Springer (1990), Springer and Laskey, (1991), Butcher (1991), Issekutz (1992), Hogg (1992), Lasky (1992), Picker (1992), Shimizu *et al.,* (1992), Bevilacqua and Nelson (1993), Hogg and Landis (1993), Bevilacqua (1994), Granger and Kubes (1994), Imhof and Dunon (1995), Bradley and Watson (1996) and Westerman *et al.*,(2001).

1.5.1 The multistep process of adhesion

CAMs and their ligands mediate the active, multistep process in the adhesion and migration of circulating leucocytes from peripheral blood into synovium (Figure 1.1). Various CAMs and ligands are upregulated on activated lymphocytes and activated ECs (Springer, 1990). The steps of extravasation are sequential but overlapping. This overlapping or redundancy ensures transmigration of T cells to the perivascular regions in many tissues, although individual CAMs may be responsible for the bias in entry into individual tissues. This redundancy may mean that notional therapies that target the activity of a key CAM may not be adequate to abolish T cell transmigration to specific target tissues (Westermann *et al.*, 2001).

Margination of leucocytes is followed by the rapid, transient interactions known as 'tethering' (Hogg and Landis, 1993; Butcher, 1991; Springer, 1991). This transient adherence is unstable under the flow conditions in the vessels (Picker, 1994), but the 'tethering' induces leucocytes to roll along the vessel wall in the direction of flow (Lawrence and Springer, 1991; Ley *et al.,* 1991). This rolling phase allows the cells to "sample" the chemoattractants, cytokines and/or other molecules associated with the EC lining.

1.5.2 Activation of integrins during rolling

Rolling may be followed by firm adhesion (Figure 1.1) and this results in arrest of the

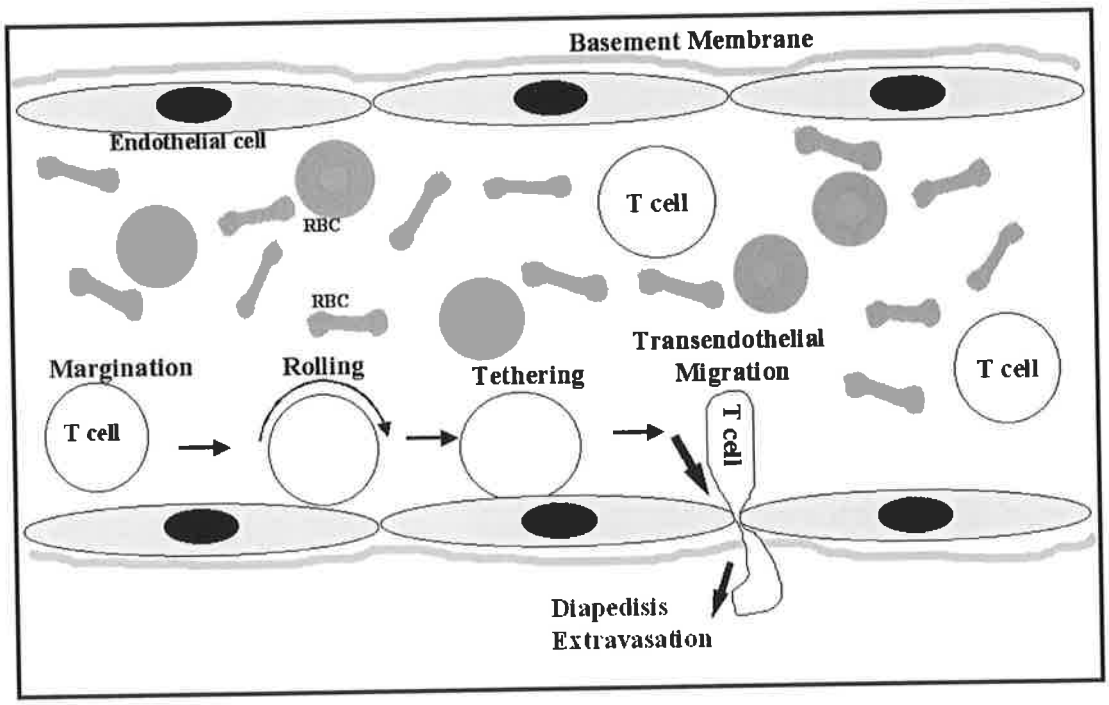
leucocyte on the EC surface (Springer, 1994). For firm or secondary adhesion to take place, the leucocyte must be 'triggered' by 'specific activating stimuli' (Picker *et al.,* 1994). Activation of the leucocyte occurs in response to specific chemokines and cell contact-mediated signals, which activate secondary CAMs (Butcher, 1991). The CAMs activated are members of the integrin family, such as $\alpha4\beta7$, $\alpha4\beta1$ and β_2 (CD18) integrins (Baggiolini, 1998). Interactions via these activated integrins bind the leucocyte firmly to counter receptors on the endothelium (Hynes, 1992). If this activation does not take place, secondary adhesion will not occur and the leucocyte is swept into the larger venules and returned to the circulating blood (Picker *et al.*, 1994).

A large number of chemoattractant / activating factors have been defined that activate the binding properties of CAMs on leucocytes (Butcher, 1991). These factors have been classified recently as ligands, belonging to structurally related chemokine superfamilies that have prefixes CXC, CC, C and CX_3C (Zlotnik and Yoshie, 2000). Activating factors include IL-8 (CXCL8), platelet activator 4 (CXCL4), RANTES (CCL5), HuMIPα (CCL3) HuMIPβ (CCL4), I-309 (CCL1), protein-1 (CCL2), platelet activating factor, leukotriene B4, IL-2 (Butcher, 1991; Szekanecz *et al.,* 2003). Cell contact mediated by E-selectin or CD44 can also activate integrins (Butcher, 1991).

On circulating leucocytes, the integrins are present in an inactive form, so that for adhesion to occur, rapid *in situ* activation must take place (Grabovsky *et al.,* 2000). Chemokines function to augment the reversible lymphocyte tethering and arrest of the leucocyte on inflamed endothelium (on the EC apical surface). This adhesion is promoted by mechanical shear forces that enhance the process of Gi protein mediated response to chemokines (Cinamon *et al.*, 2001; Grabovsky *et al.,* 2000). This leads to conformational changes at the "leucocyte-substrate contact zone", resulting in enhanced leucocyte avidity for binding to their respective CAMs (Grabovsky *et al.*, 2000).

Figure 1.1 A schematic representation of the multistep process of adhesion and migration of circulating lymphocytes from peripheral blood into tissues.

The process of adhesion and migration involves a number of cell adhesion molecules and their ligands, that interact in a cascade of events. Margination is followed by the 'tethering' of lymphocytes to EC lining the vessel wall. This adherence is transient and it induces rolling of lymphocytes along the vessel wall in the direction of flow. During this part of the cascade, 'activation' or 'triggering' of the lymphocyte may occur, depending on the presence of specific chemokines and cell contact-mediated signals. Triggering, through conformational change, increases the binding affinity of leucocyte cell surface integrins. This leads to firm adhesion and arrest of the leucocyte, a necessary precursor to transmigration, diapedesis or extravasation. Extravasation occurs between EC, allowing the leucocyte to enter the extra cellular matrix of the surrounding tissue. It probably involves interaction of leucocyte CAMs with molecules in the junctional regions of adjacent EC, and interaction with the extracellular matrix via the other CAMs and by the release of enzymes that degrade the adjacent matrix.


Basement Membrane
Endothelial cell
RBC
RBC
T cell
Margination
Rolling
Tethering
Transendothelial
Migration
T cell
T cell
T cell
Diapedisis
Extravasation

These Gi protein receptors thus mediate the induction of conformationally active "neoepitopes", which facilitate the activation of integrin adhesiveness (Tanaka *et al.*, 1993). In this process, the chemokines regulate integrins in a coordinated way which is integral to the sequential process of transmigration (Weber *et al.*, 1999).

1.6 Classification of the cell adhesion molecule families.

Most of the CAMs have been classified into five families, based on their structure. The known adhesion molecule families are the selectins, the integrins, the immunoglobulin superfamily, (Springer, 1990), sialomucins and proteoglycans (Salmi and Jalkanen, 1997). Members of the selectin family mediate transient interactions that allow tethering, but not firm adhesion (Bevilacqua *et al.*, 1994). The lectin-like nature of the selectins means that their ligands are carbohydrate structures that may be lactosamine, polysaccharides, oligosaccharides or sialomucins (Salmi and Jalkanen, 1997). Successful firm adhesion under flow conditions and subsequent migration requires involvement of immunoglobulin superfamily members and their ligands, the integrins (Springer, 1990).

1.6.1 Selectins

The selectin family of CAMs mediates the initial tethering of leucocytes to cognate ligands expressed either constitutively by EC, or upregulated by activation of EC. The transient and reversible tethering induces rolling of leucocytes along the endothelial lining and greatly slows the transit of leucocyte through the vessels (Butcher, 1991; Bevilacqua *et al.*, 1994; Ley and Tedder, 1995).

Members of the selectin family are structurally related Ca^{2+}-dependent adhesion receptors that recognise and bind carbohydrate ligands (Bevilacqua *et al.*, 1994). E-selectin, P-selectin and L-selectin have been identified as the three members of this family (Bevilacqua *et al.*, 1994; Shimizu *et al.*, 1992). E-selectin was formerly known as endothelial-leucocyte adhesion molecule-1 (ELAM-1). P-selectin was studied initially in platelets, where it was known as platelet activation-dependent granule external membrane protein (PADGEM), granule membrane protein 140 (GMP-140) or CD62. The third member of this family, L-

selectin, was identified first by mAb MEL-14 (Gallatin *et al.*, 1983) and is also known as peripheral lymph node homing receptor (Picker, 1994), peripheral lymph node addressin (PNA), LECAM, peripheral lymph-node homing receptor or LAM-1 (Issekutz, 1992; Shimizu *et al.*, 1992).

The term selectin relates to the lectin domain that is present in all members of this family. Each contains an amino terminal C-type lectin (sugar-binding) domain, an epidermal growth factor-like domain, between two and nine short consensus repeats, a transmembrane segment and a short C-terminal cytoplasmic domain (Salmi and Jalkanen, 1997; Imhof and Dunon, 1995; Lasky, 1992). The main difference between E-, P- and L-selectin lies in the individual arrangement of their short-consensus repeats (2, 8-9, and 6 respectively). The cellular distribution of individual members of this family is distinct. E-selectin is expressed by endothelium, P-selectin is expressed by platelets and endothelium, while L-selectin is expressed by all mature leucocytes, with the exception of some activated and memory lymphocytes (Bevilacqua and Nelson, 1993; Salmi and Jalkanen, 1997; Cronstein and Weissmann, 1993). E-selectin promotes adhesion of neutrophils, monocytes, eosinophils and some memory T lymphocytes to vascular endothelium. It is expressed by EC in response to factors such as bacterial endotoxin, IL-1 and TNF, and was initially characterised on HUVECS (Pober et. al, 1986). Maximal expression is reached 4-6 hours after stimulation and expression returns to basal levels by 24-48 hours (Bevilacqua *et al.*, 1994).

P-selectin is synthesised in ECs and is present in the storage granules known as Weibel-Palade bodies. This molecule is also associated with α-granules in resting platelets. P-selectin is expressed rapidly on the surfaces of these cells after activation by mobilisation from its storage granules to the cell surface (Salmi and Jalkanen, 1997; Cronstein and Weissmann, 1993). Synthesis of P-selectin is induced by the cytokines IL-1 and TNF. Inflammatory mediators such as thrombin, histamine, certain complement components and H_2O_2 can induce rapid surface expression of P-selectin (Bevilacqua and Nelson, 1993; Salmi and Jalkanen, 1997).

L-selectin is expressed on the cell surface of most leucocytes (circulating lymphocytes, neutrophils, and monocytes). However, it is shed from lymphocytes and neutrophils following their activation (Bevilacqua, 1993). The ability to shed L-selectin is thought to be a mechanism that allows detachment of leucocytes following adhesion. This adhesion molecule plays an important role in lymphocyte homing to secondary lymphoid tissues (Picker and Butcher, 1992; Gallatin, 1983; Cronstein and Weissmann, 1993), in neutrophil extravasation during acute inflammation and it participates in adhesion of neutrophils, monocytes and lymphocytes to activated endothelium (Bevilacqua, 1993).

1.6.2 Selectin Ligands

There are a number of carbohydrate ligands that are bound via the lectin domains of the selectins (Imhof and Dunon, 1995). Individual selectins bind to a number of synthetic and natural carbohydrates. The natural ligands are oligosaccharides which often contain sialylated, and /or fucosylated lactosamines, related to the isomers sialyl-Lewis x (sLe^x) and sialyl-Lewis a (sLe^a) (Springer and Lasky, 1991; Hogg, 1992). The oligosaccharides comprise a terminal sialic acid linked to galactose, which in turn is linked to an N-acetylglucosamine (GlcNAc), [sLex; Neu5Ac alpha 2-3Gal beta 1-4(Fuc alpha 1-3)GlcNAc-] (Lasky, 1992; Bevilacqua and Neslon, 1993; Salmi and Jalkanen, 1997). The isomer sLe^x and other fucosylated lactosamines are found in abundance on circulating neutrophils and monocytes and on a small subset of blood lymphocytes. Its structural isomer sLe^a is not usually expressed by blood leucocytes but it is expressed by certain cancer cells, hence it is likely to have a role in metastasis (Bevilequa and Nelson, 1993).

The mucin-type molecule, P-selectin glycoprotein ligand 1 (PSGL-1) is an important selectin ligand (Huo and Ley, 2001; Salmi and Jalkanen, 1997). All members of the selectin family can bind to PSGL-1 (Goetz *et al.*, 1997). However, the affinity of binding varies between members of the selectin family members. Furthermore, CD62P does not necessarily bind to all cells that express PSGL-1. This is due to differences in glycosylation and/or tyrosine sulphation of the core protein on activated cells. In the context of T

lymphocytes, CD62P will bind only to activated but not to resting cells. (Vachino *et al.*, 1995; Salmi and Jalkanen, 1997). Additional selectin ligands have been proposed but their role in the pathophysiology of inflammation remains unclear (Kansas, 1996). L-selectin may itself be a ligand for both P-selectin and E-selectin (Hogg, 1992; Rosen and Bertozzi, 1994).

Lipid-bound sugars have also been investigated as possible selectin ligands. The ligands contain sulfated glycosphingolipids and sulfoglucuronyl-containing neolactosylceramides (SGNL lipids). One of these is recognised by an antibody (HNK-1), raised against human natural killer cells. This antibody recognises SGNLs present on vascular ECs and nervous tissue that are ligands for both L- and P-selectin, but not E-selectin (Hogg, 1992; Imhof and Dunon, 1995; Needham and Schnaar, 1993).

Most haematopoietic cells express L-selectin (CD62L) at some stage of their differentiation. Naïve T cells express CD62L, but they shed the molecule following activation (Tedder, 1995; Tedder *et al.*, 1989; Hogg, 1992). L-selectin ligands include GlyCam-1, CD34, and MAdCam-1. Glycosylation-dependent cell adhesion molecule (GlyCam-1) and CD34 are Ca^{2+} dependent ligands of L selectin, while MAdCam-1 is also a ligand for $\alpha_4\beta_7$ integrins. GlyCam-1 is expressed by HEVs in lymph nodes, where it is involved in lymphocyte homing (Salmi and Jalkanen, 1997; Lasky, 1992). GlyCam-1 can also bind to E-selectin, but acts as an inhibitory modulator of cell adhesion (Hemmerich *et al.*, 1995).

CD34 is a vascular sialomucin that is expressed as a transmembrane cell-surface protein by EC in a range of blood vessels and on haematopoietic progenitor cells (Baumhueter *et al.*, 1993). Expression is located on the luminal aspect of EC in small blood vessels and its expression can be altered by exposure to cytokines. IL-1, IFNγ, and TNFα down-regulate expression of CD34 on EC at concentrations that up-regulate expression of selectins and integrins (Delia *et al.*, 1993). CD34 is, therefore, believed to play a role in the recirculation of leucocytes under normal conditions, rather than during inflammation. For instance, in

patients with skin lesions caused by graft versus host disease, there is massive lymphocyte infiltration in the presence of low expression of CD34 but high expression of E-selectin (Norton *et al.,* 1993).

MAdCam-1 is a transmembrane protein of the sialomucin family, that functions as a mucosal addressin (Bevilacqua, 1993; Salmi and Jalkanen, 1997). MAdCam-1 is found on EC of the HEV localised in Peyer's patches, on non-lymphoid venules in the lamina propria of the small intestine, by cells lining the marginal sinus of the spleen and in HEV of foetal lymph nodes (Streeter *et al.*, 1988a; 1988b). It plays a role in lymphocyte homing to Peyer's patches by acting as a ligand for both L-selectin and α4β7 integrin (Bevilacqua, 1993; Picker, 1992; Imhof and Dunon, 1995; Ley and Tedder, 1995).

E-selectin ligands have been identified as a series of antigens located on leucocytes that accumulate in skin. These are cutaneous leucocyte antigens (CLA), a 250-kDa glycoprotein (Picker *et al.*, 1993; Picker *et al.*, 1990), and the sialyl stage-specific embryonic antigen (SSEA-1). CLA is a molecule that is highly sialylated. It has been recognized on a population of memory lymphocytes that migrated to inflammatory lesions of the skin, where E-selectin is expressed by EC within the lesions (Picker *et al.*, 1991; Imhof and Dunon, 1995). The sialomucin PSGL-1 on lymphocytes is also an E-selectin ligand and it appears to have a role *in vivo* in traffic of lymphocyte into site of inflammation (Hirata *et al.*, 2000).

In ruminants, the 250-kDa E-selectin ligand has been located on T cells that express γ/δTCR. Induction of E-selectin expression by TNFα in skin microvascular vessels results in recruitment of γ/δ T cells to the skin. Binding of these cells to E-selectin is not inhibited by antibodies against either CLA or sLex (Walchek *et al.*, 1993), suggesting the existence of additional ligands. E-selectin also interacts with a variant 150-kDa variant of the fibroblast growth factor receptor, E-selectin ligand-1 (ESL-1). This molecule is widely expressed, but it is only active as an E-selectin ligand in its fucosylated form (Levinovitz *et al.*, 1993).

1.6.3 Integrins

The integrins are a family of CAMs that have biological roles as diverse as embryonic development, maintenance of tissue integrity and leucocyte homing. Each integrin molecule is a heterodimeric protein that comprises an α and ß subunit, both of which are transmembrane glycoproteins (Hynes, 1992). Up to 15 α- and 8 ß-chains are known to exist, and at least 21 different heterodimers have been identified. Of these, leucocyte populations express 13 different integrins and 6 are reported to be important in leucocyte-endothelial interactions. The integrins involved with leucocyte-endothelial interaction are members of the subfamilies ß1, ß2 and ß7 (Albelda and Buck, 1990; Salmi and Jalkanen, 1997; Imhof and Dunon, 1995). These include the ß1- integrin VLA-4, ß2- integrins LFA-1, Mac-1, and p150.95 and the ß7 integrin, α4ß7 (Salmi and Jalkanen, 1997).

The cations Ca^{2+} or Mg^{2+} are essential for integrin function, and can affect the affinity and specificity for ligands (Hogg, 1992). The ligand specificity of ß1 and ß2 integrins is also dependent on the associated α-chain. This is illustrated in the β1 family, where α5ß1 is a receptor for fibronectin, α6ß1 binds to laminin and α4ß1 binds to fibronectin and VCAM-1 (Delfilippi *et al.*, 1992; 1991). Biosynthesis, assembly and transportation of the alpha chain regulates cell surface expression of a particular integrin and this can be altered rapidly by the presence of inflammatory cytokines such as TNFα. This is a useful property for molecules involved in the arrest and adhesion of circulating leucocytes on vascular endothelium, where activation of the leucocytes by certain stimuli (Section 1.6.2) is followed by transendothelial migration and de-adhesion once the process of extravasation is complete. Cell to cell interaction can also bring about an increase in affinity of integrins for their ligands. For example, CD31 (PECAM) transduces activating signals to T cells when they interact with vascular endothelium. This in turn activates integrins expressed on the leucocyte cell membrane (Tanaka *et al.*, 1992). This type of activation of integrins may also be induced by interaction of certain chemokines with their transmembrane receptors. An important mechanism of integrin function appears to be the ability to rapidly up-regulate from a low-affinity (non-adhesive) state to a transient high affinity state (Springer, 1994;

Shimizu *et al.*, 1992)

1.6.3.1 Leucocyte adhesion deficiency

In nature there are two rare examples of genetic defects of cell adhesion molecules. These are leucocyte adhesion deficiency I and II (Salmi and Jalkanen, 1997; review). Type I deficiency is due to multiple genetic defects that prevents synthesis, pairing and expression of the β2 integrin common chain. The importance of the ß2 integrin subfamily can be illustrated in these immuno-compromised individuals who suffer from congenital leucocyte adhesion deficiency I (LAD-1) due to mutations in the common ß2 subunit (Hogg, 1992; Salmi and Jalkanen, 1997; Cronstein and Weissmann, 1993).

These individuals suffer from recurrent infections, because leucocytes lack the ability migrate in response to chemoattractants. The leucocytes appear to roll but do not adhere to EC (von Andrian *et al.*, 1990). They are, therefore, unable to extravasate through the endothelium to sites of infection (Etzioni *et al.*, 1992; Bunting *et al.*, 2002; Springer, 1990). This renders granulocytes unable to extravasate, but lymphocytes can still accumulate due to an alternative pathway, relying upon VLA-4-VCAM-1 interaction for transmigration (Vennegoor *et al.*, 1992). Complete lack of β2 leads to death in early postnatal life (Anderson *et al.*, 1985). The second syndrome arises from a fault in fucose metabolism, such that selectin ligands present are inappropriately fucosylated. Rolling is impaired in this syndrome (von Adrian *et al.*, 1990).

1.6.3.2 ß1 integrins

The ß1 sub-family of integrins includes structures that contain the ß1 chain with a variable alpha subunit (α1- α9). They are known as the very late antigens (VLA) because VLA-1 and VLA-2, which were the first to be identified, are expressed after T cell activation. The members of this subfamily are generally expressed on a variety of tissues and many of their ligands are molecules of the extracellular matrix.

VLA-4 (α4ß1 integrin) is found mainly on the cell surface of leucocytes and it binds to both

VCAM-1 (Bevilacqua, 1993; Cronstein and Weissmann, 1993), located on EC and fibronectin (a component of the extracellular matrix) (Hogg, 1992). Interaction of VLA-4 with its ligand VCAM-1 appears to be important in the interaction between activated T cells and EC at sites of inflammation (Hogg and Landis, 1993). Integrins α4ß1, α2ß1 and α5ß1 are involved in leucocyte spreading during adherence to extracellular matrix and to ECs (Sanchez-Mateos *et al.*, 1993). The α6ß1 integrin is expressed by ECs on their luminal aspect and it is reported to promote homing of T-cell progenitors to the thymus, as well as homing of leucocytes in normal tissues (Ruiz *et al*, 1995).

1.6.3.3 ß2 integrins

ß2 integrins are known as the leucocyte integrins, LFA-1, Mac-1 and p150, 95. LFA-1 is present on the cell surface of most leucocytes and it interacts with the ICAM members of the immunoglobulin superfamily (Springer, 1990; Salmi and Jalkanen, 1997). As well as facilitating interactions with EC, LFA-1 participates in other cell-to-cell interactions, such as T cell activation by antigen presenting cells and the interaction of cytotoxic T lymphocytes with target cells (Springer, 1990). Mac-1 and p150,95 are important in the adhesion and migration of some myeloid cells to inflammatory sites and they are involved in the binding of products of complement activation and the clotting cascades. Mac-1 and p150,95 are found at relatively low levels on lymphocytes. Therefore, these molecules are likely to play a minor role in the adhesive interactions of these cells. Mac-1 is found on macrophages and granulocytes, where it interacts with ICAM-1, but not with ICAM-2 or ICAM-3. Mac-1 can bind also to fibrinogen (Springer, 1990). P150.95, also known as CD11c/CD18, is expressed by monocytes and granulocytes. It forms the heterodimer, complement receptor 4, that binds to fibronectin (Stacker and Springer, 1991; Springer *et al.*, 1986).

1.6.3.4 ß7 integrins

The $\alpha_4\beta_7$ integrin is expressed by lymphocytes that have been activated in mucosal tissues. Its ligand in physiological recruitment of activated lymphocytes is MAdCAM-1 (Bevilacqua, 1993; Hogg and Landis, 1993). However, it has two additional ligands that

play a role during inflammation; VCAM-1 and fibronectin. This integrin mediates physiological lymphocyte homing to Peyer's patches and gut lamina propria in non-inflammatory conditions (Salmi and Jalkanen, 1997). The integrin $\alpha_E\beta_7$ is also found on lymphocytes within the GIT and it plays a role in interaction between lymphocytes and the intestinal epithelium via the immunoglobulin superfamily member E-cadherin (Bevilacqua1993; Imhof and Dunon, 1995; Salmi *et al.*, 1998a).

1.6.4 Immunoglobulin superfamily molecules

The third family of CAMs belong to the immunoglobulin (Ig) superfamily. Characteristically, each member contains multiple Ig-like domains. A number of these family members are known to be involved in leucocyte-endothelial interaction: ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), VCAM-1 (CD106), PECAM-1 (CD31), E-Cadherin, MAdCAM.

1.6.4.1 The ICAMs

ICAM-1 and ICAM-2 were both identified by their ability to bind with the integrin LFA-1 (Marlin and Springer, 1987; Staunton *et al.*, 1989; Cronstein and Weissmann, 1993). ICAM-1 has multiple ligands. In addition to LFA-1, it is known to interact with Mac-1, a group of rhinovirus serotypes (Hogg, 1992), *Plasmodium falciparum*-infected erythrocytes, as well as leukosialin (CD43), which is expressed on T lymphocytes, monocytes, neutrophils, platelets and some B cells. ICAM-1 is expressed weakly on resting endothelium, where its expression is upregulated by stimulation by IL-1, TNF or IFNγ. ICAM-2 is expressed strongly on resting EC, and its expression is not changed by stimulation. ICAM-2 has a weaker affinity for LFA-1 than ICAM-1 and it is believed that ICAM-1 controls the main inflammatory traffic of leucocytes,whereas ICAM-2 is involved in physiological transendothelial leucocyte traffic (Springer, 1990; Picker, 1994; Imhof and Dunon, 1995; Cronstein and Weissmann, 1993). ICAM-1 expression can be induced widely on leucocytes. ICAM-2 is absent from leucocytes, and ICAM-3 is expressed strongly on resting lymphocytes and monocytes. The differential expression of these molecules suggests that they each play a different role (Rothlein *et al.*, 1986).

1.6.4.2 VCAM-1

Vascular cell adhesion molecule (VCAM-1) is a cytokine-inducible adhesion molecule found on ECs. It is a ligand for VLA-4 (α4ß1 integrin) and weakly interacts with $\alpha_4ß_7$ integrin (Elices *et al.*, 1990; Salmi and Jalkanen, 1997). A soluble form of VCAM-1 has been purified from human EC culture supernatants and peripheral blood of patients with RA and systemic lupus erythematosus (SLE) (Wellicome *et al.*, 1993). In addition to its presence on activated ECs, it is expressed on some DCs from lymph nodes, skin, bone marrow and synovium of inflamed joints. Although absent from resting ECs, its expression can be upregulated by exposure to IL-1, IL-4 or TNF (Bevilacqua, 1994). It is believed that VCAM-1 and ICAM-1 play important roles in regulating lymphocyte extravasation during inflammation (Bevilacqua, 1994; Salmi and Jalkanen, 1997; Imhof and Dunon, 1995).

1.6.4.3 PECAM-1

PECAM-1 (CD31) is expressed by most leucocytes, by platelets and at intercellular junctions of normal ECs (*in vivo*). This CAM mediates endothelial-endothelial, leucocyte-endothelial and platelet-EC adhesion and it is involved in transendothelial migration (Muller *et al.*, 1993). The interactions involve homotypic or heterotypic cell-surface adhesion or adhesion to extracellular matrix components. CD31 plays a major role in regulation of leucocyte-endothelial adherence by its ability to activate ß1 and ß2 integrins (Tanaka *et al.*, 1992). CD31 is thought to play a role in EC to EC interaction both *in vivo* and *in vitro*. It may act to limit vascular permeability (Bevilacqua, 1993) through effects on the EC cytoskeleton. Several isoforms of CD31 (PECAM) are believed to exist (Salmi and Jalkanen, 1997; Imhof and Dunon, 1995).

1.6.5 Other adhesion molecules

CD44 is a widely expressed cell surface proteoglycan (Shimizu *et al.*, 1992). Multiple isoforms of this molecule have been identified on leucocytes (Bevilacqua, 1993). CD44 is known to mediate cell adhesion via interaction with hyaluronic acid (Shimizu *et al.*, 1992; Hogg and Landis, 1993; Imhof and Dunon, 1995). In addition, some isoforms bind *in vitro*

to the extracellular matrix molecules collagen, laminin and fibronectin (Jalkanen and Jalkanen, 1992). CD44 can also mediate binding of lymphocytes to EC monolayers and it has been suggested that it may be involved in lymphocyte binding to HEVs and to activated ECs (*in vivo*). A role in extravasation of leucocytes to inflamed non-lymphoid tissues, but not in normal leucocyte circulation, has been suggested (Shimizu *et al.*, 1992; Imhof and Dunon, 1995). CD44 also appears to be involved in maturation of lymphoid precursors, thymus homing and modulation of T cell responses. It may also bind leucocytes and lead to integrin activation and thus stimulate tight endothelial adhesion of leucocytes (Rigby *et al.*, 2000; Giddings *et al.*, 1999). CD44 is also reported to have a role in leucocyte infiltration in the proteoglycan induced murine arthritis (Mikecz *et al.*, 1995; Dianzani and Malavas, 1995).

Vascular adhesion protein 1 (VAP-1) is a sialoglycoprotein with dual function. It is an endothelial adhesion molecule and it also has monoamine oxidase enzymatic activity (Smith *et al.*, 1998). VAP-1 is expressed in the venules of human peripheral lymph nodes but it is not found in mucosal associated lymphoid tissue (Salmi and Jalkanen, 1992). VAP-1 shows no homology to other known CAMs. Low levels of the molecule are found in normal endothelium in brain, skin, kidney, liver and heart, and its expression is upregulated at these sites during chronic inflammation (Salmi *et al.*, 1998b). VAP-1 is believed to be important in the regulation of recirculating lymphocytes to sites of inflammation (Salmi *et al.*, 2000).

Lymphocyte-vascular adhesion protein-2 (L-VAP-2) is expressed by HUVECs and lymphocyte binding to HUVECs can be inhibited with a specific monoclonal antibody raised against L-VAP-2. L-VAP-2 is found on 20% of peripheral blood lymphocytes (mainly B cells and CD8+ cells), a sub-population of venules in lymphoid and non-lymphoid tissues, and some HEV in lymphoid tissues (Salmi *et al.*, 1998b; Salmi and Jalkanen, 1997).

1.7 Lymphocytes and rheumatoid arthritis

1.7.1 Histopathological changes in synovium with rheumatoid arthritis

Chronic, widespread synovitis is a central feature of RA (Cush and Lipsky, 1988) and inflammatory lesions also occur at extra-articular sites (Edwards, 1994). Oedema, hyperplasia and proliferation of blood vessels are evident, as well as infiltration of mononuclear and polymorphonuclear cells (within the vessels and in the perivascular region). The cellular infiltrate is composed of T lymphocytes, B lymphocytes, macrophages and plasma cells (Cush and Lipsky, 1988; Duke *et al.*, 1982; Malone *et al.*, 1984). Polymorphonuclear leucocytes are not abundant in the synovium, but are present in large numbers in the synovial fluid.

During the chronic phase of RA, significant numbers of T lymphocytes in synovium are found in the perivascular region. The majority of these are of the $CD4^+$ helper-inducer phenotype (Cush and Lipsky, 1988; Pitzalis *et al.*, 1988). In areas away from the perivascular accumulations, the $CD8^+$ T cells are more abundant (Cush and Lipsky, 1988; 1991). As the disease progresses, these infiltrating cells often form discrete aggregates, superficially resembling the lymphoid follicles found in the secondary lymph organs (Kulka *et al.*, 1955). $CD8^+$ cells and B cells are found within these cellular aggregates (Panayi *et al.*, 1992). The immuno-histology of synovitis suggests that it is a cell-mediated process of the delayed hypersensitivity type, involving T cells, antigen presenting cells (APC's) and macrophages (Cush and Lipsky, 1991).

1.7.2 Evidence for the role of lymphocytes in rheumatoid arthritis

Several studies have illustrated a role of recirculating lymphocytes in RA. Paulus and co-workers (1979), have shown in humans suffering from RA that depletion of lymphocytes by cannulation of the thoracic duct brings about a significant reduction of synovitis and symptomatic improvement (Paulus *et al.*, 1979; Wegelius *et al.*, 1970). This type of treatment also resulted in long-term remissions in rats with established adjuvant arthritis. With prolonged drainage, the body's pool of recirculating lymphocytes is gradually depleted

and this is thought to be responsible for the reduction of joint disease activity. Conversely, exacerbation of the disease was observed in patients whose autologous lymphocytes were reinfused (Paulus *et al.*, 1979). Because the cellular content of the TD lymph is essentially only recirculating small lymphocytes and some activated lymphocytes, these experiments suggest strongly that lymphocytes play a part in sustaining the inflammatory process in RA and experimental polyarthritis. In other studies, total lymphoid irradiation (Field *et al.*, 1984; Strober *et al.*, 1985), lymphapheresis (Karsh *et al.*, 1981) or treatment with cyclosporin A (Altman *et al.*, 1999; Drosos *et al.*, 1998; 2000) have produced amelioration of RA.

In the experimental animal models of collagen-induced arthritis (Williams *et al.*, 1992) and adjuvant arthritis (Whitehouse *et al.*, 1969; Spargo *et al.*, 1996), the disease can be transferred adoptively to syngeneic-naïve recipient rats by intravenous injection of TD lymphocytes from arthritic donor rats. As a historical note, it is interesting that in an early study, plasma alone or plasma with leucocytes were transferred from donor patients with RA into non-rheumatoid recipients (volunteers and prison inmates). RA did not develop in the recipients (Harris and Vaughn, 1959), who were not matched for donor HLA antigens.

RA patients, whose $CD4^+$ T cells have been depleted due to infection with human immunodeficiency virus (HIV), have shown clinical remission (Espinoza *et al.*, 1989). Treatment with monoclonal antibodies against CD4+ has induced amelioration of the RA in some studies (Herzog *et al.*, 1989) and a similar effect has been observed in the AA model (Pelegri *et al.*, 1996a; 2001). The improvement of inflammation and symptoms in these studies has been interpreted as being due to the depletion of immunocompetent T cells, or interference in their function. The findings support a role for activated T cells as major participants in the inflammatory process of RA (Cush and Lipsky, 1991) and they are consistent with the hypothesis that T lymphocytes are essential participants in the inflammatory manifestations of RA (Paulus *et al.*, 1979).

1.7.3 Immunopathology of RA

In the absence of a specific pathogen that can be linked consistently to the development of RA, several theories have been proposed to explain the aetiology of the disease. The theories that invoke a breakdown in self-tolerance commonly highlight the role of $CD4^+$ T cells either as the primary orchestrator of a cell-mediated immune responses or at least as a key participant. However, it is also evident that the innate immune system can influence the adaptive immune response (Medzhitov and Janeway, 2000). Activation of the innate immune system could result from activation of macrophages or DCs by one or more TLR (Toll-like receptor) agonists (Firestein, 2003). TLR recognise a variety of molecules expressed by micro-organisms (Medzhitov *et al.*, 1997).

Although evidence for a role of T cells in the pathogenesis of RA is substantial, the significance of their contribution is controversial (Firestein and Zvaifler, 1990; 2002; 2003; Panayi *et al.*, 1992). In particular, relatively low levels of lymphokines have been detected in samples of arthritic synovium and synovial fluid, compared with cytokines produced by cells such as macrophages and activated FBs (Firestein *et al.*, 1990; Firestein 2003). However, it should be remembered that macrophage derived cytokines (eg IL-1, TNF-α) are broadcast by the cells, whereas lymphokines exhert their actions more selectively by acting locally at privileged sites of heterotypic cell adhesion (Springer, 1990). In addition, the T cell lymphokines can potentiate the effect of IL-1 and TNF-α thereby focusing and amplifying their actions (Stamp, Cleland and James, 2004).

The major-histocompatibility-complex class II antigens have a vital function in presenting (unidentified) antigenic peptides to $CD4^+$ T cells, resulting in their activation (Choy and Panayi, 2001). CD4+ T cell activated in lymph nodes, enter joints from the circulation, a process that can be enhanced greatly by chemoattractants produced by activated synovial macrophages and FBs (Firestein, 2003). These activated CD4+ T cells can in turn stimulate monocytes, macrophages and synovial FB-like cells to produce inflammatory mediators, such as cytokines, chemokines and matrix degrading enzymes. The CD4+ T cells can also stimulate B cells to produce antibodies such as RF, and to induce proliferation of

osteoclasts.

1.7.4 Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) is an immune response mediated by Th1 cells principally via release of IFN-γ (reviewed in Mosmann and Coffman, 1989; Street and Mosmann, 1991). Following stimulation by IFN-γ, macrophages (Pace *et al.*, 1983) produce inflammatory mediators such as TNF-α, nitric oxide, free radicals and proteolytic enzymes. These mediators bring about the inflammation seen in DTH reactions. Through similar mechanisms, Th1 cells are thought to be responsible for the pathology of some autoimmune diseases, such as EAE. In contrast, Th2 cells are thought to exert a protective influence in these diseases (Kuchroo *et al.*, 1995; Das *et al.*, 1997).

1.7.5 The role of cytokines in RA

The activities of T cell derived cytokines IL-2 and IFN-γ are thought to play an important role in RA (Firestein and Zfeifler, 1990; Choy and Panayi, 2001). However, these cytokines are not as abundant in RA synovium as macrophage and FB derived cytokines, such as IL-1, IL-6, IL-15, IL-18, TNF-α, GM-CSF. CD4+ lymphocytes are involved with cell to cell interactions that involve signalling through cell adhesion molecules and directional secretion of lymphokines at sites of intercellular adhesion (Choy and Panayi, 2001).

CD4+ cells can stimulate differentiation of osteoclasts, which can contribute to periarticular erosions. Activated CD4+ T cells stimulate monocytes, macrophages and synoviocytes that produce the cytokines IL-1, IL-6 and TNF-α. They also stimulate secretion of matrix metalloproteinases. These three cytokines are key players that drive the inflammatory process in RA (Choy and Panayi, 2001). TNF-α and the TNF receptor have been targeted in the development of new arthritis therapies.

1.7.6 Lymphocytes in RA synovium; markers of activation

There is a heavy infiltration of the synovium with mononuclear cells in RA. Up to 50% of the mononuclear cells in this infiltrate are T cells, most of which have an activated phenotype (Kinne *et al.*, 1997). The majority of them are CD4+ memory T cells that have the phenotype CD45RA-, $CD29^{+}$, (Pitzalis *et al.*, 1982; Cush and Lipsky, 1991), $CD11a^{+}$ and $CD62L^{-}$ (Cush *et al*, 1992). This "memory" phenotype is not unique, in that it has been reported for activated CD4+ cells found in chronically inflammatory tissues such as the intestine, thyroid, skin, lung and liver. In contrast, in RA, Cush and co-workers reported no increase in CD25 expression but a significant increase in CD49a/CD29 (VLA-1), CD62L (L-selectin), $CD54^{+}$ and $CD58^{+}$ (Cush *et al.*, 1992). However, Panayi and coworkers (1992) reported different expression profiles and showed upregulation of CD49a (VLA-1), CD25 (IL2-receptor), CDw60, CD69 with associated downregulation of CD3, CD2 and LFA-1. Szekanecz, (1996) reviewed the literature on T cells in RA synovium and reported that the most prevalent phenotype was CD4+ CD45RO+ $CD62L^{low}$, $CD54^{+}$, $CD44^{+}$ and $\alpha_4\beta_7^{+}$.

The significance of the expression of the mucosal ligand $\alpha_4\beta_7$ integrin by some synovial T cells can be linked to the overlap of T-cell circulation compartments between gastrointestinal (GIT) mucosa and synovial tissue. T lymphocytes that express the integrin $\alpha_4\beta_7$ will home to sites that express high levels of MAdCAM-1 or VCAM-1 on their vasculature. The former molecule, MAdCAM-1 is restricted mainly to the GIT mucosa and the latter, VCAM-1 is expressed by synovial EC (Butcher *et al.*, 1999; Hamann and Syrbe, 2000).

The volume of lymphocyte homing traffic to the GIT is due to the expression by lamina propria microvascular EC of mucosal addressin (MAdCam-1) and its interactions with the integrin $\alpha_4\beta_7$ on lymphocytes that originate from the gut associated lymphoid tissues. The link between the immune regulation of the GIT and joint compartments is strengthened when one considers the pathogenesis of reactive arthritis (Salmi *et al.*, 1998a). Reactive arthritis in the joint can occur following infections at distant mucosal sites, for example, the

intestinal, respiratory or urogenital tracts (Burmester *et al.*, 1995). Examples of these triggers include infections of Yersinia, Salmonella, Shigella or Campylobacter species (Kingsley, 1993). Bacterial degradation products and bacterial DNA have been detected in synovium of patients with reactive arthritis (Toivanen., 2001), in addition to the presence of $\alpha_4\beta_7^+$ T cells.

1.7.7 Migration of lymphocytes into the tissues

As described earlier, the recruitment of lymphocytes from the blood requires the process of adhesion to, and migration through the microvascular endothelium of the synovium, followed by interaction with the synovial interstitium. Adhesion and migration are regulated by the interactions between CAMs and their ligands on the cell surface of activated EC and lymphocytes (Springer, 1990). The spectrum of CAMs expressed by synovial tissues and by synovial endothelium is, therefore, of great importance in understanding the pathogenesis of RA and for design of new therapeutic strategies that could interfere with recruitment of inflammatory cells.

Blood supply and blood flow to the tissues are integral determinants of entry of lymphocytes into specific organs and tissues (Westermann, 2001). The velocity of T cells in the venules has been estimated at approximately 500μm/ second (Salmi and Jalkanen, 1997). A large reduction of speed in a short period of time is required, therefore, for CAMs on the cell to interact with the molecules on EC. The multistep process of migration begins with margination as leucocytes exit capillaries and enter post capillary venules. The relative increase in diameter of post capillary venules and associated decrease in fluid velocity causes this movement of leucocytes towards the periphery of the vessel. Margination increases the likelihood of leucocyte contact with EC, followed by interaction between leucocyte and endothelial CAMs (Schmid-Schonbe *et al.*, 1980). Vessels at sites of inflammation are usually dilated, increasing the amount of leucocyte margination, thus facilitating adhesion of leucocytes (Shimizu *et al.*, 1992).

Adhesion of the leucocytes to endothelium may be followed by migration through

endothelial intercellular junctions and the basement membrane, leading to perivascular accumulation of lymphocytes (Ager, 1987b; Freemont *et al.*, 1983). The presence of different CAMs on naïve compared with activated T cells produces different patterns of migration and recruitment of these subsets (Bradley and Watson, 1996), while differential expression of "addressins" in various vascular beds leads to preferential recruitment of different subsets of memory T cells (Imhoff and Dunon, 1995).

1.8 Adhesion molecules in rheumatoid arthritis

In the non-inflammatory state, the processes of adhesion and migration help to maintain recirculation of lymphocytes and homing to lymphoid tissues. During inflammation, activation of endothelium leads to increased expression of adhesion molecules. There is increased adhesion of leucocytes to endothelium, an increased extravasation and increased adhesion to extracellular matrix components in the extra-vascular compartment. Together, these factors initiate and perpetuate the persistent inflammation that is the hallmark of rheumatoid arthritis.

Synovial tissue from patients with RA shows increased expression of a number of adhesion molecules. These include E-selectin, VCAM-1, ICAM-1 (Koch *et al.*, 1991; Grober *et al.*, 1993) and P-selectin (Johnson *et al.*, 1993; Grober *et al.*, 1993). Synovial fluid lymphocytes express molecules such as VLA-4, VLA-5, LFA-1 and ICAM-1 (Sfikakis and Mavrikakis, 1999). Soluble adhesion molecules, shed into the circulation, can also be detected in serum from patients with RA. ICAM-1, ICAM-3, L-selectin and P-selectin are all elevated significantly in RA serum, compared with serum from healthy controls (Littler *et al., 1997*).

E-selectin is expressed at high levels on activated EC in rheumatoid synovium (El-Gabalawy *et al.*, 1994; Kreigsmann *et al.*, 1995), while its expression is weak to patchy on synovial lining cells and mononuclear cells within the tissues (Grober *et al.*, 1993). Activated lymphocytes from synovium display enhanced capacity to bind E-selectin, compared to T cells from peripheral blood of healthy donors. (Postigo *et al.*, 1992).

Chemotaxis of human EC can be induced *in vitro* by the presence of soluble E-selectin in serum from RA patients (Koch *et al.*, 1995). However, while soluble E-selectin has been reported in the serum of RA patients (Sfikakis and Mavrikakis, 1999), others have been unable to confirm these observations (Littler *et al.*, 1997).

Expression of P-selectin has been detected in capillaries (Akin *et al.*, 2001) and post-capillary venules in arthritic synovial tissue (Grober *et al.*, 1993), where it is found on the lumenal aspect of EC lining these vessels (Johnson *et al.*, 1993). P-selectin expression is not increased in non-vascular elements of RA synovium (Grober *et al.*, 1993). Levels of serum P-selectin are elevated significantly in RA patients (Littler *et al.*, 1997). P-selectin dependent attachment of monocytes to frozen sections of RA synovium is up to 20 times greater than to sections of normal synovium. In addition, monocyte adhesion to RA synovium was blocked almost completely by monoclonal anti- P-selectin antibodies (Grober *et al.*, 1993), suggesting an important role for P-selectin in trafficking of these cells to affected joints (Sfikakis and Mavrikakis, rev, 1999). P-selectin-1 binds Sialyl-Lewis x and PSGL-1 on leucocyte cell membranes and this interaction is believed to be critical in the homing of monocytes to synovium (Springer, 1994; Salmi *et al.*, 1997a).

The third member of the selectin family, L-selectin, is expressed constitutively on the surface of leucocytes, and it is present on a variety of cell types within RA synovial tissue samples (Johnson *et al.*, 1993). Synovial fluid lymphocytes from patients with RA are reported to express lower levels of L-selectin than peripheral blood lymphocytes (Sfikakis and Mavrikakis, rev, 1999). This is consistent with an activated or memory phenotype.

The integrin family of adhesion molecules also plays a role in RA, where they are important in EC interactions that lead to lymphocyte migration to the inflamed synovium. The main integrins expressed by T cells in RA synovium are VLA-4 and LFA-1. VLA-4 (CD49d/CD29) acts a ligand for VCAM-1. T cells from rheumatoid synovial tissue and synovial fluid display higher levels of VLA-4 than their peripheral blood counterparts (Postigo *et al.*, 1992; Laffon *et al.*, 1991). Similarly, more T cells express VLA-1 and

VLA-5 in RA synovial fluid (Sfikakis and Mavrikakis, 1999). VLA-4 is believed to be important in binding of T lymphocytes to synovial vascular endothelium (van Dinther-Janssen *et al.*, 1991). Antibodies against VLA-4 and VLA-5 can block adhesion of lymphocytes to frozen sections of synovial tissue (Sfikakis and Mavrikakis, 1999), suggesting that these molecules have a role in lymphocyte accumulation in RA synovium.

LFA-1 (CD11a/CD18) is expressed by various leucocytes and it interacts with members of the ICAM family (Sfikakis and Mavrikakis, 1999). Both ICAM-1 and LFA-1 are expressed intensely in RA synovium (Akin *et al.*, 2001). ICAM-1 is expressed by a range of tissue components in normal synovium (Sfikakis and Mavrikakis, 1999). Using immunohistochemical techniques, ICAM-1 was detected on vascular EC (Szekanecz, 1994; Grober *et al*, 1993; Hale *et al.*, 1989), synovial FBs, macrophage – like type A synoviocytes (Hale *et al.*, 1989; van Dinther-Janssen *et al.*, 1994) and tissue macrophages, where it was expressed at higher levels in RA synovium (Hale *et al.*, 1989) than in normal synovium. Akin and co-workers (2001), reported high levels of ICAM-1 in RA synovium and on synovial fluid lymphocytes from patients with RA (Gerritsen *et al.*, 1993). Interactions between ICAM-1 and LFA-1 are important in the firm adhesion of leucocytes to EC in inflamed synovium (Akin *et al.*, 2001).

The adhesion molecule VCAM-1 has been detected in tissue samples from patients with RA (Postigo *et al.*, 1992; Kreigsmann et al., 1995b). VLA-4 and VCAM-1 are believed to be important mediators of binding between T lymphocytes and synovial vascular EC. Activated lymphocytes from RA synovium display enhanced capacity to bind VCAM-1 *in vitro* (van Dinther-Janssen *et al.*, 1991).

In vitro adhesion studies, using the Stamper-Woodruff assay on tissue sections, have highlighted a role for the sialoglycoprotein adhesion molecule, VAP-1 in the adherence of lymphocytes to human synovium (Salmi and Jalkanen, 1992; Salmi *et al.*, 1997b). Expression of VAP-1 is increased in synovial microvascular endothelium in RA, Lyme arthritis and inflammatory monoarthritis (Akin *et al.*, 2001).

1.9 The adjuvant induced arthritis- a rat model of rheumatoid arthritis

1.9.1 Models of arthritis

Experimental models have been used to study the pathogenesis of polyarthritis. In most models, adjuvants are used either alone or together with potential autoantigens (Kleinau *et al.,* 1994). Experimental models of polyarthritis in rodents include adjuvant arthritis (AA) in Lewis, Dark Agouti or Sprague-Dawley rats; collagen-induced arthritis (CIA) in mice and in DA and Lewis rat strains, proteoglycan-induced arthritis, Streptococcal cell wall-induced arthritis in susceptible Lewis rats (van den Broek, 1989), as well as pristane-induced arthritis in mice (Holmdahl and Kvick, 1992; Wooley, 1991) and Oil-induced arthritis (OIA) (Kleinau *et al., 1994*).

Stoerk, Bielinski and Budzilovich (1954) were the first group to report the induction of polyarthritis in rats. The arthritis was induced by subcutaneous injection of tissue extracts emulsified in Freund's adjuvant (mineral oil and heat-killed tubercule bacilli) (Stoerk *et al.,* 1954). Later, Pearson (1956) demonstrated that administration of complete Freunds adjuvant (CFA) alone could induce chronic polyarthritis in rats and this observation is the basis of the rat model of adjuvant-induced arthritis (AA). Adjuvant arthritis bears a number of similarities to human rheumatoid arthritis (Zahari *et al.*, 1969; Glenn and Gray, 1965). The disease is believed to be a manifestation of delayed hypersensitivity to unknown synovial autoantigens (arthritogens) (Waksman *et al.*, 1960; Sharp *et al.*, 1961; Pearson, 1963; Waksman and Wenersten, 1963; Pearson and Wood, 1964).

1.9.2 Induction of AA

AA is induced by injection of heat-killed *Mycobacterium tuberculosis* in an oily adjuvant intradermally (for example, at the base of the tail or in the footpad). Maximum induction of disease is achieved by direct injection into the inguinal lymph nodes (Taurog *et al.*, 1988). The resultant disease is a polyarthritis affecting mainly the ankles, wrists, digits, and spine (Waksman, 2002). The arthritis manifests as synovitis, periarthritis, peritendonitis,

periostitis, pannus formation with bone and cartilage destruction, and fibrous or bony ankylosis. Secondary changes include oedema, fibrin deposition, foci of necrosis, synoviocyte proliferation, bone destruction and bone remodelling. The disease also affects eyes, skin, mucosae of the gastrointestinal and the urogenital tract, as well as occasional granulomas in the lungs, liver or spleen.

Kleinau *et al.*, (1991) reported that the development of AA is observed on about day 14 after injection and once resolved, cannot be re-induced by a second injection of adjuvant. Resistance to reinduction of disease has been observed in several autoimmune diseases dependent on reactivity to defined antigens. Kleinau *et al.* (1991) reported also that intradermal injection of adjuvant oil without addition of antigen (Freund's incomplete adjuvant [FIA]) can trigger the development of a T-cell dependent and presumably autoimmune arthritis in DA rats. OIA is a self-limited acute disease that is less destructive than either AA or CIA. These studies indicate that the adjuvant oil itself is a critical trigger that can induce autoreactive cells to become arthritogenic (Kleinau *et al., 1994*).

1.9.3 Strain difference and susceptibility to AA

The incidence and severity of AA varies significantly, depending on the strain of rat (Billingham, 1983). A partial resistance trait in the Lewis rat has been shown to be due to a genetically dominant gene. Similarly, the susceptibility of the Dark Agouti rat is due to a recessive gene (Holmdahl *et al.*, 1992). The random bred strains Wistar, Sprague-Dawley, Lon-Evans, Holtzman and Lobund and inbred strains of Lewis, Dark Agouti, PVG, Wistar Furth, Brown Norway and WKA are susceptible to AA (Kohashi *et al.*, 1979). Some other rat strains show susceptibility to induction of AA only if they are housed in germ-free conditions disease. In addition, once the disease has run its course, convalescent rats show resistance to reinduction of the disease by subsequent inoculation of CFA (Taurog *et al*, 1988). Resistance to reinduction is likely to be due to development of antibodies to heat shock proteins (HSP), a molecule that has been implicated in the pathogenesis of arthritis in human and experimental animal models (van Eden *et al.*, 1988).

1.9.4 Adjuvant arthritis: immunologic regulation and effector mechanisms

Type II collagen and cross-reactive antigens of *Mycobacterium tuberculosis* have been proposed as arthritogens in the autoimmunity. The character of the lesions indicate that a cell-mediated immune response may be involved, similar to that seen in some other autoimmune disease models such as EAE (van Eden and Waksman, 2003). Cell mediated immune responses are mediated by the α/β TCR and AA can be transferred by adoptive transfer of α/β T cell clones sensitised with *Mycobacterium tuberculosis.*

Following injection of CFA, DCs (DC) take up and process mycobacterial antigens. In the lymph nodes that drain the site of CFA inoculation, the DC will promote the initiation of a CD4+ T-cell mediated response (Dresser *et al.*, 1970; Taub *et al.*, 1970). Van Eden (1983) and coworkers used a T cell clone to identify reactivity to joint antigens. This clone was able to transfer disease to irradiated naïve rats and to induce resistance to induction of disease in non-irradiated recipients. Later this clone was shown to be specific for the 65kD heat shock protein (HSP65)(van Eden *et al.,* 1988). HSP65 can also induce resistance to AA when administered intraperitoneally, orally (Haque *et al.*, 1996) or parenterally, and when administered as a DNA vaccine (Quintana *et al.*, 2002).

Mycobacterium tuberculosis HSP65 (also known as Hsp60) is the immunising antigen considered to be responsible for the induction of AA. The T cell epitope of this HSP is the 180-188 peptide sequence (Van Eden and Waksman 2003). It is understood that resistance to disease is due to the presence of antibodies specific for the HSP65. These antibodies may be induced by inoculation with CFA, or by segment 180-188 of the HSP65. It can be induced also by the presence of 'self' or 'natural' HSP (Ulmansky *et al.,* 2002).

1.9.5 The role of adjuvant oil and resistance to disease

Of the several experimental inflammatory joint diseases, most require immunisation with an autoantigen or immunological adjuvant in mineral oil. It has been shown that mineral oil alone causes disease in susceptible strains of rats (Kleinau *et al.*, 1991; Larsson *et al.*, 1990) and that this adjuvant oil stimulates production of IFN-γ and TNF-α at the transcriptional

level (Mussener *et al.*, 1995). 'Naturally' occuring or self-HSP may act as the immunological epitope underlying the disease in this case (Frances *et al.*, 2000). Lorentzen and coworkers (1998) have identified two susceptibility loci controlling incidence and severity of joint disease caused by adjuvant oil-alone- *Oia2* on chromosome 4 and *Oia3* on chromosome 10. Based on this information rats with an arthritis-resistant genome were made congenic for the *Oia3* (Holm *et al.*, 2001) and *Oia2* (Ribbhammar *et al.*, 2003) loci to confirm the quantitative trait locus (QTL). Conversely, arthritis-resistance was achieved in the ordinarily arthritis susceptible DA rat strain, by making a congenic with the *Oia2* allele from normally resistant strains (Ribberhammer *et al.*, 2003).

1.9.6 Histopathology of AA

Histologically, some pathologic features of AA have similarities to those of RA and Reiter's disease (a seronegative polyarthritis). The initial signs of AA appear 11 to 14 days after injection of CFA (Pearson, 1956). Clinical signs of the disease include symmetrical swelling of the tibiotarsal (ankle) joints and the metatarsophalangeal joints of the hind paws. The inflammation usually progresses to involve the entire hind paw. Joints in the front paw also become inflamed (Kleinau *et al.*, 1991).

Oedema can be identified at the site of the developing inflammatory reaction before evidence of cellular infiltration is observed (Gryfe *et al.*, 1971). Focal lesions develop in the connective tissue (CT) of the extremities, the uveal tract and the skin. In the extremities, inflammation begins in synovial and periarticular CT, and spreads directly to involve skeletal muscle and periosteum (Jones and Ward, 1963; Gryfe *et al.*, 1971). As the disease progresses, the lesions in the extremities result in arthritis, periostitis and tenosynovitis. Mast cells are the first cell type to appear in abnormally high numbers, within the inflamed tissue. These cells have been observed on day 6 after injection of adjuvant and rapidly increase in number (Gryfe *et al.*, 1971).

Burnstein and Waksman (1964) reported that lesions in early AA were seen as perivascular accumulations of medium sized lymphocytes, accompanied by venous congestion.

Lymphocytes do not accumulate within the blood vessels. As the disease develops, the number of lesions increase and individual lesions become larger and more diffuse. During this time, monocytic or histiocytic cells progressively become the major inflammatory cell population. These cells are concentrated centrally within the foci. Infiltration of polymorphonuclear cells, proliferation of FBs, synoviocytes and epidermis adjacent to the lesions appear as secondary alterations. These changes were not evident in early lesions (day 9). It is noteworthy that these changes were found only in the presence of mononuclear cell infiltrates and were observed at the clinical onset of the disease. Destruction of bone and cartilage, as well as pannus formation, were observed as late changes (Burstein and Waksman, 1964).

T lymphocytes play a major role in the pathogenesis of adjuvant arthritis (Taurog *et al.*, 1983; 1984; Holoshitz *et al.*, 1983; 1984; Larsson *et al.*, 1985; Yoshino and Cleland, 1992; Spargo *et al.*, 1996). AA is ameliorated by depletion of T lymphocytes *in vivo* (Billingham *et al.*, 1990; Yoshino and Cleland, 1992; Pelegri *et al.*, 1996a) or by treatment with non-depleting anti-CD4 mAb (Pelegri *et al.*, 1995b) and it can be adoptively transferred by lymphocytes from TD lymph of animals that are developing AA (Whitehouse *et al.*, 1969; Spargo *et al.*, 2001) and by T cell clones derived from rats with the disease (Holoshitz *et al.*, 1983, 1984.

A number of studies specifically implicate the CD4+ T cell subset in the pathogenesis of AA. It has been demonstrated that the cells of the CD4+ subset (Pelegri *et al.*, 1996a; Hoffman *et al.*, 1974; Spargo *et al.*, 2001), but not the CD8+ subset (Larsson *et al.*, 1985; Spargo *et al.*, 2001) are pivotal. It has been shown that lymphoblasts in TD lymph from arthritic donor rats are recruited to synovium in significant numbers after intravenous injection into normal or arthritic recipients (Spargo *et al.*, 2001).

1.10 Studies on adhesion molecules

1.10.1 Circulation and Migration

In order to be involved in the pathogenesis of an autoimmune disease, lymphocytes need to leave the circulation and enter the tissues. Through trafficking, the lymphocyte effector cells disperse and are recruited preferentially to specific sites. Although the molecular details of tissue selective migration have been studied for a number of years, there remain aspects that are not fully understood. It is known, however, that "regulation and expression of functional adhesion molecules is pivotal to both physiological surveillance and all types of inflammatory processes" (Salmi and Jalkanen, 1997).

Early *in vivo* migration studies were fundamental to the formulation of the views that are held currently about lymphocyte recirculation. The experiments by Gowans and Knight (1964) were pioneering, and showed for the first time that lymphocytes could leave the blood in the peripheral lymph organs and return to the systemic circulation via efferent lymph. These early experiments used lymphocytes harvested from the thoracic duct of rats (efferent lymphatics). After radiolabelling *in vitro*, they were reinjected intravenously and their distribution studied by autoradiography and scintillography. Within this thoracic duct lymph population, Gowans and Knight observed differential circulation of large and small lymphocytes. They showed that small lymphocytes migrated to the secondary lymphoid tissues, while many of the larger activated lymphoblasts homed specifically to mucosal sites (tertiary tissues) (Gowans and Knight, 1964).

Following this work, other studies of lymphocyte homing utilised large and small animal models. This included work in sheep (Cahill *et al.*, 1977; Hall, 1979; Chin and Hay, 1980), pigs (Binns *et al.*, 1988), rats (Griscelli *et al.,* 1969; Goldschneider and McGregor, 1968a; 1968b; Ford and Simmonds, 1972; Husband and Gowans, 1978; Issekutz *et al.*, 1982) and mice (Lance and Taub, 1969; Guy-Grand, 1974; Parrott and Ferguson, 1974; Rose, 1976). These studies led to the general concept of lymphocyte recirculation. (see Section 1.4.)

1.10.2 Investigation of lymphocyte-endothelial cell interactions

Since these early *in vivo* studies, a number of methods have been developed to investigate the molecular interactions between lymphocytes and ECs. These methods include the *in vitro* adhesion assay developed by Stamper and Woodruff (1976), *in vitro* adhesion assays involving EC from large vessels such as human umbilical cord (Haskard *et al.*, 1986; Chin *et al.*, 1991; Oppenheimer-Marks *et al.*, 1991; Kavanaugh *et al.*, 1991) or rat aorta (Issekutz, 1993; Issekutz and Wykretowicz, 1991), and EC from the microvasculature of the rat heart (Issekutz and Wykretowicz, 1991; Issekutz, 1992b; Wykretowicz and Issekutz, 1993).

1.10.3 Discovery of the first lymphocyte homing molecule

It was not until 1983 that Gallatin, Weissman and Butcher identified the first molecule involved in lymphocyte homing. The discovery of this molecule was a highly significant development in the study of tissue selective tissue homing. This group reported the development of mAb, MEL-14, an antibody that was specific for a molecule found on the surface of most circulating lymphocytes in mice. They found that this molecule was involved in lymphocyte homing to peripheral lymph nodes in mice, (Gallatin *et al.*, 1983). Notably, mAb MEL-14 had no effect on homing to mucosal associated lymphoid tissue.

The MEL-14 antigen was the first putative homing receptor and its identification helped to explain the phenomenon of lymphocyte recirculation that had been reported some 20 years earlier. This molecule is now known as L-selectin (see Section 1.7.1 for details). The second lymphocyte homing receptor identified was recognised by anti-human mAb Hermes-1 (Jalkanen *et al.*, 1996). This antibody is specific for CD44, which binds hyaluronic acid and has an accessory role in binding to HEVs. Some five years after the discovery of the MEL-14 antigen, a EC counter receptor for this molecule was identified (Streeter *et al.*, 1988a; Streeter *et al.*, 1988b) and the term vascular addressin was coined to describe the adhesion ligands present on EC. Later, this the mAb MECA-79 was found to recognise L-selectin ligand, the peripheral LN addressin (PNAd) on HEVs in normal LN and on EC at sites of chronic inflammation. MECA-79 binds to the PNAd by reacting with

glycoproteins, such as CD34, Podocalyxin, Sulphated glycoprotein (Sgp200) and GlyCAM-1, all of which fall under the heading of this addressin (Rosen, 1999).

With the characterisation of these adhesion molecules (homing receptors), Butcher proposed a simple model to explain the mechanism of tissue specific trafficking of lymphocytes. This simple 'lock-and-key mechanism was, however, an oversimplification and Butcher (1986) recognised "an additional level of complexity" highlighted by the discovery of soluble factors present in lymph and in LN cell culture supernatants. These were shown to enhance *in vitro* interactions of lymphocytes with HEVs (Butcher, 1986).

Following the discovery of multiple adhesion related molecules, and the accumulation of data regarding adhesive interactions between lymphocytes and EC, a more complex model of "multi-step adhesion" was adopted (Butcher, 1991; Springer, 1994) consisting of at least 4 distinct steps (see Section 1.5.1). During the 1990s three structurally different adhesion molecule families were recognised (Springer; 1990; Imhof and Dunon, 1995). There are now five such families (Salmi and Jalkanen, 1997), adding further to the complexity of the model.

1.10.4 Methods to investigate lymphocyte-endothelial cell interactions

1.10.4.1 Adhesion to HEVs; the first adhesion assay

During the mid-1970s a method was described to investigate lymphocyte-EC interactions *in vitro* (Stamper and Woodruff, 1976). Much of the early work investigating the role of HEV in lymphocye migration was carried out by Woodruff and co-workers (Chin and Hay., 1980; Woodruff and Woodruff, 1980; Woodruff *et al.*, 1987) using this simple *in vitro* assay. The assay showed that lymphocytes adhere to HEV in a specific and reproducible manner. This was significant because it helped confirm that lymphocyte-EC recognition is essential for lymphocyte recirculation. However, it was recognised that factors, some not yet identified, existed that selectively direct the trafficking of particular lymphocyte populations through specific tissues (Butcher, 1986) and that a surface receptor system allowed discrimination between HEV in peripheral LN (Jalkanen and Butcher, 1985), and

mucosal associated lymphoid tissue (Butcher, 1979) and in inflamed synovium (Jalkanen *et al.*, 1986).

The Stamper-Woodruff assay has been an important model because these specialised vessels are a major *in vivo* route of lymphocyte extravasation. It facilitated the first investigations into the possible mechanisms of adhesion molecule interactions that precede the cascade of events that occurs in migration of leucocytes to specific tissue sites, such as inflamed synovium. The HEV *in vitro* binding assay has continued to be a useful model, especially for human tissues from various inflammatory pathologies, where small tissue biopsy samples are utilised. In addition to examination of leucocyte adhesion to inflamed synovium (Salmi and Jalkanen, 2001; Salmi *et al.*, 1997a; Grober *et al.*, 1993), adhesion to non-synovial tissues such as gastrointestinal mucosa (Hamann *et al.*, 1994; Kadioglu and Sheldon, 1995), pancreatic islets of Langerhans (Hanninen *et al.*, 1993), salivary gland (O'Sullivan and Montgomery, 1993), central nervous system (Sasserville *et al.*, 1994), lymph node (Sawada *et al.*, 1993), middle ear (Spilman *et al.*, 1995), nasal tissues (Symon *et al.*, 1994) and renal tissue (Turunen *et al.*, 1994) has been examined. Studies of lymphocyte adhesion to human synovium using the Stamper-Woodruff *in vitro* assay are discussed in Section 1.10.5.1.

This assay has its limitations. Identification of these limitations has led to questions about how well the assay mimics the *in vivo* situation. An important issue is the non-vital state of the tissue, and the inability of the EC to facilitate further activation of lymphocytes, as might occur *in vivo* or in assays using cells cultured *in vitro*. Furthermore, in tissue sections, molecules are not only presented on the EC surface. Intra-cytoplasmic antigens are also exposed for interaction with potentially adherent lymphocytes. Other concerns are the performance of the assay at non-physiological temperatures and the absence of shear stress that is present in blood vessels. Finally adhesion assays, only examine adhesion and do not address transmigration or the stage when cells detach after adhesion.

1.10.4.2 Endothelial cell monolayers from human umbilical vein

The successful isolation and *in vitro* culture of HUVECs reported in the early 1970s by Jaffe and coworkers (1973) was the next milestone in the examination of lymphocyte-EC adhesive interactions. Much valuable information regarding EC biology and adhesion molecules was gained by the use of HUVEC monolayers for the purpose of *in vitro* adhesion assays. There are several advantages in the use of living monolayers in adhesion studies. The cells in the monolayers are a pure EC population, only the molecules expressed on the cell surface are available for interaction with leucocytes, and the HUVECs can be manipulated or stimulated to mimic physiological or pathological aspects of leucocyte-EC interactions. Finally, an advantage of *in vitro* assays using EC monolayers is that they may be adapted to address transmigration.

1.10.4.3 HUVEC monolayers; adhesion molecule investigations

HUVEC monolayers have been stimulated to upregulate the surface expression of adhesion molecules that are involved in the adhesion of lymphocytes (Haskard *et al.*, 1986; Yu *et al.*, 1986; 1986; Cavender *et al.*, 1986). Studies have been carried out using mAbs with specificity to adhesion molecules as blocking agents. A number of research groups have investigated the role of adhesion molecules in adhesive interactions between HUVECs and lymphocytes. Adhesion molecules such as LFA-1 (Haskard *et al.*, 1986; Shimizu *et al.*, 1992; Oppenheimer-Marks *et al.*, 1988; Kavanaugh *et al.*, 1991), VCAM-1 (Shimizu *et al.*, 1992; Vonderheide and Springer, 1992, Kavanaugh *et al.*, 1991), E-selectin (Shimizu *et al.*, 1992; Graber *et al.*, 1990), VLA-4 (Vonderheide and Springer, 1992), CD44 (Oppenheimer-Marks *et al.*, 1990; Kavanaugh *et al.*, 1991) and ICAM-1 (Kavanaugh *et al.*, 1991) have been investigated and found to play a role in adhesion and migration. In *in vitro* cultures of HUVECs have been adapted to examine also the role of blocking mAbs in transmigration of lymphocytes through endothelial monolayers (Oppenheimer-Marks *et al.*, 1990; Van Epps *et al.*, 1989).

A major drawback of studies using HUVECs the origin of these EC from large vessels that would not normally support transmigration in inflammation. In addition, HUVECs lack

surface expression of certain adhesion molecules, eg VAP-1 and MAdCAM-1. The heterogeneity of expression (as discussed in Section 1.3.5) and regulation of adhesion molecules on EC in different vascular beds does not allow direct extrapolation of results to specific tissues and pathologies, such as synovium in arthritis. This model has however, facilitated the development of methods for measuring *in vitro* adhesion and migration. This has provided a platform from which more specific inquiries have been made into the role of adhesion molecules in individual systems and specific pathologies.

1.10.4.4 High endothelial cell monolayers.

HEVs are known to play a major role in directing lymphocyte trafficking (Butcher, 1986). Following the successful use of EC monolayers from large vessels to investigate adhesion molecules *in vitro*, a method was developed to isolate EC from the HEV of rat lymph nodes (Ager, 1987a; Ager and Mistry, 1988). Culture of EC from HEV, known as high ECs (HEC), has facilitated the investigation of lymphocyte adhesion and also the transendothelial migration of lymphocytes across the HEV wall (Ager and Mistry, 1988). Although HEVs from rats have been cultured *in vitro*, those from humans have proved more difficult.

Cell adhesion molecules, LFA-1 (Faveeuw *et al.*, 1997) and α4 integrins (Faveeuw *et al.*, 1997; Szekanecz *et al.*, 1992) have been implicated in the adhesion and transmigration of T lymphocytes and lymphoblasts across HEV. Blocking of lymphocyte or lymphoblast adhesion individually with mAbs against α4 integrin, VCAM-1 or LFA-1 lead to similar levels of inhibition, with reduction of approximately 40% of lymphocyte and around 23% of lymphoblast adherence. Remarkably, a combination of antibodies to α4 integrin and LFA-1 inhibited adherence of both lymphocyte or lymphoblast adherence by greater than 90% (Faveeuw *et al.*, 1997).

1.10.4.5 Gene targeted animals or gene-knockouts

In nature, two rare examples of genetic defects of cell adhesion molecules have been recognised. Both lead to susceptibility to infection. As described in Section 1.6.3.1, these

are leucocyte adhesion deficiency I and II (Salmi and Jalkanen 1997). The type I deficiency interferes with expression of the β2 integrin common chain and the second syndrome involves a fault in the selectin ligand interactions (see Section 1.6.3.1).

Site directed mutagenesis of targeted genes in mice has lead to the development of a number of knockout models that have null alleles of gene encoding specific adhesion molecules. Genes L-selectin, E-selectin and P-selectin have been targeted (Salmi and Jalkanen, 1997). L-selectin knockout mice have been shown to exhibit impaired rolling and migration of leucocytes (Arbonés *et al.*, 1994; Ley *et al.*, 1995) and an altered immune response (Tang *et al.*, 1997). E-selectin-deficient mice do not show impairment of leucocyte migration in inflammation (Labow *et al.*, 1994; Kwee *et al.*, 1995; Ley *et al*, 1995). Leucocytes in P-selectin-deficient mice can roll without this molecule (Mayadas *et al.*, 1993; Ley *et al.*, 1995; Kwee *et al.*, 1995; Yamada *et al.*, 1995) and surprisingly, a greater number of extravasated cells have been observed in manipulated mice compared with unmanipulated controls (Salmi and Jalkanen, 1997). Knockout mice specific for the L-selectin ligands, CD34 (Suzuki *et al.*, 1996) and GlyCAm-1, as well as ICAM-1 knockouts (Sligh *et al.*, 1993; Kwee *et al.*, 1995), have also been developed. However, homogeneous β1, α4 and VCAM-1 knockouts have an embryo-lethal phenotpe (Kwee *et al.*, 1995; Salmi and Jalkanen, 1997).

It is significant, considering results of adhesion molecule blocking experiments, the knockouts that successful knockouts tend to exhibit a less compromised phenotype than anticipated. The findings highlight, therefore, the redundancy and overlap of function of most adhesion molecules (Salmi and Jalkanen, 1997). Other problems in the interpretation of results obtained in genetically modified mice have been reported. For example, the absence of a specific molecule may not be the only difference between the knockout mouse and the wildtype that affects T cell migration (Westermann *et al.*, 2001). A study investigating the effects of CCR7 deficiency on lymphocyte migration found that the observed reduction in migration was due not only to the absence of CCR7 receptor on T cells, but also to a significant reduction in L-selectin expression on T-cells (Forster *et al.*,

1999). In addition, it has been reported that the density of the T cell population in LN of genetically modified mice can be reduced, compared with wild-type (Chaplin and Fu, 1998). This change in cellularity can affect T cell survival (Pilling *et al.*, 2000) and the rate of migration through LN (Stekel *et al.*, 1997), possibly contributing to differences between the lymphocyte migration observed in genetically manipulated models compared with other models of migration (Westermann *et al.*, 2001).

1.10.4.6 Models of microvascular endothelial cell culture

EC from the microvasculature of a wide range tissues have been cultured *in vitro*, in part to address the issue of the known heterogeneity between EC from different sized vessels (for a discussion of heterogeneity in EC refer to Section, 1.3.5). Human microvascular EC have been cultured but because of the difficulties in obtaining human tissue samples, most studies have used material from rat, bovine, ovine, primate, guinea pig, and mouse. Microvascular EC have been grown from tissues such as the the central nervous system (Hess *et al.*, 1996; Greenwood *et al.*, 1995; Male *et al.*, 1995; Hughes *et al.*, 1988; Diglio *et al.*, 1982), skin (Issekutz 1992b; 1992c; Swerlich and Lawley, 1993; Haskard *et al.*, 1987; Lee *et al.*, 1995; McCormick *et al.*, 1997), retina (Mesri *et al.*, 1994; 1996), ovary (Mayerhofer *et al.*, 1992), heart (Wysocki and Wykretowicz, 1993; Issekutz, 1990; Issekutz and Wykretowicz, 1991; Issekutz, 1992; Nishida *et al.*, 1993; Grafe *et al.*, 1994), lung (Magee, 1994; Taylor *et al.*, 1997; Muller *et al.*, 2002; Gerritsen *et al.*, 1995), gastrointestinal tract (Haraldson *et al.*, 1996; Binion *et al.*, 1997), adipose tissue (Kern *et al.*, 1983; Hewett *et al.*, 1993), kidney, (Muczzynski *et al.*, 2003) and lymph node (Masinovsky *et al.*, 1990).

1.10.4.6a Synovial EC

Human synovial EC have also been cultured (Jackson *et al.*, 1989; Abbot *et al.*, 1992; Gerritsen *et al.*, 1993; To *et al.*, 1996) and surface expression of adhesion molecules by these cells has been investigated. To my knowledge *in vitro* adhesion assays have not been carried out on EC from this tissue. Furthermore, there do not appear to have been any

studies on adhesion to microvascular EC from inflamed joints in the AA model of polyarthritis.

1.10.4.6b Microvascular EC and adhesion studies

Published studies on lymphocyte adhesion to microvascular EC from normal animals have required expansion of cell numbers *in vitro* and stimulation of the cells prior to use to induce or increase expression of surface CAMs. Surface expression of CAMs in response to bacterial lipopolysaccharide or proinflammatory cytokines is used to mirror expression of the molecules in inflamed tissues. *In vitro* experimental models have allowed the use of blocking antibodies to determine importance of specific CAMs in lymphocyte-EC adhesion.

EC cultured from normal rat heart microvasculature have been used to investigate lymphocyte adherence to either stimulated or non-activated cells (Issekutz, 1990). As with HUVECs, increased lymphocyte adhesion was observed after *in vitro* stimulation of the EC. This *in vitro* model has been used also to investigate the functional implications of VLA-4 expression by lymphocytes, using a mAb against these rat CAMs (Issekutz and Wykretowicz, 1991) and LFA-1 (Issekutz, 1992b; 1992c). As discussed earlier, the advantage of using microvascular cells, compared with those from large vessels, is that they respond differently to inflammatory mediators and, unlike HUVECs, they are cells from vessels that are commonly subjected to inflammatory pathological processes. It is evident that EC from different vascular beds display heterogeneous responses to stimulation by inflammatory mediators (Section 1.3.5).

In vitro investigations have shown that blockade of VLA-4 reduces adhesion of small peritoneal lymphocytes to cytokine stimulated cardiac microvascular EC by 60%. The antibody also inhibited the adherence of lymphoblasts to stimulated EC by 40% (Issekutz and Wykretowicz, 1991). Lymphocytes from LN adhered poorly to unstimulated cardiac microvascular EC, but lymphoblasts showed some adhesion that was inhibited by the VLA-4 mAb (Wysocki and Issekutz, 1992). Adhesion of peritoneal exudate T lymphocytes to rat aortic macrovascular EC was partially inhibited by blockade of VLA-4, but to a lesser

extent than adhesion to microvascular EC (Wykretowicz and Issekutz, 1993).

The source of the lymphocytes used in assays is, therefore, an important factor that influences dependence on particular CAMs for adhesive interactions with EC. Antibodies against LFA-1 reduced adhesion of small peritoneal lymphocytes to cytokine stimulated microvascular EC by just 10%, while adhesion of splenic lymphocytes was inhibited by 50-60%. The same antibody blocked adhesion by splenic lymphocytes to unstimulated EC by only 30% (Issekutz, 1992c). *In vitro* studies by Haskard *et al.* (1996), using HUVECs, has established that LFA-1 is important in the adhesion of phorbol-ester stimulated peripheral blood lymphocytes.

1.10.5 Studies of adhesion molecule functions in arthritis

The following discussion focuses specifically on investigations into adhesion or cell migration in synovial tissues from patients with RA, or in rat models of arthritis. Comparison with non-articular tissues such as skin will be used as appropriate, including studies of non-articular tissues in rats inoculated with CFA.

1.10.5.1 The use of the Stamper-Woodruff in vitro adhesion assay in arthritis.

The Stamper Woodruff assay has been used to study lymphocyte adhesion to synovial tissues from patients with RA (Stamper and Woodruff, 1976). Salmi and Jalkanen carried out a number of such studies, investigating the adhesion of lymphocytes to inflamed human synovium. They showed differences in lymphocyte binding between HEVs in synovium from arthritic joints, compared with HEVs in peripheral LNs. They identified the adhesion molecule VAP-1 in synovial vessels (Jalkanen *et al.*, 1986) and have shown that blockade of VAP-1 had a marked inhibitory effect on the adhesion of activated lymphocytes from the inflamed gut to synovial vessels (Salmi and Jalkanen, 2001; Salmi *et al.*, 1997b).

1.10.5.2 The use of rodent models in arthritis

Rodent models have long been used to study lymphocyte migration and adhesion in inflamed tissues. Blocking studies using monoclonal antibodies are widely utilised to

investigate these processes, as a range of mAbs have been raised against rodent CAMs (Kulidjian *et al.*, 1999). *In vivo* studies of this kind can only be carried out in small animals, as large animals would require prohibitive amounts of antibodies. Studies contrasting the migration of lymphocytes to skin and joint, using lymphocytes from sources such as peritoneal cavity, spleen, LN or Peyers patch, have shown that the origin of the lymphocytes is an important determinant of migration to joints (Issekutz and Issekutz, 1991a; 1992b).

1.10.5.3 In vivo migration studies in rat models of arthritis

Issekutz and co-workers have carried out a number of studies *in vivo,* investigating migration of lymphocytes to sites of inflammation. These studies include migration of lymphocytes to joints in rats with CFA induced AA, with comparisons to traffic to skin in response to cytokine or intradermal inoculation of CFA. Because skin is easily accessible, it is a convenient organ for comparison in lymphocyte migration studies (Issekutz and Issekutz, 1988). The assessment of the effects of experimental manipulation on lymphocyte migration has been either by observation of clinical outcomes of disease or by the detection of radiolabelled cells that have migrated to the tissues (Issekutz and Issekutz, 1991a; 1991b). Blocking studies with mAbs against CAMs has allowed the identification of individual molecules that are participants in the process of lymphocyte migration in these models.

1.10.6 Identification of CAMs in arthritis by the use of blocking antibodies; in vivo and in vitro models of arthritis.

1.10.6.1 Selectins

The selectins have been implicated in the early adhesive events that precede lymphocyte migration. Studies with L selectin deficient mice have established a major role for L-selectin in T cell migration to inflamed peritoneal tissues (Tedder *et al.,* 1995) and inflamed gut mucosa (Salmi and Jalkanen, 2001). Anti-L-selectin antibody caused significant (but not complete) inhibition of adhesion by peripheral blood lymphocytes to endothelium in inflamed, human synovium (Fischer *et al.,* 1993). The latter findings indicate that L-

selectin ligands are expressed by venules in RA synovium, and that this molecule may have a role in lymphocyte migration to the inflamed joint. However, other adhesion molecules may also be involved.

A role for E-selectin in joint inflammation has also been indicated. Earlier studies have shown that lymphocytes isolated from inflamed synovial membrane or synovial fluid bind recombinant soluble E-selectin *in vitro* (Postigo *et al.*, 1992). The proportion of synovial lymphocytes from patients with RA that bind soluble E-selectin, was significantly higher than in lymphocytes from peripheral blood. Significantly, treatment with mAb against E-selectin early in the pathogenesis of AA inhibited development of the disease (Issekutz *et al.*, 2001). Although this demonstrates a role for E-selectin, further work is needed to fully define the function of the molecule in lymphocyte adhesion and migration to synovium.

In contrast to E-selectin, treatment with mAb against P-selectin had no effect on the clinical course of AA (Issekutz *et al.*, 2001). However, Grober *et al* (1993), using the Stamper-Woodruff assay, did show that leucocyte adherence to venules in inflamed synovium was blocked by 90-95% by antibodies against P-selectin (Grober *et al.*, 1993), this suggests a role for P-selectin in the early stage of the adhesion cascade. P-selectin has also been reported to have a role in the recruitment of monocytes and PMN to inflamed joints in active AA, possibly in concert with VLA-4 or LFA-1.

Blockade of both P-selectin and VLA-4 together inhibited monocyte migration by 83%, while blockade P-selectin together with LFA-1 blockade led to 85% inhibition of PMN migration to AA joints (Issekutz *et al.*, 2003). Walter and Issekutz (1997) showed that antibody against P-selectin, but not antibody against E-selectin, blocked migration of peripheral blood monocytes to joints in AA by approximately 50% and Birner and co-workers demonstrated approximately 40% reduction of migration of PMN cells (Birner *et al.*, 1999). They, like the other members of the selectin family of CAMs, appear to have a role in migration of leucocytes to joints, particularly in concert with other CAMs, such as LFA-1 and VLA-4.

At non-articular sites, such as skin, the role of the selectins in leucocyte migration to skin is well established. In dermal inflammation, the selectins are believed to play a major role in the migration of PMN and monocytes. Blocking studies show that P and E-selectin, in combination with L-selectin, appears to account for approximately 90% of monocyte and PMN cell migration to inflamed skin (Walter and Issekutz, 1997). However, as in synovium, it appears that P- or E- selectin play minor roles in the migration of activated T cells to the skin, although they appear to synergise VLA-4 (Issekutz and Issekutz, 2002).

1.10.6.2 Integrins

VLA-4 (CD49d/29), a member of the β1 integrin CAM family is expressed by a number of different leucocytes. VLA-4 appears to synergise with other CAMs in migration of leucocytes to inflamed joints. Investigations using gene knockout technology has not been possible because knockout of the gene VCAM-1 is lethal. However, a role for VLA-4, in the pathogenesis of RA has been suggested by observations using the Stamper-Woodruff binding assay, applied to sections of RA synovium (van Dinther-Janssen *et al.,* 1991). Blocking studies using mAb in the rat, have demonstrated a role for VLA-4 in the trafficking of T lymphocytes to joints affected by AA. Blockade by anti-VLA antibodies reduced recruitment of peritoneal exudates T lymphocytes to inflamed joints by 40-50% (Issekutz and Issekutz, 1991b).

The reported effect of blockade in combination with other CAMs, have been variable. For example, blockade of VLA-4 complemented E-selectin blockade in suppressing the development of AA (Issekutz *et al.*, 2003) and reduced the severity of adoptively transferred AA by greater than 65%. This treatment resulted in a reduction of leucocyte infiltration and limited degradation of articular cartilage. In contrast, blockade of VLA-4 and P-selectin together, had the effect of exacerbating severity of clinical disease, compared to blockade of VLA-4. When VLA-4 and E-selectin were blocked simultaneously, there was delayed onset of disease, but no significant reduction in disease severity. Surprisingly, blockade of VLA-4, P-selectin and E-selectin together led to an almost complete abrogation

of the disease (Issekutz *et al.*, 2003). These findings indicate that combinations of CAMs act in concert to facilitate adhesive interactions and migration of luecocytes to inflammatory sites.

At non-articular sites, VLA-4 appears to be involved in the migration of lymphocytes to sites such as the skin. The adhesion molecule VLA-4 is important in migration of lymphocytes from peritoneal exudate to inflamed skin in response to a DTH response induced by *M. butyricum* in mineral oil (Issekutz, 1991; Issekutz, 1993; Issekutz and Issekutz, 1993). It has been implicated also in lymphocyte migration to allergen induced inflammation of the respiratory tract (Nakajima *et al.,* 1994), complement-induced lung injury (Mulligan *et al.,* 1993a;1993b), EAE (Yednock *et al.,* 1992) and migration of lymphocytes from spleen to mucosal tissues (Palecanda *et al.,* 1999).

In the case of LFA-1, mAb TA-3 (directed against the α-chain, CD11a), administered to rats with AA blocked migration of polymorphonuclear cells to joint by 50%. This mAb did not affect migration of spleen lymphocytes to inflamed joints (Issekutz and Issekutz, 1993). However, a later study by the same group showed that another mAb (WT.3) (directed against the common β-chain of the β2 integrins, CD18), reduced migration of spleen T lymphocytes to inflamed joints by 56-75%. Furthermore, simultaneous treatment with antibodies against VLA-4 and CD18 led to amelioration of clinical disease (Issekutz *et al.*, 1996). Although T cell migration to inflamed joints in rats is only inhibited partially by antibody against CD18, therapeutic blockade in rabbits inhibited synovitis and ameliorated joint damage in experimentally induced arthritis (Jasin *et al.*, 1992). On the other hand, in adoptively transferred AA, blockade with antibody against LFA-1 alone did not affect the severity of the disease or the time of onset. Furthermore, it did not add to the inhibitory effect of VLA-4 blockade (Issekutz *et al.*, 2003). This suggests that the effect on blockade of the LFA-1/ICAM-1 interaction affects the initiation of the immune response that leads to AA, rather than the recruitment of effector T cells to the synovium.

At non-articular sites blockade of LFA-1 by mAb TA3 inhibited migration of lymphocytes

to inflamed skin. The antibody blocked the migration of lymphocytes derived from an inflammatory exudate to cutaneous DTH reactions by 80% (Issekutz, 1993). In support of the role of LFA-1, administration of antibody against ICAM-1, which blocks one of the ligands of LFA-1, led to suppression in the development of AA (Iigo *et al.*, 1991). Again, this observation may not reflect only interactions between effector T cells and EC but could also involve LFA-1/ ICAM-1 interactions between T cells and antigen presenting cells.

It is evident that various adhesion molecules play a role in adhesion and /or migration of leucocytes and that there is substantial overlap of CAM actions (Section 1.6.1). This notion is supported by numerous studies that have shown that more than one adhesion molecule is responsible for adhesion of leucocytes to synovial vessels and migration of leucocytes into synovium. There is evidence for differences in migration of lymphocytes through various tissue beds, for example the different contributions of VLA-4 and LFA-1 to adhesive events in traffic to skin compared with joint tissues and for differences in migration patterns between leucocytes of different origins. In addition, at least one CAM shows some specificity to the synovial vessels (VAP-1). The fact, that mucosal lymphocyte homing to gut is influenced by expression of $\alpha_4\beta_7$, which may also be involved in recruitment of lymphocytes to synovium, shows that adhesion molecules can favour recruitment to more than one tissue site. The example of $\alpha_4\beta_7$ may help explain the well-recognised clinical association between bowel inflammation and arthritis.

1.11 Focus of project

CD4+ T cells that arrive in joints from the blood circulation appear to play an important role in the inflammatory processes of RA. A primary focus of this study is the interaction of CD4+ T cells with synovial microvascular ECs during synovial inflammation.

An animal model of poly-arthritis, adjuvant arthritis has been used to gain further

understanding of interactions between lymphocytes and ECs during the inflammatory process. AA is reliable and robust as a model of polyarthritis. It is highly reproducible in both the severity of the disease in timing the onset and the peak of inflammation, and in the appearance of arthritogenic cells in the central lymphatics.

Various approaches have been used to investigate the adhesive interactions between leucocytes and vascular EC, and the subsequent migration of the leucocytes. These include *in vivo* migration studies, using experimental models of arthritis; *in vitro* adhesion assays, utilising EC from large vessels (for example, HUVECs, or EC from rat or bovine aorta), or adhesion to frozen tissue sections of inflamed synovial tissue, the Stamper-Woodruff assay. Adhesion assays using monolayers of EC are useful model systems, although it is now accepted widely that there is heterogeneity in the expression of adhesion molecules on EC between different sized vessels, between different vascular beds and after various periods of culture *in vitro*. An alternative approach is to investigate interactions between leucocytes and EC isolated freshly from the microvasculature of the inflamed joint. Furthermore, the availability of TD lymphocytes from rats with established AA provides lymphocytes that have proven arthritogenic potential for studies of adherence to syngeneic synovial microvascular EC from the same disease model.

1.12 General hypotheses and aims

Hypotheses:

- Rat synovial microvascular EC can be isolated and cultured and used in assays for adherence of syngeneic lymphocytes.

- Monoclonal antibodies (mAb) can be raised in the mouse against antigens on synovial EC isolated from arthritic rats.

- One or more murine mAbs, raised against rat synovial EC can inhibit adhesion of rat lymphocytes to synovial microvascular EC *in vitro*.

- One or more murine mAbs selected on the basis of the above properties can inhibit expression of actively-induced AA or the adoptively transferred disease, as assessed clinically and by quantitative immunohistochemistry.

Aims

- To identify a suitable tissue from which to purify synovial microvascular EC

- To develop a method to purify and culture vascular endothelium isolated from the microvasculature of rat synovium.

- To examine adhesive interactions between lymphocytes (from normal and arthritic donors) and freshly isolated EC in micro-culture.

- To use lymphocytes, collected by thoracic duct cannulation of normal rats or rats in the prodromal period of AA, in adhesion assays.

- To examine the phenotypes of migrated T cells found in the inflamed synovial-rich tissues using flow cytometry.

- To examine the nature of the receptor-ligand interactions responsible for adhesion of leukocytes, using mAb directed against known adhesion molecules.

- To raise further mAbs against synovial ECs from inflamed joints.

- To screen the ability of the new mAbs for their ability to block lymphocyte-endothelial interactions, and to identify novel homing receptor/addressin interactions that are involved in lymphocyte recruitment to synovium.

Chapter 2

Materials & Methods

2.1 Animals

Female inbred specific pathogen-free Dark Agouti (DA) rats were obtained at the age 7 weeks from the Gilles Plains Animal Resource Centre (Adelaide, Australia). Within each experiment, all rats were from the same source and the same weaning. Prior to use, the animals were housed in the Institute of Medical and Veterinary Science animal holding facility. Standard rat pellets and water were available *ad libitum*.

2.2 Induction of adjuvant arthritis

Adjuvant-induced arthritis was initiated in 7-10 week old DA rats by subcutaneous injection of 0.1 ml of Complete Freund's Adjuvant (CFA), just caudal to the base of the tail. The adjuvant consisted of Incomplete Freunds Adjuvant (IFA) (Difco, Detroit, MI), containing 10mg/ml of heat-killed *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). Essentially all rats that received this treatment developed polyarthritis.

2.3 Preparation of arthritic hind paws for histological survey

Thirteen to 14 days after induction of arthritis, the rats were euthanased by intraperitoneal (ip) injection of 0.1ml Lethabarb (pentobarbitone sodium 325mg/ml) (Virbac, Peakhurst, NSW, Aust). The tibia and fibula were cut above the ankle just distal to the soleus muscle, such that approximately the distal half of the hind limb, including the paw was detached. The skin along the medial and lateral aspects of the limb and of the plantar and dorsal surface of the paw was perforated using a scalpel blade, taking care not to damage the underlying tissues. The detached limbs were fixed in 10% formalin for 24 hours. Following fixation, the feet were washed in 3 changes of 0.1M phosphate buffered saline

(PBS) at pH7.2. Decalcification was carried out at 4°C in TEP decalcifying solution, comprising 0.1M Tris buffer, 10% EDTA and 7.5% polyvinylpyrrolidone (Jonsson *et al.*, 1986). The decalcifying solution was changed every 48 hours. Decalcification took approximately 4 weeks to complete, with the endpoint of the process detected by x-ray photography.

Decalcified whole feet were washed in two changes of PBS and then dehydrated through a graded series of ethanol solutions, from 50% through 70%, 85% and 90% to absolute ethanol. The feet remained in each ethanol solution for 24hrs, with 2 solution changes each day. Following dehydration, the tissue was cleared in cedar wood oil for 48hrs at room temperature. Infiltration of molten Paraplast-plus tissue embedding medium (Oxford Labware, St Louis, MO, USA) was achieved after 8 hours under vacuum at 20mmHg at 57°C and the tissues were then embedded in the same medium.

Transverse and longitudinal sections of paraffin embedded whole feet were cut at 8μm using a standard rotary microtome. Sections were collected at 80μm intervals, mounted on albumin-coated glass slides and dried overnight at 37°C. The sections were then de-waxed, re-hydrated and stained with routine Harris' haematoxylin (BDH Chemicals) and eosin (Gurrs, BDH, Vic) (H&E) as described by Drury and Wallington (1980). Finally, the slides were dehydrated in ethanol, cleared in xylene (BDH, Vic, Aust) and mounted in Gurrs DePeX mounting medium (BDH, Vic, Aust).

2.4 Immunohistochemistry

2.4.1 Harvest of synovial tissue for immunohistochemistry

2.4.1.1 Normal synovium

Synovium from the supra-patella pouch was collected from normal female 6-8 week old DA rats. Rats were euthanased by intraperitoneal injection of Lethabarb (sodium pentabarbitone). Firstly, the skin was removed from the hind limbs, to reveal the patella region. In order to expose the supra-patella pouch, the patella tendon was cut just distal to

the patella. The tendon was then grasped firmly with a large pair of toothed forceps and retracted in a proximal direction. The quadraceps muscle was cut longitudinally, in order to reflect the tissue and reveal the suprapatella pouch. The quadriceps muscles were cut transversely approximately 1 cm above the patella, releasing the infra-patella tendon, the patella and the synovium of the suprapatella pouch attached to the distal fragment of the quadraceps. The supra-patella region was defined further by trimming away excess muscle tissue from the anterior surface of the quadraceps and trimming away the distal ¾ of the patella. The remaining tissue, just superior to the patella was snap frozen in OCT compound (Tissue-Tek, Miles Inc., Elkar, IN) using liquid nitrogen cooled isopentane (BDH Chemicals). Cryostat sections were cut as described in below Section 2.4.2., for use in immunohistochemical studies.

2.4.1.2 Synovium from arthritic paws

An area of tissue that could be reproducibly isolated was located on the posterior aspect of the ankle joint and the tibia (sub-Achilles region). Its position is posterior to the tibia, superior to the calcaneum and distal to the musculo-tendinous junction of the Achilles tendon and the soleus muscle. A small amount of hyperplastic tissue was also harvested from the lateral aspect of the ankle, adjacent to and surrounding the lateral maleolus. The tissue was harvested as described in Section 2.13.2 and embedded in OCT as described above.

2.4.2 Preparation of frozen tissue sections

Tissue blocks were stored at –70°C until required. Sections were cut on a Leica CM 3000 modular cryostat (Leica Instruments GmbH, Nussloch, Germany. Cryostat sections of the tissue were cut at 5-7μm thickness, mounted on chrome alum /gelatin coated glass slides and air dried for 2 hours at room temperature. Frozen sections were then be stored at –20°C for up to one week in a sealed slide box containing Silica gel, (BDH, Poole, England) as a desiccant.

2.4.3 Indirect immunoperoxidase –staining of tissue sections

Stored frozen sections were brought to room temperature and encircled with a (water-repellent) wax pen (Pap Pen, Zymed Laboratories, San Francisco, CA, USA). Sections were fixed by immersion in 96% ethanol (AR grade) at 4°C for 10 minutes, then washed in 3 changes of cold PBS. The final wash contained 0.1% (w/v) bovine serum albumin (BSA) (Multicell Cytosystems, Castle Hill, NSW). Primary antibody, containing 10% heat inactivated normal rat serum (NRS), was applied to the section and the slides were incubated in a sealed, humidified container at 4°C for 1hour. The slides were then washed in 3 changes of cold PBS. The secondary antibody, sheep anti-mouse Ig, horseradish peroxidase (HRP) conjugated (Amersham), that had been diluted 1:20 in PBS with 10% NRS, was added to each section and incubated at 4°C for 1 hour.

Localisation of bound antibody was visualised by production of a coloured, insoluble product from 3,3'-diaminobenzidine peroxidase (DAB), 1.1 mg/ml (Sigma Fast™, Sigma, St Louis, Missouri, USA) by oxidation with reactive oxygen species released from H_2O_2 by the action of horseradish peroxidase. DAB was prepared just prior to use. Each section was flooded with the DAB solution using a transfer pipette and incubated for 10 minutes at room temperature. The slides were washed in 2 changes of fresh PBS and then counterstained lightly with haematoxylin for 30 seconds. Following this step, the slides were washed in running tap water, dehydrated in 3 changes of absolute ethanol for 2 minutes each, cleared in 2 changes of xylene and mounted with glass coverslips using Gurrs DePeX clear mounting media (BDH, Poole, England).

2.4.4 Immunoperoxidase staining of cell monolayers

Monolayers of adhered cells were labelled with monoclonal antibodies using a method modified from that used for frozen sections. Cells were seeded into fibronectin or gelatin coated wells of an 8 well Lab-Tek™ Chamber Slide™ System (Nalge Nunc Int, Denmark) and allowed to adhere to the surface at 37°C with 5% CO_2 in a humidified incubator.

Prior to washing, monolayers of cells were first allowed to equilibrate to room temperature. Cell growth media was removed and the wells were washed twice with PBS at room temperature. The cells were then fixed with 96% ethanol (AR grade) (AnalaR, Merck Pty Ltd, Vic, Aust) at 4°C for 10 minutes and washed in 3 changes of cold PBS. The fixed cells in each well were incubated with primary antibody containing 10% heat inactivated NRS for 1hour at 4°C with horizontal rotation and then washed with 3 changes of cold PBS. Sheep anti-mouse peroxidase conjugated (HRP) secondary antibody (Amersham), diluted 1:20 in PBS containing 10% NRS was applied to each well and incubated at 4°C for 1 hour with horizontal rotation.

Localisation of bound antibody was visualised by DAB, as described in section 2.4.3, except that following counterstaining, the slide chamber was removed before further processing could occur. Once the Labtek chambers were removed from the slides, the silicone sealant was peeled away and the slides were washed gently in tap water, dehydrated in 3 changes of absolute ethanol for 2 minutes each, cleared in 2 changes of xylene (BDH, Merck Pty Ltd, Vic, Aust) and mounted with glass coverslips using DePeX clear mounting media (Gurrs) (BDH, Merck Pty Ltd, Vic, Aust).

2.4.5 Cell Smears

An aliquot of approximately $1x10^6$ cells was pelleted by centrifugation, the media removed and the cells resuspended in 30µl of heat inactivated fetal calf serum (FCS). A volume of 10µl of this cell suspension was placed on a clean glass slide and the suspension spread in a circular motion to approximately 20mm diameter with a wooden orange stick. The cells were deposited onto the slide by holding one end of the slide and flicking it with downward force. This ensured a good separation and flattening of cells in the smear preparation. The smear was then allowed to air dry at room temperature and the cells were stained as for tissue sections (as described in Section 2.4.3.) or frozen tissue sections as in the case of immunoperoxidase (as described in Section 2.4.3.).

2.5 Microscopy

Sections stained with H&E or by the immunoperoxidase technique were examined using either an Olympus BH2 light microscope, or an Olympus dissecting light microscope (to view sections of the whole foot). Light photomicrographs were taken using Ektachrome 64T ASA colour reversal film (Kodak, UK).

An Olympus BH-2 microscope with a BH2-RFL epi-illumination fluorescence attachment (Olympus, Tokyo, Japan) was used to examine cells labelled for immunofluorescence. CFSE-labelled cells were observed using 530nm barrier filter and fluorescent labelling of EC using low density lipoprotein that has been labelled with the fluorescent probe, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL), (Biomedical Technologies, Stoughton, MA, USA), as described in Section 2.13.7 were observed using a 590nm barrier filter and a green dichroic mirror. Cultures of live, unstained cells were observed and photographed using an Olympus CK2 inverted microscope with a phase ring attachment. Live or fixed cells that have been fluorescently labelled were observed and photographed using an Olympus IMT-2-RFL (fluorescence attachment). Phase contrast or light photomicrographs were taken using Ektachrome 64T ASA colour reversal film (Kodak, UK), while fluorescence photo micrographs were captured using Ektachrome 400 ASA colour reversal film (Kodak, UK) or T-Max 400 ASA for black and white photomicrographs.

2.6 Cell culture

2.6.1 General

All procedures for collection, isolation, culture and manipulation of cells were performed under aseptic and pyrogen-free conditions. Solutions and media were prepared using water purified in a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, USA) and sterilised by filtration through 0.22μm (Millex, Millipore) filters, 0.2μm Acrocap Filter Unit (Pall Gelman, Ann Arbor, MI, USA) or by autoclaving. Disposable plastic culture-ware and pipettes were used throughout. Glassware was washed in E-toxa-clean (Sigma-

Aldrich, Irvine, UK), rinsed extensively in MILLI-Q water and sterilised by autoclave prior to use. The condition of cell cultures was examined using an Olympus CK2 phase contrast inverted microscope (Olympus, Tokyo, Japan). All cell cultures were maintained at 37°C in a humidified atmosphere incubator containing 5% CO_2 in air.

2.6.2 Preparation of Fetal Calf Serum

Prior to use, fetal calf serum (FCS), normal rat serum (NRS), normal mouse serum (NMS), normal human serum (NHS) and normal sheep serum (NSS) were all heat inactivated. This was achieved by heating the serum in a water bath at 56°C for 45 minutes. This treatment removes complement and reduces the cytotoxic actions of immunoglobulins without affecting polypeptide growth factors.

2.6.3 Preparation of culture medium

Roswell Park Memorial Institute (RPMI-1640) culture medium was used in all cell culture work, except where indicated. RPMI-1640 was prepared by dissolving a sachet of RPMI-1640 powder (Life Technologies, Gibco, Grand Island, NY, USA) and 2g $NaHCO_3$ (Univar, Ajax Chemicals, Aust.) in 900ml of Milli-Q-purified water (MILLI-Q H_2O). Sterile stock solutions of N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer Mannheim, Germany), pH7.2, penicillin (CSL, Parkville, Vic, Aust.) and gentamicin (DBL, Fauldings, Adelaide, Aust) were added to give final concentrations of 10mM, 1.2mg/ml and 1.6mg/ml respectively. The pH was adjusted to 7.4 with 1M HCl. The volume was made up to 1 litre and the medium was filter-sterilised using an AcroCap™ 0.22μm filter unit with filling bell (Pal-Gelman Sciences, Ann Arbor, MI, USA).

Prior to use, medium was supplemented with 2mM L-glutamine (Gibco, Vic, Aust) and either 1%, 10% or 20% v/v heat inactivated (45mins @ 56°C) FCS (Gibco, LIFE Technologies, NZ). Medium was re-supplemented with L-glutamine after 7 days storage at 4°C.

2.6.4 Conditioned Medium

Conditioned media support vigorous growth, providing factors not present in synthetic media. Conditioned medium, containing a mixture of undefined growth factors, was used as an additive to culture medium when culturing difficult-to-grow cell types (eg hybridoma cells).

Conditioned medium for use as a supplement in growing hybridoma cells, was produced by culturing murine macrophage cell line J774 (obtained from ATTC.org, USA) in RPMI-1640 10% FCS complete with antibiotics and L-glutamine. The cells were grown until the media was acidified, observed by change in colour of the neutral red pH indicator. The medium was harvested and cells were pelleted by centrifugation. The conditioned spent medium was collected asceptically and stored at –70°C until required. Initially, conditioned medium was used as an additive to usual growth medium (RPMI-1640 10%FCS) at a concentration of 10% J774 additive (v/v). Once vigorous cell growth was established, the concentration of J774 was reduced over a period of days to 5% and then further reduced until, within a 7 day period, the cells were 'weaned off ' the additive completely.

2.6.5 Culture of endothelial cells

2.6.5.1 Culture media

Rat EC were cultured under conditions developed for long-term culture of HUVECS (personal communication, J. Gamble; Gamble and Vadas, 1988) with the exclusion of EC growth supplements. These EC grew well and replicated in growth media composed of RPMI-1640 including L-glutamine, antibiotics and 20% heat inactivated fetal calf serum. It was found that primary cultures of rat EC do not require presence of endothelial growth supplements (ECGS) or heparin. Cultures of EC that were passaged did have ECGS (20μg/ml) and heparin (20μg/ml) added to their culture medium.

2.6.5.2 Fibronectin

To provide a matrix for EC adherence, culture wells were coated with fibronectin (Boehringer Mannheim) purified from human plasma. Wells of Terasaki plates were coated with 2µl of fibronectin per well (at a concentration of 50µg/ml in sterile PBS). After 45 minutes at room temperature, excess fibronectin was removed from the wells, they are washed with sterile PBS and filled with RPMI-1640 20% FCS.

2.6.5.3 Gelatin

To provide a matrix for EC adherence, culture wells were coated with gelatin type B (Sigma, USA) purified from bovine skin. The flasks or wells were coated with gelatin at a concentration of 25µg/ml in sterile PBS. After 15 minutes at room temperature, excess gelatin was removed from the wells with a sterile PBS wash, followed by RPMI-1640 20% FCS.

2.7 Antibodies

2.7.1 Primary antibodies

A large number of antibody-secreting hybridoma were raised against cells isolated from the arthritic rat joint, as described in Section 2.13. In addition, a number of existing mouse anti-rat monoclonal antibodies (mAbs) were used in adhesion assays, flow cytometric and immunohistochemical analysis. The existing mAbs used are listed in Table 2.1. They were either produced from hybridomas in a collection maintained by the Department of Microbiology and Immunology (Molecular Biosciences), or were purchased from commercial sources.

Hybridomas were stored in liquid nitrogen in media containing 10% DMSO. When required, cell lines were cultured in 75cm^2 Polystyrene Tissue Culture Flasks (Corning Costar Corp, Cambridge, MA, USA) in medium RPMI-1640/10% FCS at 37°C, 5% CO_2 in air in a humidified incubator.

2.7.2 Detection of primary antibodies

To prevent residual cross-reactivity with rat Ig, 10% NRS was added during incubation with the secondary anti-mouse Ig conjugates. FITC-conjugated affinity purified goat anti-mouse IgG (Pharmingen, USA) was used for immunofluorescence and affinity purified F(ab')2 sheep-anti-mouse Ig conjugated to horseradish peroxidase (Amersham Life Sciences, Buckinghamshire, England) was used in the indirect immunoperoxidase technique for immunohistochemistry.

2.7.3 Directly labelled mAbs

In order to carry out 2-colour flow cytometric analyses, several phycoerythrin (PE) – conjugated mouse anti-rat mAbs were purchased from Pharmingen. These are R73-PE (anti-rat α/β TCR), OX38-PE (anti-rat CD4), OX8-PE (anti-rat CD8α) and 10/78-PE (anti-rat CD161). PE-conjugated mAb 107.3 (anti-trinitrophenol [TNP]) was utilised as a negative control. Labelling of cells with antibody for both flow cytometry and immunocytochemistry was carried out in the presence of 10% heat inactivated NRS to block non-specific binding through Fc receptors.

2.7.4 Dialysis of monoclonal antibodies

Azide is used routinely as an anti-microbial agent to preserve supernatants of monoclonal antibodies. Prior to use in functional assays on live cells, the azide was removed from culture supernatants by dialysis. Approximately 150mm of dialysis tubing (size 8; 25.4mm; MWCO-12-14000 Daltons) (Medicell International Ltd, London, UK) was cut and prepared by boiling in RO water for 10 minutes. The distal end was tied carefully in a knot and 3-5ml of antibody supernatant was added. A firm knot was then tied in the proximal end of the tubing. Dialysis pouches were then placed in a 5 litre, standard laboratory glass beaker filled with cold PBS. Dialysis was carried out over a 4 to 5 day period at 4°C with continuous stirring using a magnetic stirring bar. The PBS was replaced each day with fresh cold PBS. After dialysis the antibodies were filter-sterilised using a syringe operated 13mm Millex-GV_{13} unit 0.22μm (Millipore Products Div, Bedford, MA, USA) and stored at –70°C until required.

2.7.5 Concentrating monoclonal antibodies

To concentrate antibodies for cell adhesion assays, a Centriprep 30, Centrifugal Filter Device (Amicon Inc, Beverly, MA , USA) was used. Approximately 16mls of azide free (dialysed) mAb was placed in the Centriprep 30. Concentration was carried out by centrifugation for 20 minutes at 1500rpm at 4°C using a Beckman GS-6R (swinging bucket) Centrifuge (Beckman, Palo Alto, CA, USA). Antibodies were concentrated about 4 fold, to a volume of approximately 4ml. Concentrated antibodies were then aliquoted at 50µl per tube, snap frozen in liquid nitrogen and stored at –70°C until required.

2.8 Preparation of a cell suspension

2.8.1 Sterile lymphocyte suspensions from lymph nodes

Rat lymph nodes were removed aseptically and cells were prepared within the sterile environment of a lamina flow hood. Sterile tissue culture grade glassware or plastic disposable tissue culture items and sterile surgical grade instruments were used throughout. Filters consisted of the barrel of a 10ml single-use plastic syringe that was packed loosely with absorbent cotton wool prior to autoclave sterilisation.

Rats were euthanased by intraperitoneal injection of 0.1ml Lethabarb (pentobarbitone sodium 325mg/ml) (Virbac, Peakhurst, NSW, Aust). Using aseptic technique, lymph nodes from cervical, axillary, superior mesenteric, iliac, inguinal, popliteal, para-aortic, brachial and caudal regions were harvested using curved forceps and placed in 1ml sterile RPMI-1640 1% FCS. Tissues were diced finely with curved scissors and the resulting cell suspension was transferred to a loose fitting glass homogeniser, where it was dissociated

Table 2.1 Mouse anti-rat monoclonal antibodies used in this study. Target antigens of monoclonal antibodies, their reported distributions on rat cells and tissues, the isotypes of the antibodies and their sources are listed.

Abbreviations:

SN	-antibody culture supernatant	EC	-endothelial cells
P	-purified antibody	FB	-fibroblasts
Asc	-ascites	mϕ	-monocytes and macrophages
Hybr.	-hybridoma	DC	-dendritic cells
R	-receptor	NK	-natural killer cells

mAb V65 was kindly provided by Dr T. Hunig, Wurzburg, Germany

mAb OX85 was kindly provided by Dr P. Holt, Perth, Australia

mAbs 3D3 & 1D4 were kindly provided by Dr L. Ashmann, Adelaide, Australia

Table 2.1 Mouse anti-rat monoclonals used throughout this study in flow cytometry and immunohistochemistry

Ab clone	Target Antigen		Distribution	Form	Isotype	Source	Reference
R73	α/β TCR		α/β T cells	SN	IgG_1	Hybr.	Hunig et al.,1989
V65	γ/δ TCR		γ/δ T cells	Asc	IgG_1	T Hunig	Kuhnlein et al., 1994
OX19	CD5		peripheral T cells, thymocytes	SN	IgG_1	Hybr.	Dallman et al.,1984
OX34	CD2	(LFA-2)	peripheral T cells, thymocytes, NK cells, macrophages	SN	IgG_{2a}	Hybr.	Jeffries et al., 1985
W3/25	CD4		T cell subset, thymocytes, Mϕ, DC	SN	IgG_1	Hybr.	Williams et al., 1977
OX8	CD8α-chain		T cell subset, thymocytes, NK cells	SN	IgG_1	Hybr.	Brideau et al., 1980
341	CD8β-chain		T cell subset, thymocytes	SN	IgG_{2a}	Hybr.	Torres-Nagel et al., 1992
OX33	CD45RA		B cells	SN	IgG_1	Hybr.	Woolett et al. , 1984

MARK1	rat Ig κ-chain		B cells	SN	IgG_1	Hybr.	Bazin et al., 1984
10/78	CD161		NK cells, T cell subset, Mϕ, DC	SN	IgG_1	Hybr.	Kraus et al., 1996
OX1	CD45	rat L-CA	leucocytes	SN	IgG_1	Hybr.	Sunderland et al., 1979
OX7	CD90	(Thy1.1)	thymocytes, nerves, perivascular cells, fibroblasts, immature B cells	SN	IgG_1	Hybr.	Mason and Williams, 1980
OX22	CD45RC		CD8+ T cells, NK, CD4+ T cell subset,	SN	IgG_1	Hybr.	Woolett et al. 1985
OX6	MHC Class II		B cells, mϕ,DC, activated T cells	SN	IgG_1	Hybr.	McMaster and Williams, 1979
OX26	CD71	(transferr in R)	dividing/proliferation cells	SN	IgG_1	Hybr.	Jefferies et al. 1985
OX39	CD25	IL-2R α	Activated T cells, activated B cells	SN	IgG_1	Hybr.	Paterson et al., 1987
OX40	CD134		activated CD4+T cells	SN	IgG_{2a}	Hybr.	Paterson et al., 1987
WT-1	CD11a		most leucocytes	SN	IgG_{2a}	Hybr.	Tamatani et al., 1991
WT-3	CD18			SN	IgG_1	Hybr.	Tamatani and Miyasaka,1990
WT-5	CD11b	Mac-1 α-subunit	NK cells, mϕ,DC, granulocytes	SN	IgA	Hybr.	Tamatani et al., 1993
W3/13		leukosialin	T cells, plasma cells, NK cells, granulocytes	SN	IgG_1	Hybr.	Kroese et al., 1985
OX50	CD44	Hyaluronate R	Most leukocytes (not on a B cell subset)	SN	IgG_{2a}	Hybr.	Paterson et al., 1987
Mrα4-1	CD49D (VLA-4)	α4 Integrin	T cells, B cells, thymocytes, mast cells, mϕ	P	IgG_{2a}	Pharmingen	Yasuda et al., 1995
1A29	CD54 (ICAM-1)	ICAM-1	B cells, subset of T cells	SN	IgG_1	Hybr.	Tamatani and Miyasaka, 1990
OX85	CD62L	L-selectin	B cells, subset of T cells, neutrophils	SN	IgG_1	P. Holt	Seddon et al., 1996
UA009	CD36		mϕ, endothelial cells, RBC, neutrophil subpop	SN	IgG_1		Zhang et al., 2003
UAO11	Similar CD36	unknown	As above	SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
3A12	CD31		EC, platelets, monocytes, granuloc, T cell subset	Asc	IgG_1	Serotec	Flaris et al. 1993; Male et al. 1995
UA015	PECAM-1 like	unknown	EC	SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
UA016	PECAM-1 like	unknown	EC	SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
UA017				SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
SG1A1				SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
MCA970	RECA-1	Pan EC	Pan endothelial	SN	IgG_1	Serotec	Duijvestein et al. 1992
1B5	Giardia surface antigen	Giardia Neg con		SN	IgG_1	Hybr.	Prof. G. Mayrhofer (unpub)
3D3		Neg con		P	IgG_1	L.Ashman	
1D4	L	Neg con		P	IgG_{2A}	L.Ashman	

gently further by manual homogenisation. The suspension was then filtered through cotton wool. The resulting lymphocyte suspension was washed in 50ml of RPMI-1640 1%FCS and after centrifugation at 1200 rpm for 7 minutes the pellet was resuspended in 10 ml of RPMI-1640 10%FCS. A viable cell count was performed in a standard haemocytometer (Weber Scientific, Teddington, Middlx, UK) using Trypan blue exclusion (0.4% (stock) Trypan Blue Solution, Sigma-Aldrich, Irvine, UK).

2.8.2 Stimulation of Lymphocytes

Lymphocytes harvested from lymph nodes of normal rats were stimulated with 5μg/ml concanavalin A (Con A) (Sigma, St Louis, MO, USA) in RPMI-1640 10% FCS) for periods of time ranging from 24-96 hours. Following stimulation, the cells were washed three times in RPMI-1640 1%FCS. A viability count was carried out in a standard haemocytometer, using Trypan blue exclusion. The cell density was then adjusted to $1x10^7$/ml in pre-warmed RPMI-1640 1%FCS.

2.8.3 Blocking Con A with methyl α-D-mannopyranoside

Incubation of lymphocytes in methyl α-D-mannopyranoside, (a sugar bound by Con A), after stimulation with Con A was used to inhibit binding of residual Con A to cell-surface glycoproteins. After Con A stimulation, the cells were washed and then incubated in 50mM methyl α-D-mannopyranoside (Sigma, StLouis, MO, USA) in RPMI-1640 1%FCS. The lymphocytes were not washed again prior to use and so remained in media containing methyl α-D-mannopyranoside throughout the procedure.

2.8.4 Labelling of lymphocytes with the vital stain, CFSE

Lymphocytes from lymph nodes, or those collected by thoracic duct cannulation, were labelled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA,SE) (more commonly known as CFSE) (Molecular Probes, Oregon USA). A stock solution of 5mM CFSE in Dimethyl sulphoxide (DMSO) (AnalaR, BDH Chemicals, Kilsyth, Vic, Aust) was prepared and stored at –20°C under desiccant. Each aliquot of stock CFSE was thawed a maximum of two times to avoid the loss of activity.

Lymphocytes were labelled by incubating 2mls of cell suspension ($1x10^7$/ml) in RPMI-1640 1%FCS containing 1µl of 5mM CFSE stock. Cells incubated with CFSE solution were incubated for 15 min in the dark at 37°C with continuous rotation. The process was terminated by addition of 20mls of cold RPMI-1640 10%FCS followed by centrifugation. The cells were washed twice in RPMI-1640 1%FCS and resuspended at $1x10^7$/ml in the same medium.

2.9 Thoracic duct cannulation

2.9.1 Construction of cannulae

Surgical procedures carried out in this Section were performed by Ms Sarah Wing, Research Assistant, Arthritis Research Lab, RAH. Cannulae for use in this procedure were constructed from polyethylene tubing (internal diameter 0.5mm & outside diameter 1.0mm) (Dural Plastics & Engineering, Dural, NSW, Australia). For the construction of cannulae, a U-bend was formed at one end of a 50cm length of tubing. The U-bend shape was made permanent by the application of heat to the tubing while held in position. A bevelled edge was then cut at the leading end of the U-bend to facilitate insertion of the cannula into the thoracic duct. The cannula was slightly curved to enable the distal end of the tubing to exit through the dorsal abdominal wall of the rat.

2.9.2 Preparation of the rat

The rats were anaesthetised with isofluorane-nitrous oxide. The ventral surface and the left

side of the body was shaved with electric animal clippers. An intravenous drip, using a 23G needle, was then inserted via the tail vein to deliver 1U/ml heparin (Sigma, St Louis, MO, USA) in PBS.

2.9.3 Surgical procedure

An incision was made through the abdominal wall, approximately 0.5cm caudal to and parallel with the left costal margin and extending from near the midline to the mid-axillary line. The left kidney and adrenal gland were exposed and cotton buds (Johnson & Johnson) were used to reflect these organs to the contra-lateral side of the abdomen, in order to expose the aorta. The temporary relocation of the kidney, adrenal gland and other viscera was secured with saline moistened gauze and stabilised with a retractor. The thoracic duct was separated carefully from the adjacent psoas muscle and aorta, then a tie of surgical silk was passed around it. Following this a 12G needle was passed through the dorsal abdominal wall, adjacent to the tie and this was secured loosely in place by a second tie through the dorsal muscle fibres. The needle was used to pass the cannula through the body wall and then removed. The cannula was primed with 1U/ml heparin in PBS and the distal end clamped. The proximal end of the cannula was inserted and secured firmly into position by knotting the first silk tie. Once flow of lymph was observed, the cannula was further secured by the addition tightening the second tie and securing the knot. The gauze was then removed and the displaced organs were returned to their anatomical position. The wound was closed in layers by suturing the muscle and skin with continuous sutures of surgical silk.

2.9.4 Overnight collection of thoracic duct lymph (TDL)

After surgical insertion of the cannula, the rat was housed overnight in a Bollman metabolic cage and provided with standard rat chow and isotonic saline to drink *ad libitum*. Lymph was collected overnight into a sterile 300ml tissue culture flask, containing 5ml of 20U/ml heparin in PBS at room temperature. The following morning, the collected lymphocytes were resuspended, the lymph filtered through cotton wool and the cells washed twice with RPMI-1640 1%FCS. A cell count and viability assessment was carried out, using a

haemocytometer and Trypan blue exclusion.

2.9.5 Preparation of lymphocytes from inflamed synovial tissue

An area of inflamed synovial tissue from the hind limbs of 6 rats with AA was excised, as described in Section 4.3.2. A single cell suspension was prepared as described in Section 2.13.3. The resulting suspension consisted mainly of single cells and relatively small cell aggregates. The cells were a mixed population, and included inflammatory cells of haematopoietic origin, red blood cells, FBs and EC. The mixed cell suspension was overlayed upon the density gradient, Lymphoprep and centrifuged in order to remove cellular debris and increase the relative numbers of mononuclear cells.

2.10 Preparation of density gradients

2.10.1 Percoll enrichment of lymphoblasts from TD lymph

The osmolality of Percoll was adjusted with PBS to prepare a stock solution. Stock Percoll was prepared by combining 9 parts (v/v) of Percoll to one part (v/v) 10x concentrated PBS. Stock Percoll solutions were diluted to lower densities by diluting (v/v) with Dulbecco's PBS containing Ca^{+2} and Mg^{+2}. Specific concentrations (30, 40, 45, 50, 55%) of Percoll were prepared and discontinuous gradients formed by adding the lightest (lowest density) first and carefully underlayering successively heavier layers in the bottom of V-bottomed 25ml poly-carbonate tubes. This was done using a 10ml syringe fitted with a mixing cannulae (Maersk Indoplas Pty Ltd, Sydney Australia).

The cells to be separated were resuspended at $1x10^8$/ml in the lowest density (30%) Percoll and 2mls of this suspension was layered carefully on top of the gradient. The cell preparation was applied to the surface by running it gently down the side of the tilted tube from a 10ml syringe barrel fitted with a 21G hypodermic needle. The reservoir was suspended from a retort stand, ensuring that the tip of the needle remained against the wall of the tube, just above the surface of the liquid.

Centrifugation of Percoll density gradients was carried out in a bench-top Beckman GS-6R centrifuge (Beckman, Palo Alto, CA, USA) at 400g (1400rpm) for 25 minutes. The cells that had settled to their isopycnic position were collected using a plastic transfer pipette. The Percoll medium was diluted with wash medium, RPMI-1640 1%FCS, 5 volumes to 1 volume of cell suspension. After centrifugation, the washing process was repeated twice and the cells were collected by centrifugation at 1000rpm for 5-7 minutes. The isolated cells were resuspended finally into wash medium and a viable cell count and viability assessment was carried out using a haemocytometer and Trypan blue exclusion.

2.10.2 Lymphoprep enrichment of mononuclear cell preparations from crude synovial cell suspensions from rats in the prodrome of AA

Lymphoprep ™ (Nycomed Pharma AS, Oslo, Norway) is a solution for the isolation of pure lymphocyte suspensions in "a one step centrifugal technique for isolation of lymphocytes". The solution contains 9.1% sodium diatrizoate (w/v) and 5.7% polysaccharide (w/v) with a density of 1.077 and osmolarity of 280mOsm. The procedure was carried out following manufacturer specifications, however a brief description follows.

The crude cell suspension of sub-Achilles synovial cells from arthritic rats prepared by enzymatic digestion (described in Section 2.13.3) was diluted in an equal volume of 0.9% NaCl. The diluted cell suspension was layered over Lymphoprep in a Falcon 50ml centrifuge tube. The tube was centrifuged at 800g for 20 minutes at room temperature. After centrifugation the mononuclear cells that formed a distinct band at the sample /Lymphoprep medium interface were collected using a plastic Pasteur pipette. The harvested cells were diluted with RPMI-1640 containing 1% FCS (wash medium) and centrifuged at 1000rpm for 7 minutes. The supernatant was discarded and the pellet resuspended in was medium. This wash step was repeated 3 times, and the cells counted (as described in Section 2.10.1).

2.10.3 Ficoll-Paque (Nycodenz) cell preparations

Cells were suspended in PBS with FCS before layering over (Nycodenz) Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 500g for 20 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). Mononuclear cells that had formed a band at the interface, were aspirated carefully, then washed and counted (as described in Section 2.10.1).

2.11 Labelling cells for flow cytometric analysis

2.11.1 Antibodies

Monoclonal antibodies used in flow cytometry and their sources are listed in Table 2.1. All hybridoma were either raised in the Department of Microbiology and Immunology, University of Adelaide using Balb/c mice or obtained from other sources such as European Cell Culture Collection, American Cell Culture collection (ATCC), gifts or from commercial sources. Supernatants were used undiluted in the presence of heat inactivated 10% NRS.

2.11.2 Labelling cells for indirect single fluorochrome immunofluorescence

Cells to be analysed by flow cytometry were washed and resuspended at a density of $1x10^6$ cells/ml in RPMI-1640-1%FCS,. Aliquots of 1ml were placed into 5ml polystyrene round-bottom tubes (Falcon, Becton Dickinson, NJ, USA). The cells were pelleted by centrifugation at 1200 rpm for 7 minutes at 4°C and the supernatant was aspirated from the tube. The cells were then resuspended by vortexing and incubated in 50µl of primary antibody, containing 10% NRS, for 1 hour on ice. Unbound primary antibody was removed by washing the cells twice in 2ml aliquots of cold IF buffer (PBS with 1% FCS and 10mM sodium azide [NaN_3]. Following washing, the cells were incubated in 50µl of FITC-conjugated $(Fab^1)_2$ goat anti-mouse IgG secondary antibody containing 10% NRS. The cells were incubated on ice for 45 minutes after vortexing. Finally, they were washed twice with 2 ml aliquots of IF buffer, prior to fixation in 500µl of FACS fix (1% formalin, 2% glucose, 0.2% sodium azide) and stored in the dark at 4°C for subsequent flow cytometric analysis.

2.11.3 Labelling cells for dual fluorochrome immunofluorescence

The method of dual labelling cells for flow cytometry combines both direct and indirect immunofluoresence labelling procedures. The first stage is the same as that described for single colour indirect labelling by the indirect technique (2.11.2). However, after the last wash but prior to fixation, the cells were resuspended in 5µl of normal mouse serum (NMS) and incubated for 10 minutes on ice, to block free valances of the FITC goat anti-rat Ig. Following this, without washing, the cells were labelled directly by the addition of 50µl of PE-conjugated purified antibody containing 10% NRS and incubation on ice, for one hour. The cells were washed twice with 2ml aliquots of IF buffer and fixed in 500µl FACS fix. Labelled and fixed cells were stored in the dark at 4°C for subsequent flow cytometric analysis, as described in 2.11.2.

2.12 Flow cytometry

2.12.1. Acquisition and analysis of data

Labelled cells were analysed using a FACScan (Becton Dickinson, San Jose, Ca, USA) with CellQuest software, version 3.1f. Lymphocyte populations were gated on the basis of their characteristic forward scatter (FSC) and side scatter (SSC). Events were collected from a gate containing lymphocytes. Separate gates were drawn to delineate small from large lymphocytes, on the basis of FSC and SSC characteristics of the lymphocytes. Cells from TDL, LN and the sub-Achilles tissues from arthritic rats were analysed.

2.12.2. Single fluorochrome flow cytometry

Cell suspensions obtained from tissue harvested from inflamed sub-Achilles region, peripheral lymph nodes or from TDL were analysed by flow cytometry. Where necessary, lymphocytes were gated from the mixed population of cells by first identifying their position in the plot of forward scatter and side scatter. This was assessed by back-gating to identify events in the analysis of forward and side scatter that were T cells labelled by the

mAb R73 (αβTCR). B lymphocytes within the lymphocyte gate were identified using the mAb OX33. Cells labelled with this antibody could then be excluded from the analysis of the lymphocyte population within the lymphocyte gate.

Instrument settings were optimised and background fluoroescence levels were determined using the negative control. The background fluorescence was defined as that part of the negative peak that contained 98-99% of the recorded events that occurred within the lymphocyte gate.

2.12.3 Dual fluorochrome flow cytometry

The setting up procedure involved the use of two negative control antibodies that were labelled with the appropriate fluorochromes (FL-1: FITC signal and FL-2: PE signal). Gates were set up and the background fluorescence levels for each channel were adjusted using the FL-1 versus FL-2 dot plot. Compensation was made for the overlap of the FL-1 signal into the FL-2 channel using a brightly stained (FITC) positive control sample. A similar adjustment was made to allow for the overlap of the FL-2 signal into the FL-1 channel using a brightly stained (PE) positive control sample.

2.13 Purification of endothelial cells from synovium

2.13.1 Isolation of synovial tissue from rats with adjuvant arthritis

An histological survey of the lower hind limb (see Section 3.3.1.2) identified a suitable area from which to harvest inflamed synovium from the ankle joint regions of DA rats with adjuvant arthritis, during the period 13 to 14 days after inoculation with CFA. This area of tissue is described in Section 4.3.2. It is found posterior to the tibia, superior to the calcaneum and distal to the musculo-tendinous junction of the Achilles tendon soleus muscle. A small amount of hyperplastic tissue was also harvested from the lateral aspect of the ankle adjacent to and surrounding the lateral maleolus.

2.13.2 Harvesting inflamed sub-Achilles tissue

The rat was prepared as described in Section 4.3.1. Using aseptic technique, the skin was then excised from the lower limb of the rat using the method described in Section 4.3.2.

2.13.3 Disagregation and enzyme digestion of synovial tissue

Disagregation and enzymatic digestion was carried out as described in Section 4.4.4.

2.13.4 Isolation of endothelial cells

2.13.4.1 'Mouth pipette'

In this study, a mouth pipette was used to collect dissociated segments of vessels of microvascular origin. This instrument is used routinely in reproductive biological research to physically manipulate gametes and embryos. A mouth pipette was constructed from a sterile Pasteur pipette, drawn over a flame to fine diameter and connected to plastic tubing.

2.13.5 The optimised method for isolating endothelial cells

The optimised method for isolating EC is described below. The experimental details that lead to this method are described as Results in Chapter 4.

2.13.5.1 Enrichment of endothelial cells using immunomagnetic beads

Endothelial cells were isolated from the mixed cell suspension in two stages. The first step was to enrich the relative numbers of EC in the mixed population. This was achieved by depleting cells of haematopoietic origin, using Dynabeads (Dynal, Oslo, Norway) coated covalently with sheep anti-mouse IgG, M450 Dynal beads as described in Section 4.7.6.6.

The mixed suspension of approximately 2-4x10^7 cells was washed 3 times in wash medium (RPMI-1640 1%FCS) centrifuged at 800rpm for 10 mins. The pellet of cells was resuspended in 2mls of sterile mAb OX1 (anti-CD45: leucocyte common antigen) culture supernatant and transferred to a 6ml disposable, capped polypropylene Falcon tube (Becton Dickinson, NJ, USA). The cells were incubated at 4°C for 25 minutes, with gentle end to

end rotation. They were then washed 3 times with RPMI-1640 1%FCS wash media and resuspended in 3mls of wash media.

Just prior to use, all Dynal beads were washed twice by resuspension in endotoxin free PBS and collected by retaining the beads in the tube with the Dynal magnetic particle concentrator while discarding used PBS. An aliquot containing 200µl of pre-washed Dynal beads at $1x10^8$ beads per ml ($2x10^7$) were added to the cell suspension and were distributed evenly throughout the suspension by the use of a 200µl micropipette tip. This gave a bead to cell ratio, in the mixed population of approximately 1-2 beads per OX1 positive cell.

Cells and Dynal beads were incubated together at 4°C for 30 minutes, with gentle end to end rotation. The $CD45^+$ cells, bound to the Dynal beads via the antibody-antigen complex, were then be trapped over 4 minutes. The $CD45^-$ cells were then removed from the tube with a transfer pipette (Samco Scientific Corp, CA, USA) and placed into a clean 10ml centrifuge tube. The wash process was repeated 5 times using fresh wash media and collecting those not trapped on the magnet, to release cells trapped between the magnet and the Dynal beads. Approximately 40% of the cell population was removed attached to the immunomagnetic beads ($CD45^+$ cells).

During the second stage of this process, EC were positively selected as detailed in the following section.

2.13.5.3. Positive selection of microvascular endothelial cells

The cells remaining in the mixed population after removal of $CD45^+$ cells were coated with mouse anti-rat CD36 (mAb UA009). The cells were resuspended in 2mls of UA009 supernatant. The cells were incubated at 4°C for 25 minutes, with gentle end-to-end rotation. They were then washed 3 times with and resuspended in 8mls of wash medium. A 100µl aliquot ($6.6x10^7$) of pre-washed Dynal™ beads from a Dynal Human IgG_4 anti-mouse CELLection kit™ (stock $6.6x10^8$/ml) were evenly distributed with a micropipette

and 1ml aliquots were distributed into 8 sterile 1.6ml Eppendorf tubes. These tubes were incubated at 4°C for 45 minutes, with gentle end-to-end rotation. UA009+ve cells were collected from the mixed cell population using a Dynal MPC. The cells, with Dynal beads attached, adhered to the wall of the Eppendorf tube and allowed the removal of any unbound UA009 -ve cells. The Dynal beads were resuspended in RPMI-1%FCS wash buffer, placed on the magnet for 4 minutes and the cells remaining in suspension discarded. This process was repeated five times to remove any unattached cells, trapped between Dynal bead and the magnet. Prior to being discarded, any used wash buffer was also placed on a MPC for 5 minutes to improve the yield of UA009+ve cells.

The final stage of this process was to release the beads from the UA009+ve cells. Beads and cells were resuspended into 1200µl of wash buffer and divided into equal volumes in two Eppendorf tubes. A 20µl aliquot (1000U) of DNase solution was added to each Eppendorf tube and the beads were resuspend gently (the DNase cleaves the DNA that links the captive antibody to the magnetic bead in this system). The tube was capped and incubated for 45 minutes at 37° C with tilting and gentle rotation (keeping the cells in the bottom of the tube). The Eppendorf tube was placed onto the Dynal MPC for 4 minutes. The released cells were collected in the supernatant and the process was repeated 5 times to collect cells trapped with beads and maximise the EC yield. The released cells were collected by centrifuging the Eppendorf tube at 1000rpm for 3 minutes.

2.13.6 Culture of endothelial cells

The $CD36^+$ ($UA009^+$) EC released from the Cellection beads were resuspended in 500µl of RPMI-20%FCS. A cell count was performed and the cells were adjusted to approximately $1.5x10^4$/10µl ($1.5x10^6$/ml) with RPMI-20%FCS. The cells were seeded onto fibronectin (Boehringer Mannheim) coated Terasaki plates (Nunc), at 10µl/well. They were incubated overnight at 37° C with 5% CO_2.

2.13.7 Identification of endothelial cells

2.13.7.1 Fluorescent conjugate low density lipoprotein

Acetylated low density lipoprotein (Ac-LDL) conjugated with the fluorescent probe, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL), (Biomedical Technologies, Stoughton, MA, USA), can be used to identify EC. DiI-Ac-LDL was added to EC cultures (RPMI-1640-20%FCS) at a concentration of 10μg/ml and the cells were incubated at 37°C with 5% CO_2 for between 4 and 8 hours. After incubation, the media containing DiI-Ac-LDL was removed and the cells were washed 3 times with LDL free media. Cells where either visualised live or after fixation with 3% formaldehyde/ PBS for 20 minutes at room temperature. Cells where either visualised live with an Olympus inverted fluroscence microscope IMT-2-RFL or after fixation using an Olympus BH2 fluoroescence microscope with standard settings for rhodamine fluorescence (Excitation filter:514nm, Emission filter:550nm).

2.13.7.2 Monoclonal Abs for CD36, PECAM-1 and RECA

Immunohistochemical methods were used to confirm identity and assess purity of EC purified from rat synovium. Monolayers of cells were stained with the mAbs UA009 for CD36, 3A12 for PECAM-1 (Serotec, USA) and RECA-1 pan endothelial marker (Serotec, USA) using the method as described in Section 2.4.4.

2.14 Production of antibodies against cells from rats with AA

2.14.1 Immunisation of mice

Inflamed tissue was harvested from the sub-Achilles region of 6 DA rats, 13-14 days after inoculation with CFA (as described in Section 2.13.2-2.13.4). The resulting mixed cell population was washed in wash media and resuspended at a density of $1x10^7$ in 2ml RPMI-1640.

Fresh preparations were made for each booster injection. Balb/c mice were injected ip with 0.5ml aliquots of the single cell suspension, 4 times over a period of six months. A final

aliquot of cells was administered, half of the volume by a tail vein injection and half by ip injection, 4 days before collection of spleens. Two mice were euthanased by CO_2 asphyxiation and soaked in 70% ethanol. The spleens were removed aseptically in a laminar flow hood and minced finely with scissors in a sterile disposable Petri dish. The fragments were transferred with serum free medium to a glass homogeniser and dissociated by gentle homogenisation. Debris was removed from the suspension by filtration through cotton wool and the cells were pelleted by centrifugation.

The pellet was resuspended in 2ml of cold Tris buffered 0.14M NH_4Cl, made up to 10ml with the same buffer and left on ice for 10minutes in order to lyse the RBCs. (Tris/NH_4Cl made from stock solutions A and B, 1ml of A and 9ml of B) The lymphocytes were collected by centrifugation at 1000rpm in a benchtop Beckman GS-6R centrifuge. The supernatant was aspirated and the cells were resuspended in 10mls of serum-free medium. A cell count and assessment of viability was carried out using a haemocytometer using the Trypan blue exclusion method.

2.14.2 Preparation of X63 myeloma cells

Myeloma X63 cells were harvested at log-phase and pelleted by centrifugation. They were resuspended in serum-free media to a volume of 30mls. A cell count and assessment of viability was carried out using a haemocytometer and the Trypan blue exclusion method. The cell concentration was adjusted to $1x10^7$ viable cells per ml.

2.14.3 Cell Fusion

The washed spleen cells in serum-free medium were transferred into a sterile 50 ml tube and added them to viable X63 cells at a ratio of 4:1. The volume was made up to 30ml with further serum-free RPMI-1640 medium and the cells were pelleted by centrifugation. The cells were resuspended in serum-free medium and centrifuged at 1000rpm for 2 minutes. Molten polyethylene glycol (PEG) (Sigma, St Louis, MO, USA) was diluted by adding 1 part of molten PEG plus 1 part of serum-free medium. One millilitre of prewarmed diluted PEG was added slowly but evenly over a 1 minute period to the cell pellet. During addition

of PEG, the cell pellet was stirred gently with the pipette tip used to mix the PEG with the cells. The mixture was incubated for 2 minutes at 37°C and then diluted with 2ml pre-warmed serum-free medium was added, over a 2 minute period with constant stirring. A further 7ml of serum-free medium was added over 2-3 minutes, with stirring. The cells were then centrifuged at 800rpm for 5 minutes. The pellet was resuspended in 2ml of HAT medium (RPMI-1640-10%FCS plus 2ml of hypoxanthine-aminopterin-thymidine (HAT) (Sigma, USA) per 100ml, and diluted with further HAT medium. The cells were dispersed in 100µl aliquots to the wells of 96 well flat-bottomed culture plates (Corning Costar Corp, Cambridge, MA, USA).

2.14.4 Growth of hybrids from the fusion mixture

The cells were cultured initially in conditioned HAT medium containing 10% v/v of conditioned medium from the mouse macrophage cell line J774 (see Section 2.6.4). Hybridoma growth was observed daily using an Olympus inverted CK-2 microscope with phase contrast. Fresh growth medium was replenished approximately every 2-3 days, as required. The supernatants were sampled from culture wells after they were assessed to contain macroscopically visible colonies or after colour change of the medium caused by acidification.

2.14.5 Screening hybridoma culture supernatants

Supernatants from wells containing hybridomas were screened for the presence of antibodies reactive with tissue antigens, using an indirect immunoperoxidase technique on ethanol-fixed frozen sections (Section 2.4.3). Tissues employed to screen for antibodies of interest were lymph node and small intestine (including Peyers patch) from normal female DA rats and inflamed sub-Achilles tissue obtained from rats 13 days after inoculation with CFA (Section 2.4.1).

2.14.6 Cloning of hybrid cells producing specific antibodies

Clonal populations of some selected antibody-secreting hybridoma cells were produced by

limiting dilution. Cells were plated out at dilutions of approximately 2.0, 1.0 and 0.5 cells per well. Supernatants from wells at a dilution, where less than one third of the wells produced growth were screened using the indirect immunoperoxidase technique on frozen sections as described in Section 2.4.3. and 2.14.5. Hybrid cells from antibody-containing wells were expanded by growth in HAT medium and samples were frozen for storage in liquid nitrogen.

2.15 Lymphocyte-endothelial cell adhesion assay

2.15.1 Preparation of $CD4^+$ lymphoblasts for use in adhesion assays

2.15.1.1 Enrichment of lymphoblasts from TDL

Thoracic duct lymph was collected overnight from 3 female DA rats, 9 days after inoculation with CFA (as described in Section 2.9). The lymphocytes were enriched for the larger, blast cell population using Percoll density gradients (as described in Section 2.10.1). Briefly, discontinuous gradients of 30, 40, 50 and 55% Percoll solutions were layered in 30ml centrifuge tubes, with the highest density at the bottom. Lymphocytes that had been washed previously were resuspended in 30% Percoll and layered on top. The tubes were then centrifuged at 400g for 25 minutes, with the centrifuge brake dis-engaged. After centrifugation, cells had settled to their isopycnic position in the gradient. The cells at each interface were collected separately for subsequent flow cytometric analysis in order to identify and isolate which layers were enriched for the larger blast cells.

2.15.1.2 Depletion of $CD8^+$ T cells and B cells

Cells at the interfaces on top of 50% and 55% were enriched for the larger blast cell component of the TDL. Cells from these two levels were pooled then depleted of B cells and $CD8^+$ cells. This was accomplished by negative selection, utilising the Dynal Immunomagnetic bead system M450 (indirect). The procedure followed that described in Section 2.13.5.a, except a cocktail containing supernatants of mAbs OX33 (CD45RA/AB) and OX8 (CD8a) were used instead of the mAb OX1.

2.15.2 Summary of adhesion assay method

Monolayers of EC were prepared, as described in Section 2.13.6. The isolated EC were resuspended in RPMI-1640 with 20% FCS and seeded at a sub-confluent density of $1x10^4$ per well into fibronectin coated replicate wells of a Terasaki plate (Section 2.6.5). Ten hours after seeding, the monolayers were washed with PBS at a rate of 2 drops per second for a total of 20 seconds per well. Washing was carried out using a modified disposable sterile saline drip infusion set filled with sterile warm (37°C) wash buffer, RPMI-1640 with 1% FCS. The bag was suspended 55cm above the bench surface and the flow standardised 2 (see Section 6.3.2.4). Each well was washed for a total of 20 seconds. The EC in these monolayers occupied approximately 60 percent of the surface area of the flat bottoms of the Terasaki plate wells, as determined by video-image analysis of ethanol fixed, toluidine blue stained preparations of EC monolayers. Estimations were made on 5 replicate culture wells. Three different areas were assessed and in each well a percentage area covered by EC was determined.

Adhesion experiments utilised LN lymphocytes from normal rats that had been stimulated for 24 hours with Con A or CD4+ lymphocytes isolated from TDL. Con A stimulated lymphocytes were pre-incubated in α-methyl-D-mannopyranoside and this sugar was present throughout the assay (Section 2.8.3). Lymphocytes were applied to each well at a density of $0.5x10^5$ (5µl at $1x10^7$/ml) and were allowed to settle at unit gravity. LN lymphocytes were prepared as described in Sections 2.8.1 to 2.8.4. CD4+ T cells from TD lymph were prepared as described in Sections 2.9.4 and 2.10.1. Lymphocytes were labelled with CFSE prior to use in the assay (method after Lyons and Parish (1994) see Section 2.8.4), to allow identification and enumeration of adherent cells.

The adhesion assays were carried out in the presence or absence of mAb against adhesion molecules or control mAbs. Purified mAbs were used at a final concentration of 50µg/ml, and supernatants were pre-concentrated four-fold, to allow for dilution in the assay (Section 2.7.5). Aliquots of lymphocyte suspensions were pre-incubated with antibodies against cell surface adhesion molecules that are known to be expressed by lymphocytes. Monoclonal

Ab against adhesion molecules that are expressed by EC, or Abs reactive with unknown antigens expressed by EC were incubated with the EC before the addition of lymphocytes. In all experiments the antibody being investigated was present throughout the assay.

In the presence of test or control mAbs, lymphocytes were applied to each well and incubated for 1hr at 37° C. Following the incubation period, non-adherent lymphocytes were removed by washing, identical to that described above for washing EC monolayers (Wash buffer at 2 drops per second, for 20 seconds). Following this the monolayers with adherent lymphocytes were fixed with 2.5% glutaraldehyde in PBS.

2.15.3 Analysis of adhesion assay results

CFSE labelled lymphocytes that had adhered to the endothelial monolayers were quantified by manual counting of fluorescent cells from photomicrographs. Counts were performed manually over a standard area of the well, comprising 40% of the total area of the flat surface of the well utilising photographs prepared using an inverted fluorescence microscope.

The number of lymphocytes adhering to the monolayers, in the presence of a range of anti-adhesion molecule antibodies was compared with control wells. Statistical analysis of the data was carried out using ANOVA with post-hoc Tukeys test. The results were also analysed for inter-experiment variation in lymphocyte adherence by Main Effects ANOVA (2-way) analysis performed in the General Linear Models module of Statistica Version 6. To make allowance for random effects in replicate experiments carried out on different days, the Main Effects ANOVA analysis, calculated the regression coefficients for each antibody, with the coefficient for RPMI (assay buffer) set at zero (adhesion calculated as a percent of the total number of adherent lymphocytes detected in the absence of antibody).

Chapter 3

Identification of suitable site for harvest of inflamed tissue

3.1 Introduction

An histological survey was carried out of the hind paws from rats assessed to have severe or moderate AA. The aim of this survey was to identify an accessible area of tissue, from which EC from the microvasculature of inflamed synovium could be isolated and purified. These cells would then be used as an investigative tool to study *in vitro* interactions between synovial microvascular EC and lymphocytes.

A mAb specific for EC was required in order to identify, assess and select these cells. Monoclonal Abs that were candidates for this purpose were drawn from a panel raised in this laboratory and screened by immunohistochemical staining of a limited range of tissues. The mAbs UA009, UA011, UA015 and UA016 were raised by immunisation of mice with rat TD lymphocytes. From a preliminary investigation, they were known to stain EC in tissue from rat small intestine. An immunohistochemical survey of the specificities of mAbs UA009, UA011, UA015 and UA016 was carried out in a larger range of tissues from female DA rats.

Three other EC specific mAbs OX43, RECA-1 and TLD-3A12 were also examined. Monoclonal Ab OX43 has been reported to stain rat vascular endothelium with the exception of those in cerebral blood vessels (Robinson *et al.*, 1986). It binds to a 90 kD molecule, detected in Western blot analysis of rat macrophage membrane proteins. A mAb against rat EC antigen (RECA-1 or HIS52) which was also investigated (Serotec) reacts with an antigen expressed by endothelium in all blood vessels in rats (Duijvestijn *et al.*, 1992) and mAb TLD-3A12 (Serotec), binds the adhesion molecule PECAM-1 in the rat (Male *et al.*, 1995).

In anticipation that FB would be a major cell-type contaminating preparations of EC, an immunohistochemical investigation was made also of the mAb OX7 (anti-rat Thy-1.1, CD90) in a range of tissues from arthritic and normal rats. mAb OX7 is reported to stain FB, thymocytes, peripheral nerve fibres and vascular pericytes (Williams and Gagnon 1982; Mason and Williams 1980; Williams *et al.*, 1977).

3.2 Hypotheses and aims

3.2.1 Hypotheses:

- Rat synovial tissues can be isolated and harvested in sufficient amounts to enable investigation and development of a method to purify EC from this tissue.

- An EC-specific mAb can be identified that will allow the identification and selection of EC for subsequent *in vitro* investigations.

- A FB specific mAb can be identified and used *in vitro* to identify FB and to remove them selectively.

3.2.2 Aims:

- To identify a suitable area of tissue from which to harvest EC for purification and tissue culture.

- To identify specific mAbs that are suitable for identification of EC and for the process of selection/purification of EC.

- To identify a specific mAb that is suitable for identification of FB and for the process of selective eradication of FB from cell suspensions.

3.3 Results

3.3.1 Identification of inflamed synovial tissue

3.3.1.1 Surface anatomy of severe AA

With the exception of occasional small foci, redness and swelling that are characteristic of CFA-induced AA were not observed in the paws of normal DA rats (Figure 3.1.A). The onset of AA was observed after 9-10 days, however, severe AA (Figure 3.1.B) develops and reaches a plateau between 12 to 14 days after inoculation with a single injection of 0.1ml CFA into the tail base. The arthritis was widespread and showed marked swelling and redness around the calcaneum (heel), tibiotarsal (ankle) and metatarso-phalangeal joints. The medial and lateral aspects of the ankle region were also red and swollen. In most rats, by day 13 or 14 post-inoculation, the calcaneum had lost its surface definition, when compared with the normal foot. In normal rats, the shape of the calcaneum from its posterior and its plantar aspect could be easily defined macroscopically because the skin tends to follow the contour of the bone. In arthritic rats, however, this region of the foot appears markedly swollen and red, making it difficult to define the bony surface anatomy of the heel and ankle regions.

The posterior aspect of the ankle joint, superior to the calcaneum but distal to the musculotendinous junction of the Achilles tendon into the gastrocnemius muscle, was markedly swollen and red in comparison to the same region of the normal rat. The plantar surface of the paw and the region of the dorsum of the foot adjacent to the tibio-talar and talar-tarsal joints were also swollen. Macroscopic examination of the forepaws revealed swelling and redness of the palmar aspects, the metacarpo-phalangeal joints and the tissue surrounding the carpal bones (wrist). Usually the arthritis was more severe in the hind paws and for this reason the hind paws were chosen for further study.

3.3.1.2 Histopathology of severe AA

Hind paws obtained from rats with severe AA were decalcified, embedded in paraffin wax and stained with haematoxylin and eosin (H&E) (described in Section 2.3). These paws showed

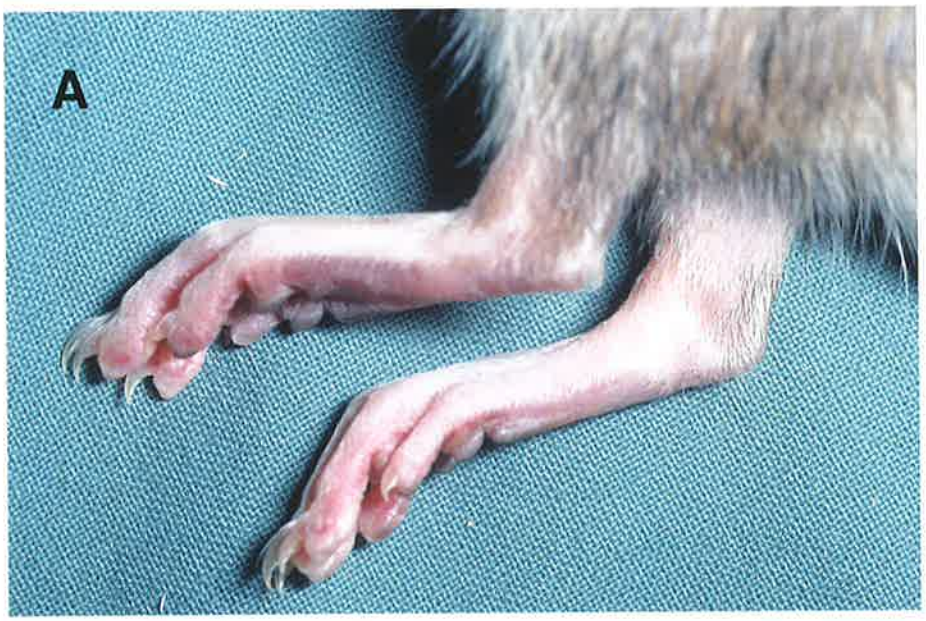
marked swelling and redness (Figure 3.2.B), not seen in hind paws from normal rats (Figure 3.2.A).

In the normal rat ankle, the connective tissue surrounding the joint cavities consists of fatty, fibrous and areolar tissue. This type of tissue can be observed posterior to the tibia, in the sub-Achilles region of the normal foot (Figure 3.3A), where significant amounts of loose areolar connective tissue, with a predominance of adipocytes are present (Figure 3.3 C). The synovial intima lines, the non-cartilaginous joint cavities and its surface may be flat, or may be folded into tubular or villus-like projections (Henderson and Edwards 1987). The region of the ankle joint anterior to the distal end of the tibia shows a typical, relatively thin synovial intima composed of flattened synoviocytes overlying the sub-synovium (Figure3.3A & B). In the normal rat, the surface observed microscopically, in the region of the joint adjacent the tibia has been thrown into folds and projections that have a villus-like appearance. The fatty sub-synovium appears somewhat vascular and underlying the adipose tissue is a region of fibrous connective tissue (Figure 3.3B). Once the inflammatory process is initiated with leucocyte migration evident, the adipocytes appear to reduce in their predominance (Figure 3.3D).

As the process of inflammation continues, the fatty tissue is swollen by an inflammatory exudate (Figure 3.4A and B) and a dense inflammatory exudate. This tissue is highly vascular and contains large numbers of small vessels (Figure 3.5.C). In rats that were assessed macroscopically to exhibit a more moderate AA, (where the extent of the inflammation was not as advanced as in those assessed to have severe arthritis) histological examination showed the presence of inflammatory cells, but in these rats, the adipocyte-rich connective tissue was less densely infiltrated with inflammatory cells (Figure 3.3.D).

Figure 3.1 Macroscopic view of hind paws of normal and arthritic foot DA rats: Surface appearances of severe AA

(A) In the normal hind paw, there were no areas of redness and swelling characteristic of AA. Macroscopically, the shape of the calcaneum is easily defined from its posterior and plantar aspects as the skin follows the contour of the calcaneum. (B) In the feet from rats 13 to 14 days after induction of arthritis, swelling was typically marked and widespread. Swelling and redness are present around the calcaneum (heel), the tibio-tarsal joint (ankle), the metatarso-phalangeal joints and the plantar surface of this paw. In addition the medial and lateral aspects of the ankle region are severely red and swollen and the calcaneum has lost its surface definition. The posterior aspect of the ankle joint, superior to the calcaneum distal to the musculotendinous junction of the Achilles tendon into the soleus muscle is also swollen.


A

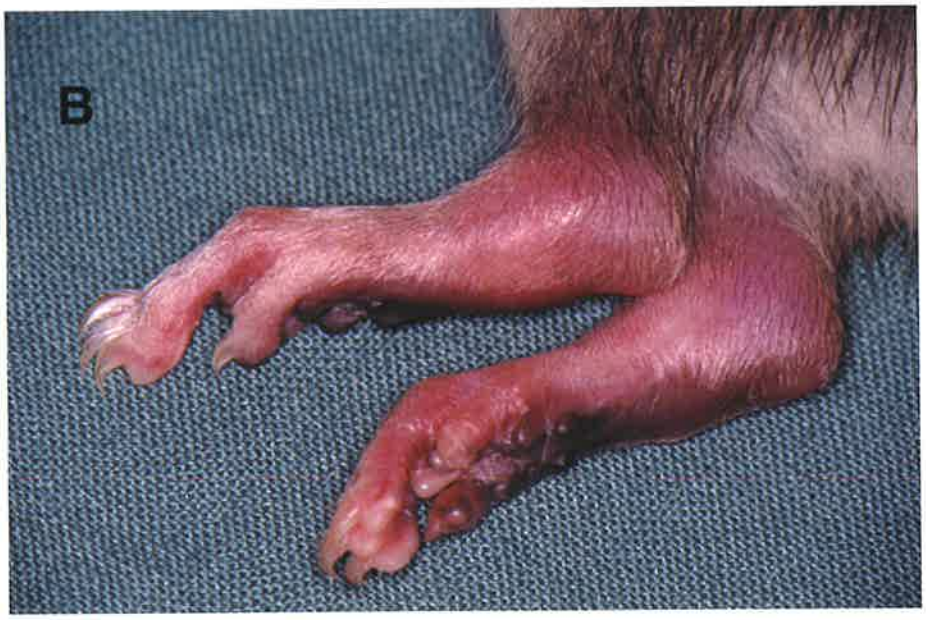

B

Figure 3.2 Low power light micrographs of hind paws from normal and arthritic rats

Sagittal sections through the decalcified hind feet from normal and arthritic rats (8 weeks old). Note the increased longitudinal-sectioned area of the arthritic foot (B) when compared to the normal foot (A). The presence of inflamed connective tissues is evident, particularly at sites anterior and posterior to the tibia (Tib). The synovium and sub-synovial tissues in the region posterior to the tibia (Tib), deep to the Achilles tendon (TC) and superior to the calcaneum are markedly oedematous. The joint space of the ankle is distended with fluid. Synovial swelling can also be seen at the dorsal aspect of the joint between the lateral cuneiform and the metatarsal bone (MTB). The section of normal foot passes through a more lateral sagittal plane compared to the section in the micrograph depicting the arthritic foot. Low power (original magnification 4.5x), H&E stained, paraffin embedded.

A Normal

B Arthritic

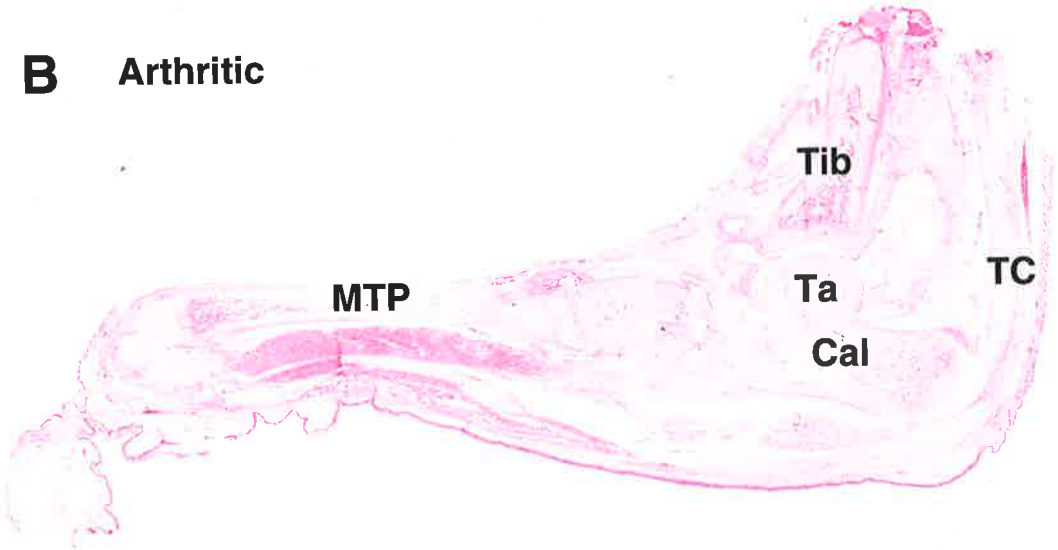


Figure 3.3 Histology of the normal hind foot

Micrograph A displays the histology of the normal hind foot in more detail (original magnification 7x). Two different areas of synovial intima and sub-synovium are highlighted. The region of normal synovium indicated by the asterisk (*), observed anterior to the calcaneum, is represented in micrograph B (original magnification 100x). The synovial intima (SI), indicated by the arrow, is a thin layer of cells that cover the sub-synovium (SS). The sub-synovium (SS) is composed of a loose connective tissue with abundant adipocytes. Small blood vessels can be seen in this region. At the bottom of this micrograph, the joint capsule is seen as a region of fibrous or dense irregular connective tissue, with a number of blood vessels. A portion of the region of the sub-synovium within the area surrounded by the broken line in micrograph A is represented in micrograph C, where the most abundant cells visible are adipocytes (original magnification 100x). For comparison, micrograph D shows a view of synovial tissue from a rat with moderate AA taken from a region similar to micrograph B (normal rat). By comparing micrograph D with micrograph B above, it is possible to easily identify the early changes that have occurred, where the adipocytes within the sub-synovium have been infiltrated by inflammatory cells (original magnification 100x).

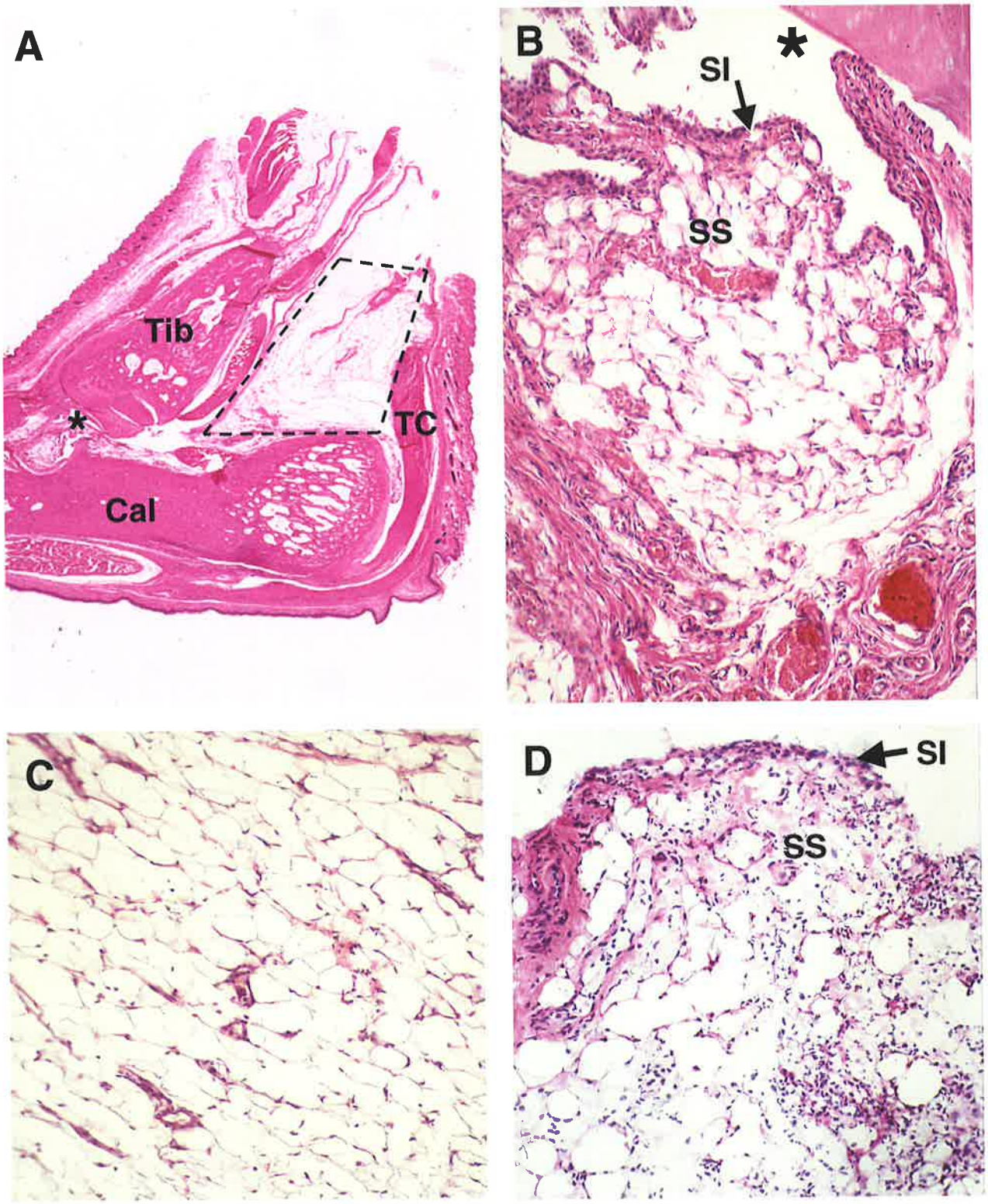

A
Tib
TC
Cal
*
B
*
SI
SS
C
D
SI
SS

Figure 3.4 Histology of the arthritic hind foot (low magnification)

Micrograph A shows a more detailed look at the histology of the arthritic foot from a DA rat (original magnification 7x). Two different areas of synovial intima and sub-synovium are highlighted. The region indicated by the double asterisk (**), observed anterior to the calcaneum, is represented in Figure 3.5.A. The region of synovium within the area surrounded by the broken line is represented in micrograph B. The adipose tissue observed within a similar region in the normal foot (Figure 3.3.C) has been infiltrated by inflammatory cells. The nuclei seen in this tissue belong mainly to cells that have infiltrated from the peripheral circulation as part of the inflammatory process (original magnification 100x). The area enclosed by the dashed line in micrograph A, between the tibia (Tib), Achilles Tendon (TC) and superior to the calcaneum is depicted in micrograph B (original magnification 20x). The large space (indicated by small closed arrows) is the distended posterior extension of the ankle joint cavity (associated with the tibio-talar joint). A smaller distended space associated with the sub-talar joint, is visible just superior to the calcaneum (Cal). These spaces are distensions of the cavities of synovial joints and are thus lined by synovial intima (deep to which lies the sub-synovium).

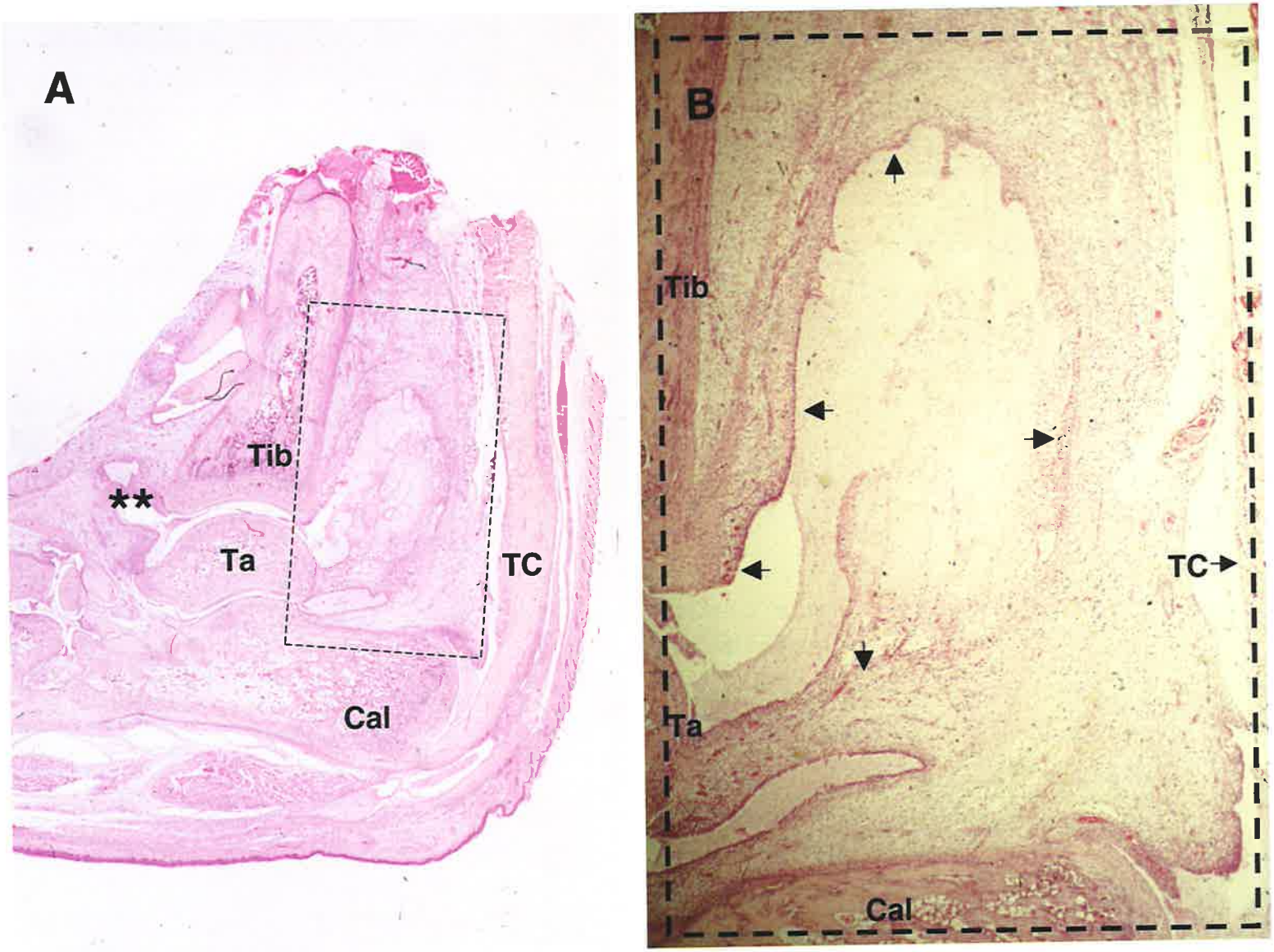
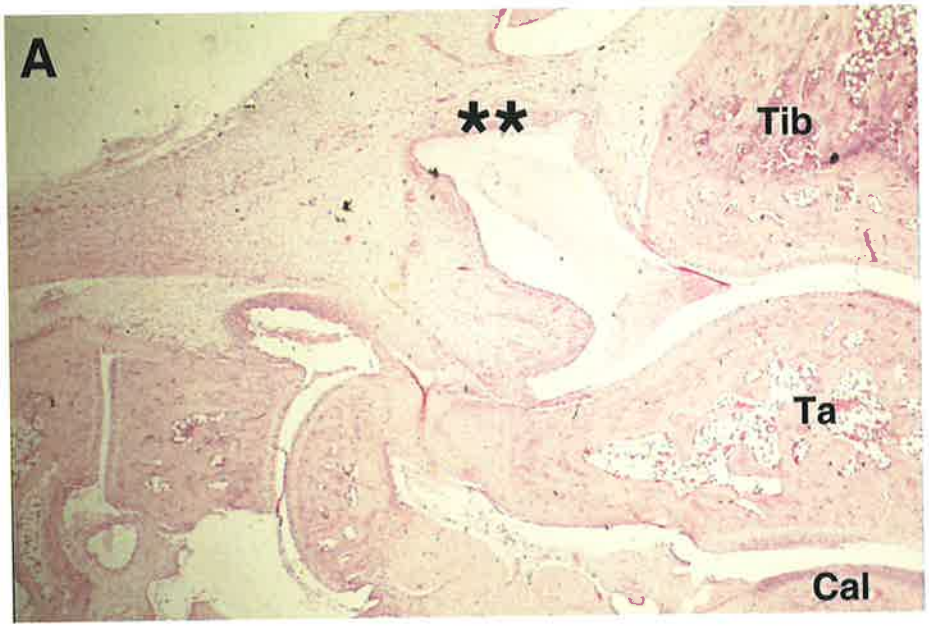

A
Tib
**
Ta
TC
Cal
B
Tib
Ta
TC
Cal

Figure 3.5 Histology of the arthritic hind foot (higher magnification)

The micrographs in this figure are enlargements of areas shown in Figure 3.4.B. A continuation of the distended ankle joint cavity is visible in Figure 3.5.A, just anterior to the talus (Ta) and is indicated a double asterisk (**). The space contains a proteinaceous inflammatory exudate (original magnification 20x). Micrograph B shows the distension of the joint cavity of the sub-talar joint. The margins of the space, lined by synovial intima, are indicated by 2 small arrows (original magnification 40x). Micrograph C, a higher magnification from micrograph B, shows the sub-intimal or sub-synovial tissue to be highly vascular. Numerous small blood vessels are present, indicated by small arrows. The synovial intima is indicated by 2 large arrows. Note also the pitted surface of the bone, where the arthritis has brought about erosion of the surface of the talus (Tal) (original magnification 100x).


A
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Tib
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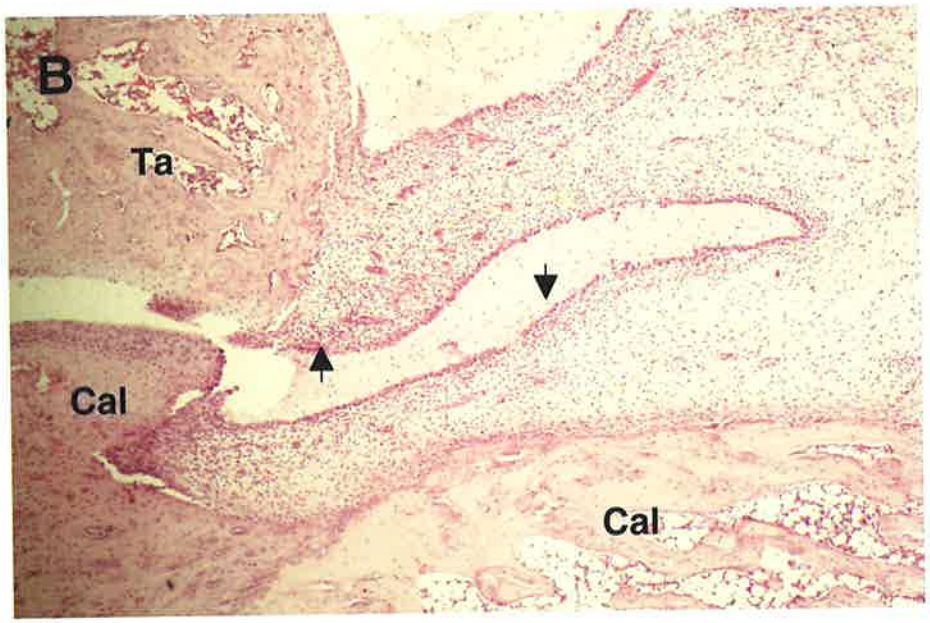
B
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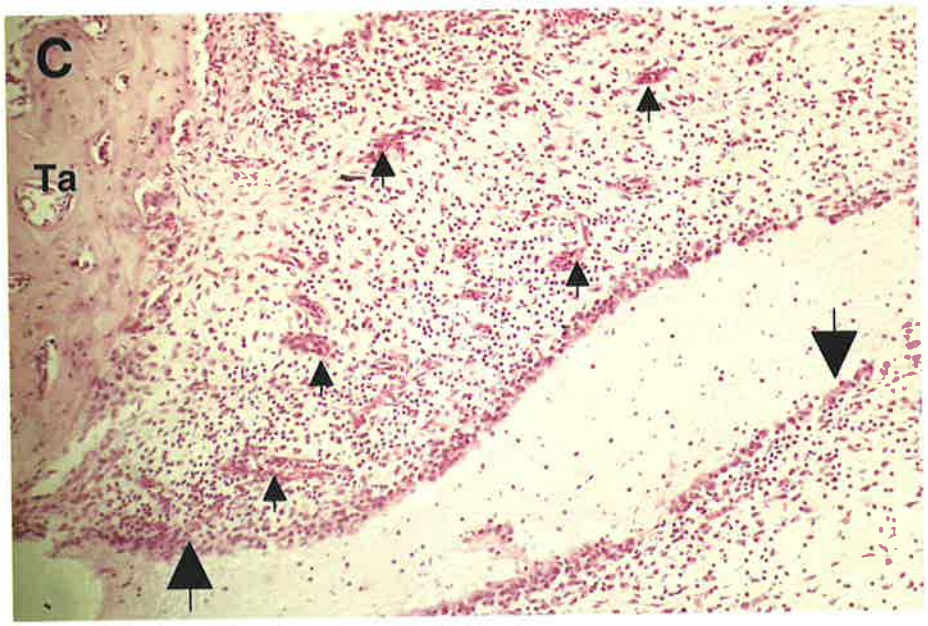
Cal


C
Ta

In the arthritic rat, the joint spaces of the tibiotarsal joint and subtalar (talar-calcaneal) joint were observed to be distended, with a proteinaceous cellular effusion. This is visible posterior (Figure 3.4A-B) and anterior to these joints (Figure 3.5A-C). Posterior to the tibia, the distended space is observed to have ballooned out from the tibiotarsal joint well into the sub-Achilles space (Figure 3.4A-B). Connective tissue underlying the dermis on the dorsal aspect of the paw also displays an inflammatory exudate (Figure 3.5A). Anterior joint spaces in this region, the tibio-talar, subtalar (talar-tarsal) and inter-tarsal joint spaces are all enlarged or distended (Figure 3.5.A) compared with those in the normal rat foot (not shown). The synovial intima lining these distended spaces (Figures 3.4 & 3.5) was hyperplastic and the sub-intimal synovium or sub synovium was infiltrated with inflammatory cells (Figures 3.5B & 3.5C).

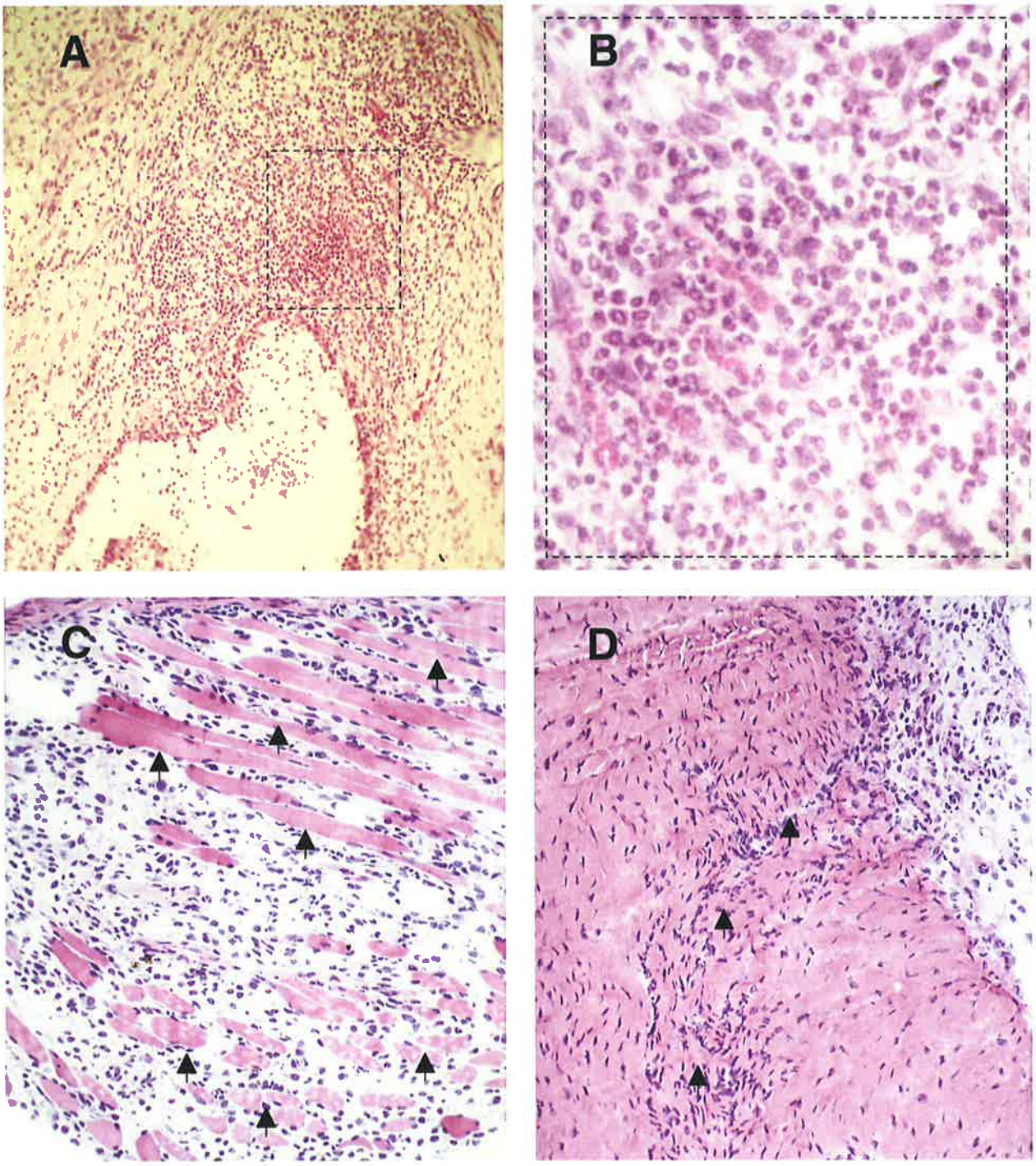
In some areas isolated foci consisting of high-density aggregations of inflammatory cells were observed, especially in areas adjacent to synovium (Figures 3.6.A-B). Erosion of bone was also evident, where the surface of the talar bone had lost its smooth appearance and small pits were observed with invasion by inflammatory tissue (Figure 3.5.C). Inflammatory cells appear to have infiltrated into skeletal muscle as shown (Figure 3.6.C), where a significant number of inflammatory cells were observed between the myocytes. Inflammatory cells, as shown in Figure 3.6.D have also infiltrated tendons.

3.3.1.3 Sub-Achilles synovial tissue

This histological survey helped to identify a location for harvesting substantial amounts of inflamed synovium from rats with AA. Small blood vessels were abundant in this tissue. The area of tissue chosen for further study (shown boxed in Figure 3.4A) is bordered anteriorly by the tibia, has the Achilles tendon at its posterior margin and the calcaneum at its inferior margin. It extends proximally to the musculotendinous junction of the Achilles tendon with the soleus muscle. The boundaries of the tissue are well circumscribed anatomically in a way that allows reproducible excision of the tissue for immunohistochemical studies and for isolation of cells associated with inflamed synovium in AA.

Figure 3.6 Inflamed tissues in AA

Micrograph A shows inflamed sub-synovial tissue. In some areas of the sub-synovium, high density aggregations of inflammatory cells form isolated foci, as indicated within the dashed box (3.6.A) (original magnification100x). Micrograph B shows a higher magnification view of the inflammatory cells within the area indicated in micrograph A. Both polymorphonuclear and mononuclear cells are visible within the focus (original magnification 200x). Micrograph C shows an area where inflammatory cells were observed to have infiltrated into skeletal muscle. A substantial number of inflammatory cells were observed between the myocytes, which are indicated by small arrows (original magnification 100x). Micrograph D shows infiltration of a tendon by inflammatory cells, indicated by small arrows (original magnification 100x).


A
B
C
D

3.3.2 Screening of monoclonal antibodies with known specificity for antigens expressed by endothelial cells or fibroblasts.

In the quest for a mAb that recognised rat endothelium, the following questions were asked: "Is the antibody lineage specific?" or "Is the antigen also present on non-EC?" "In which tissues does staining occur?" "Does extracellular material stain?" It was also important to examine whether the mAbs stained EC in vessels of all sizes and types, for example, in large and small vessels; in all vessels including arteries, arterioles, veins, venules, capillaries; in specialised vessels such as high endothelial vessels (HEV) or whether it stained microvascular EC preferentially. In each region of tissue, the proportions of the vessels that were stained by the uncharacterised mAbs UA009, UA011, UA015 or UA016 were compared with those stained by the mAbs OX43, RECA-1 and TLD-3A12. Since a major aim of this study was to isolate and purify EC from the microvasculature of inflamed synovial tissue from arthritic rats, it was preferable that the antibody should be specific for the microvasculature, and that its antigen should be expressed on the surface of EC at a density sufficient to allow the antibody to be used to select the cells, when used in conjunction with immuno-magnetic beads.

Each antibody was investigated separately. However, a number of the antibodies showed similar patterns of binding. For this reason, some of the antibodies have been grouped for the purposes of discussion, in the results section. The mAbs UA009, UA011 and OX43 for example, have been shown subsequently to recognise the same antigen (CD36) by Dr X Zhang in this laboratory (PhD thesis 2001; Zhang *et al.,* 2003). The mAbs UA015 and UA016 stain vascular endothelium in a pattern similar to that of the commercially available mAb TLD-3A12, an anti-PECAM-1 antibody but the antigen(s) recognised by these mAbs have not been investigated further.

Figure 3.7. Immunohistochemical survey of distribution of staining by mAb UA009 in normal DA rat tissues.

Micrograph A shows distribution of staining by mAb UA009 in normal lymph node (original magnification 100x). The endothelium lining small blood vessels, including the specialised post-capillary venules (not shown) are stained strongly by mAb UA009. Strong staining within the medullary region is probably due to the presence of numerous macrophages. Micrograph B shows normal liver (original magnification 40x). This pattern of staining was observed throughout the liver. The parenchyma surrounding the central vein was stained strongly, but the hepatocytes adjacent to the portal triad were unstained. Endothelial cells lining the vessels within the liver were also stained strongly. Micrograph C shows skeletal muscle (quadraceps) (original magnification 100x). The myocytes were not stained, but small vessels in the endomysium between bundles muscle fibres were positive. Micrograph D shows a low power image of normal small intestine (original magnification 40x). The HEV and small vessels within the Peyer's patch (PP) are stained strongly. In addition, small vessels in the lamina propria and muscularis externa were stained. The apical surfaces of mature enterocytes were stained moderately by the antibody. Micrograph E shows the luminal (Lu) aspect of the abdominal aorta (original magnification 100x). The endothelium of large arteries, including the aorta, were not stained by mAb UA009. Micrograph F shows normal cardiac muscle (original magnification 100x). The antibody stained cardiomyocytes, the endocardium and endothelium of vessels within the myocardium.

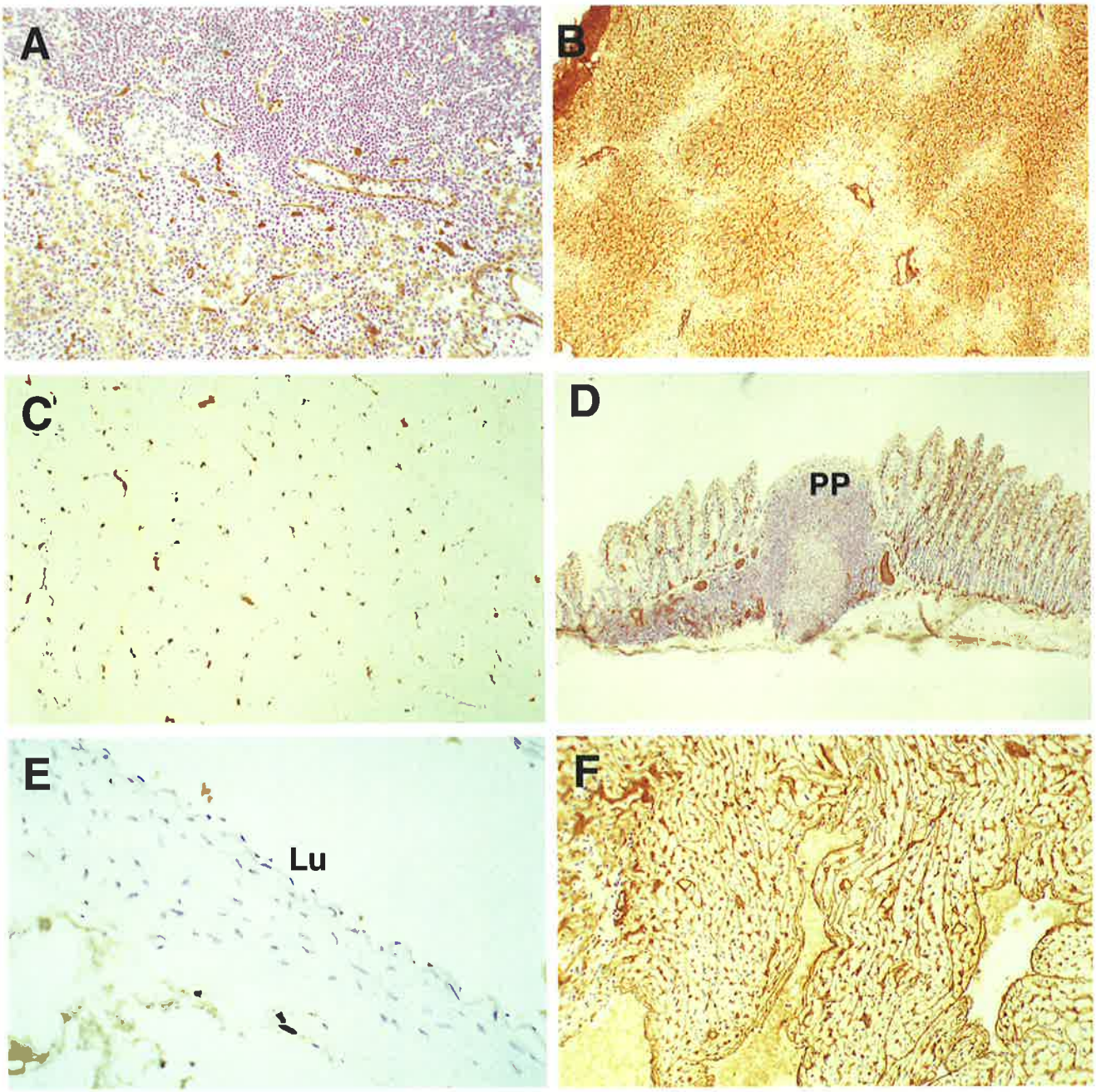

A
B
C
D
PP
E
Lu
F

Figure 3.8 Confocal images of UA009 antigen distribution on vessels in normal thymus.

Confocal images of vessels in normal rat thymus stained with mAb UA009 by an indirect immunofluorescence technique. Micrograph A shows a low power image of small and medium sized vessels. Image B shows a high magnification image of capillaries. Endothelium is stained along the length of the vessel. A nucleus is visible as an unstained area near the middle of the micrograph. (Photographed in this laboratory by Professor Peter Groscurth, unpublished material, 2000).

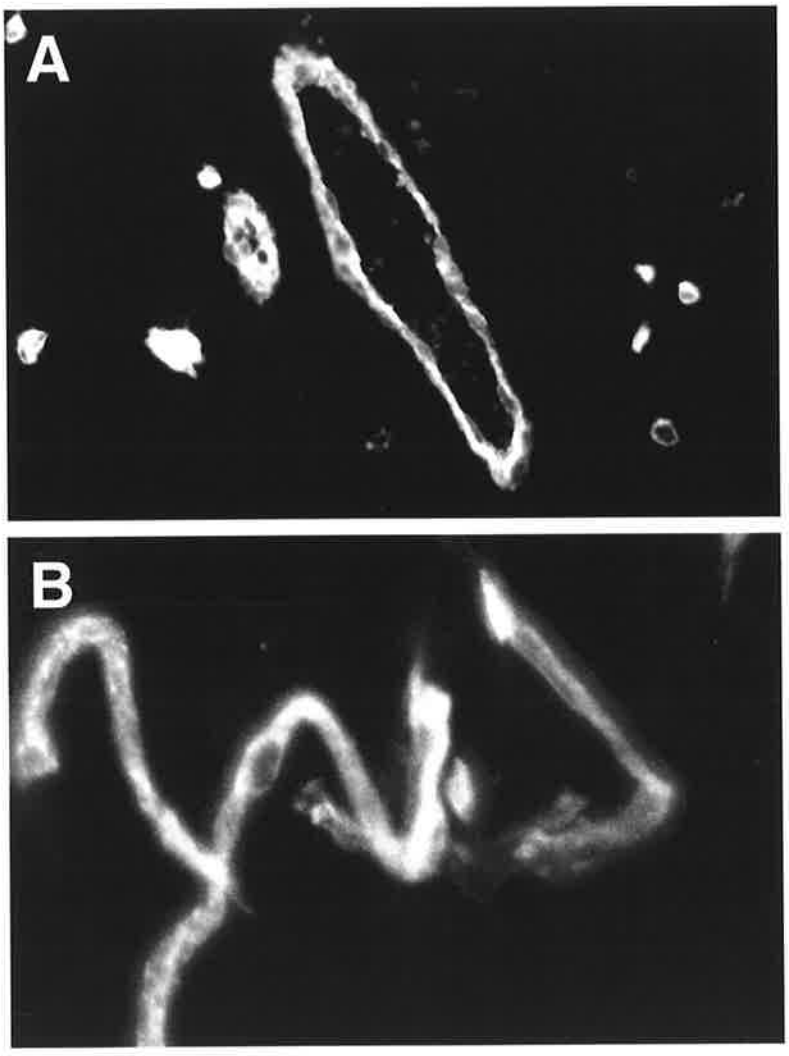

A
B

Table 3.1 Summary of distribution of the antigens recognised by mAbs UA009,UA011 and OX43 in DA rat tissues

Cell/Tissue Type	**Distribution Of Antigen**
Lymphoid	HEV, LN sinuses, spleen marginal zone, marginal sinus and red pulp.
Surface epithelium	Enterocyte brushborder, respiratory epithelium.
Glandular epithelium	Sebaceous gland, central hepatocytes, renal proximal and distal convoluted tubules.
Mesothelium	Serosa of small intestine, visceral pleura of lung, pericardium.
Neural	None (small vessels in endoneurium).
Muscle	Cardiomyocyte sarcolemma and intercalated disc; Some skeletal muscle fibres.
Haematopoietic	Macrophages, RBCs.
Connective	Adipocytes, pericytes.
Endothelium	Capillaries (++++), venules and veins (+++), small arteries(+), medium and large arteries (-), aorta and vena cava (-)
Synovium	Endothelium, tissue macrophages, (but not macrophage-like cells of synovial intima).

3.3.2.1 An immunohistochemical survey of staining with mAbs UA009 and UA011.

a Procedure

Indirect immunohistochemistry was carried out on a range of tissues from DA rats as described in sections 2.4.2 and 2.4.3. Tissues harvested from the normal rat included ileum (including Peyers patch), jejunum, inguinal lymph node, spleen, kidney, lung, brain, liver, skin, aorta, inferior vena cava, cardiac muscle, skeletal muscle, thymus, salivary gland, and synovium of the suprapatella pouch. Inflamed synovium was taken from the sub-Achilles region of the ankle of a DA rat, 14 days after inoculation with CFA. The mAbs UA009 and UA011 detected antigens with essentially identical distribution patterns. The distribution pattern of the mAb UA009 antigen is shown in Figure 3.7 and the results obtained with the two mAbs are summarised in Table 3.1.

b. Vasculature.

Expression of the antigen recognised by mAbs UA009 and UA011 was observed mainly on small vessels. Staining appeared strongest on capillaries (Figures 3.8 and 3.9) and was also observed on the endothelium of venules, most veins, and arterioles, but to a lesser extent, small arteries. The staining on small arteries was relatively weak, compared with capillaries and was uneven in its distribution. On medium sized and large arteries, there was no staining, except in the case of endothelium of large pulmonary arteries. The endothelial lining of the aorta was not stained by either mAb (Figure 3.7E) but the vasa vasorum within the wall of this vessel were strongly positive. The endothelium lining the vena cava was an exception to the otherwise uniform staining of veins of all sizes.

Generally, staining by mAbs UA009 and UA011 appeared stronger on EC than on other cell types. However, the intensity of vascular staining did show variability between EC from different vascular beds. The strongest staining was observed on endothelium from lymphoid tissues and skeletal muscle. In the cerebrum, some limited expression of the antigen was noted on vessels within the pia mater and those associated with choroid plexus. In the kidney, staining of endothelium in the renal glomeruli and vessels within the renal cortex was strong, staining of interlobular and arcuate arteries was moderate but staining

was weak in the medulla and absent from the afferent arterioles. Endothelium lining the peripheral lymphatic vessels was not stained.

c. Lymphoid tissues, including the mucosa associated lymphoid tissues (MALT).

Within lymph nodes (Figure 3.7A) and Peyer's patch (Figure 3.7D), the endothelium of specialised post-capillary venules known as high endothelial venules (HEV) stained strongly. In the lymph nodes, HEV near the cortico-medullary junction stained most strongly while those in the periphery of the cortex were weakly positive or did not show staining at all (not shown). The endothelium of the lymphatic sinuses and of the efferent lymphatics were stained. The simple squamous endothelium that lines the sub-capsular, peritrabecular and medullary sinuses was also moderately positive for the antigens detected by mAbs UA009 and UA011. Strong expression of the antigen observed within the LN medullary sinuses was thought to be due to the presence of numerous large macrophages (Figure 3.7A).

The spleen is composed of white and red pulp. Central arteries, continuous with the splenic artery, enter the white pulp and are surrounded by numerous lymphocytes. This cuff of lymphoid cells forms the periarterial lymphatic sheath (PALS). Red pulp is composed mainly of splenic cords and sinuses. The antigens detected by mAbs UA009 and UA011 were expressed at high levels in the red pulp of the spleen. The PALS were unstained, except for endothelium associated the central arteriole and its branches. The marginal zone was stained strongly by the antibodies and staining of the endothelium lining the marginal sinuses was prominent (not shown). The thymus was unstained by either mAb, except for the endothelium of small blood vessels and capillaries (Figure 3.8).

d. Surface epithelia

In the small intestine, the lumen is lined with enterocytes and goblet cells. The surfaces of the enterocytes are covered with microvilli, which are projections of the apical cell membrane. The apical regions of the enterocytes in particular the region of the brush border, were stained moderately (Figure 3.7D). In the adult large intestine, villi are absent.

In large intestine, the apical enterocyte border was moderately positive especially on the upper 1/3 of the crypt but became weaker along the length of the crypt moving toward the basal region. The antigen recognised by these mAbs are expressed most strongly at the apical membranes of mature enterocytes (adjacent the lumen), the apical enterocyte surface in the middle 1/3 of the crypt is weakly stained and the basal 1/3 appears unstained for both antigens.

e. Glandular epithelium

The liver parenchyma surrounding the portal triad was unstained by mAbs UA009 and UA011, but the, parenchyma adjacent to the central vein was strongly stained. This striking pattern of staining was observed throughout the liver (Figure 3.7B). Mucous and serous acini in salivary gland were unstained. The basal regions of cells lining the proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) in the kidney were stained moderately but Bowman's capsule of the glomeruli and large collecting ducts of Bellini were unstained. In the skin, there was moderate staining of sebaceous glands associated with hair follicles.

f. Mesothelium & duct epithelium

The mesothelium lining the pericardium, visceral pleura and the serosa of the small intestine were all stained by mAbs UA009 and UA011. Of the other epithelia examined, only the ciliated, pseudostatified columnar epithelium lining of the airways and the epithelium lining the alveoli of the lung were stained by mAbs UA009 and UA011. There was no staining of epidermis, pelvi-calyceal system of the kidney, the epithelium lining the ureters, interlobular and intralobular ducts of the salivary glands or the epithelium of the ducts of the resting mammary gland.

g. Neural tissue

Neural tissue in the cerebrum including nerve cell bodies, neuroglial or microglial cells were not stained by mAbs UA009 and UA011. Peripheral nerves (Figure 3.11.B, D & F)

and associated nerve cell bodies including Meissner's plexus in the muscularis externa of the gastrointestinal tract, were also unstained. Pale staining was present on endothelium lining vessels associated with the pia mater. A small number of vessels found deeper within the cerebrum also exhibited low expression.

h. Muscle

In cardiac muscle (Figure 3.7F), there was pale staining of the sarcolemma, with moderate staining of the communicating intercalated discs. However, there was strong staining of the endothelium of small and medium sized vessels in the myocardium. Myocytes of skeletal muscle (quadraceps) were unstained, but there was staining of the endomysium at the periphery of each bundle of fibres. Small vessels, between the myocytes were stained strongly (Figure 3.7C). No staining was observed on smooth muscle cells in the muscularis externa of the gastrointestinal tract (Figure 3.7D).

i. Haemopoietic cells

As noted above, large cells within the medullary sinuses of lymph nodes were stained by mAbs UA009 and UA011. These are thought to be macrophages. Red blood cells in the lumen of large vessels were also stained by antibodies and this staining was not due to the activity of pseudoperoxidase.

j. Connective tissues

Adipocytes in peri-renal fat, in fat surrounding lymph nodes and within non-lactating mammary glands showed peripheral staining, suggesting expression of the antigen recognised by mAbs UA009 and UA011 at the cell membranes. Adipocytes in normal and arthritic synovium stained strongly. Pericytes from perimysium and vascular pericytes surrounding the blood vessels also were strongly stained, but FB, fibrocytes, osteocytes, osteoblasts, chrondocytes, chrondoblasts were all unstained.

k. Normal synovium

Small vessels were numerous and were stained strongly with mAbs UA009 (Figure 3.9A). Adipocytes were stained also. It was of interest to note that the macrophage-like type 2 synoviocytes that comprise 30% of the cells that form the synovial intima (Barland *et al.*,1962; Roy and Ghadially 1967) were not stained by mAbs UA009 or UA011.

l. Arthritic synovium

The avascular synovial intima (synovial lining) did not stain with either mAb. The sub-intimal or sub synovial tissue, however, was highly vascular and large numbers of capillaries, venules and small veins, were stained strongly (Figures 3.9B,C & D). In contrast, endothelium of arteries was unstained. Distributed throughout the tissue were relatively large cells that were stained by both mAbs (Figure 3.9D). These cells are probably macrophages. However, the macrophage-like type 2 synoviocytes were unstained as was the case in normal synovium (Figure 3.9A).

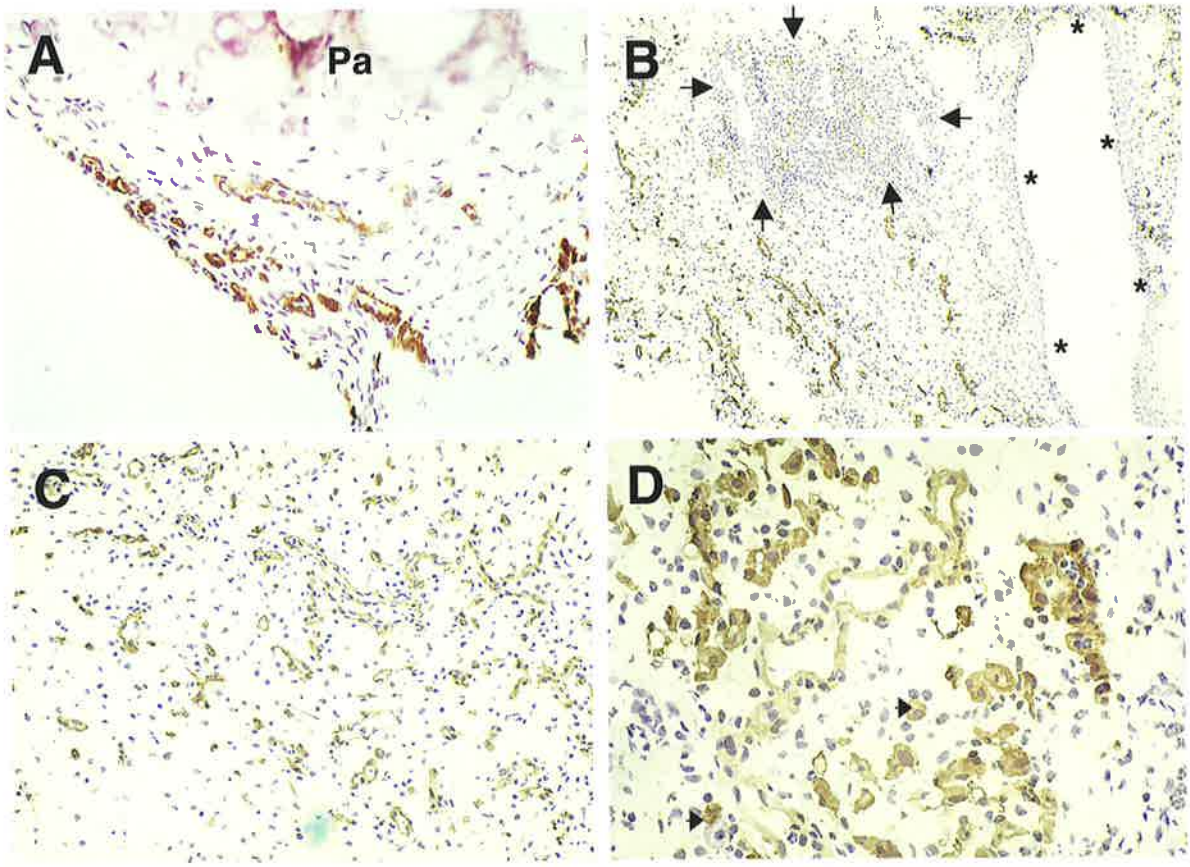
3.3.2.2 An immunohistochemical survey of staining by mAbs UA015 and UA016 in the DA rat.

An immunohistochemical survey of staining by mAbs UA015 and UA016 was carried out in a range of normal tissues from DA rats. Indirect immunohistochemistry was performed as described in Sections 2.12.2 and 2.12.3. Tissues harvested from normal rats included small intestine, heart, kidney, lung, spleen, LN and liver. In addition, synovium was harvested from the sub-Achilles region of a DA rat 14 days after inoculation with CFA. The mAbs UA015 and UA016 demonstrated essentially the same pattern of staining, which will be illustrated by reference to the staining with mAb UA015.

Expression of the UA015 antigen was observed on endothelium of veins, arteries and capillaries in all of the tissues surveyed, including EC lining HEV in lymphoid tissues. However, the intensity of endothelial staining was greater in small and medium sized vessels than in larger vessels. Staining was absent in large arteries. Staining by the mAb was associated with EC to cell

Figure 3.9 Staining of normal and arthritic synovium by monoclonal antibody UA009

Micrograph A shows normal synovium from the supra-patella pouch of the knee joint. The haematoxylin (purple) stained region at the top of the field is hyaline cartilage of the patella. The cellular synovial region below the patella shows presence of small vessels that are stained strongly by UA009 antigen (original magnification 200x). Micrograph B shows a low power view of arthritic synovium from the sub-Achilles region, stained with mAb UA009 (original magnification 40x). Note the perivascular region, indicated by arrows, which shows a high density of inflammatory cells (arrows). Note that the synovial intima (indicated by asterisks), which contains macrophage–like type II synoviocytes is not stained. Micrograph C shows synovium from an arthritic rat. Small and medium sized vessels are stained by mAb UA009 (original magnification 100x). Micrograph D shows the same area of tissue as in the previous image (C). Vessels with plump endothelium are stained for UA009. Note the even distribution of staining along the vessel. Macrophages (indicated by arrows) also are strongly stained with this mAb (original magnification 200x).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D

junctions. MAb UA015 also stained the EC of the endocardium. Neither mAb stained other cell-types in any of the tissues surveyed.

3.3.2.3 Staining with commercially available endothelial markers OX43, TLD-3A12 and RECA-1.

The mAb: OX43, TLD-3A12 (anti-PECAM-1) and RECA-1 were investigated because their antigens were known to be present on rat EC. Accordingly, they could be used to identify vessels within the region of the synovium to be utilised for harvest and to assist in the development of a method for isolation of EC and to assess the purity of the isolated EC.

MAb OX43 was raised against rat peritoneal macrophages (Robinson *et al.* 1986). The distribution of the OX43 antigen in rat tissues was assessed and the distribution of staining was identical to that described for mAb UA009 and UA011 (see Table 3.1). At the time that this work was carried out, these antigens were uncharacterised, but it was evident from these immunohistochemical investigations that they may recognise the same antigen. Further work carried out in this laboratory by Dr Xingqi Zhang has shown that the three mAbs recognise the same antigen and that antigen is CD36, also known as fatty acid translocase (Abumrad *et al.*, 1983).

a. PECAM-1

PECAM-1 (CD31) is a well-characterised adhesion molecule. The mAb TLD-3A12, which recognises PECAM-1 in rat was raised by inoculation of mice with rat microglial cells (Flaris *et al.* 1993; Male *et al.* 1995). The distribution of PECAM-1 antigen was investigated using indirect immunohistochemistry on tissue sections of normal heart and arthritic synovium. In normal myocardium (Figure 3.10.B) and arthritic synovium (Figure 3.10.E), it was expressed on the endothelium of capillaries, veins and arteries. The distribution of PECAM-1 in tissues is restricted to vascular endothelium associated with the region of the intercellular junction and the distribution and appearance of staining with mAb TLD-3A12 was identical with staining by mAbs UA015 and UA016. The antigen recognised by these mAb has not been characterised but appears likely to be PECAM-1.

b. RECA-1

The mAb RECA-1 (rat endothelial cell antigen-1) was raised against stromal cells from rat lymph node. The antibody is reported to be reactive with all vascular endothelium in the rat, including both small and large vessels (Duijvestijn *et al.*, 1992). The distribution of RECA-1 antigen was investigated using indirect immunohistochemistry on tissue sections of normal heart (Figure 3.10.C), lymph node, small intestine and arthritic synovium (Figure 3.10.F) from the DA rat. The antibody appeared to stain EC lining vessels of all sizes, including arteries, arterioles, veins, venules, capillaries, as well as the specialised endothelium lining HEV. However, the intensity of staining was low compared with that obtained with mAbs in the OX43/ UA009 (Figure 3.10A & D) group or the TLD-3A12/ UA015/ UA016 group (Figure 3.10B & E). The RECA-1 antigen was not detected on non-EC in normal tissues or in arthritic synovial tissue (Figure 3.10F).

3.3.2.4 Staining of endothelium in synovium by mAbs UA009, TLD-3A12 and RECA-1

The number of capillaries and small blood vessels detected by mAbs UA009 and TLD-3A12 in inflamed synovium was similar, but staining by UA009 was the stronger of the two mAbs. The mAb RECA-1 appeared to detect fewer vessels than mAbs UA009 or TLD-3A12. The staining intensity produced by mAb RECA-1 was weaker than in the case of the other two mAbs and this may account for its lower sensitivity in detecting small blood vessels (Figure 3.10).

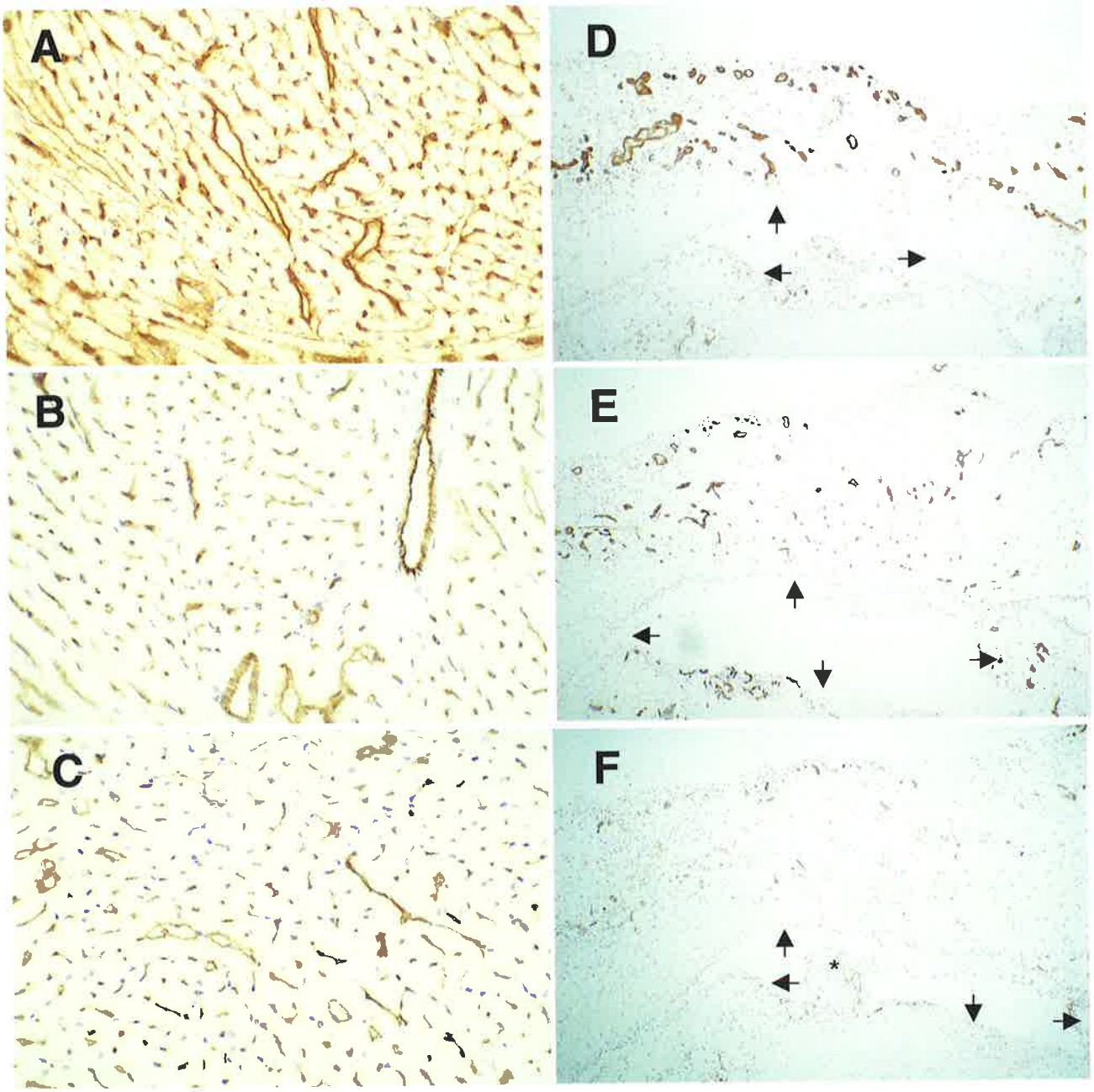
3.3.2.5 An immunohistochemical survey of Thy-1.1 antigen in the DA rat.

A mAb was sought to differentiate between EC and FB from arthritic synovium. It can be difficult to differentiate between FB and EC *in vitro*, since they both adhere to gelatin- or fibronectin-coated culture vessels. From the literature, it has been evident that the major difficulty in culturing EC is ensuring that no FB contaminate the cultures. This is important because FB typically grow more rapidly than EC and tend to outgrow them.

Thy-1.1 antigen is expressed by thymocytes, nerve cells and vascular pericytes, but it has

Figure 3.10 Comparison of the staining of capillaries and small blood vessels by monoclonal antibodies UA009, RECA-1 and TLD-3A12 (anti-PECAM-1) in cardiac muscle and synovium from an arthritic rat.

In each case, fresh frozen tissues were stained by indirect immunoperoxidase technique. Micrographs A, B and C, cardiac muscle stained with UA009 (A), TLD-3A12 (B) and RECA-1 (C) and photographed on original magnification (200x). Note that both cardiomyocytes and blood vessels are stained with mAb UA009 (A), while mAb TLD-3A12 -1 (B) and RECA-1 (C) stain only endothelium. Micrographs D, E and F, arthritic synovium stained with mAbs UA009 (D), TLD-3A12 (E) and RECA-1 (F) and photographed at original magnification (40x). The arrows are situated within the synovial space and point towards the synovial intima. In arthritic synovium, mAb UA009 stains vessels strongly. MAb TLD-3A12 stained a similar number of vessels but less strongly, while mAb RECA-1 stained fewer vessels and at a lower intensity. MAb UA009 appears to stain a larger range of vessels than RECA-1. The macrophage-like type II synoviocytes are not stained by mAb UA009 (D). The pattern of staining observed with mAb OX43 was identical to that with mAb UA009 (not shown).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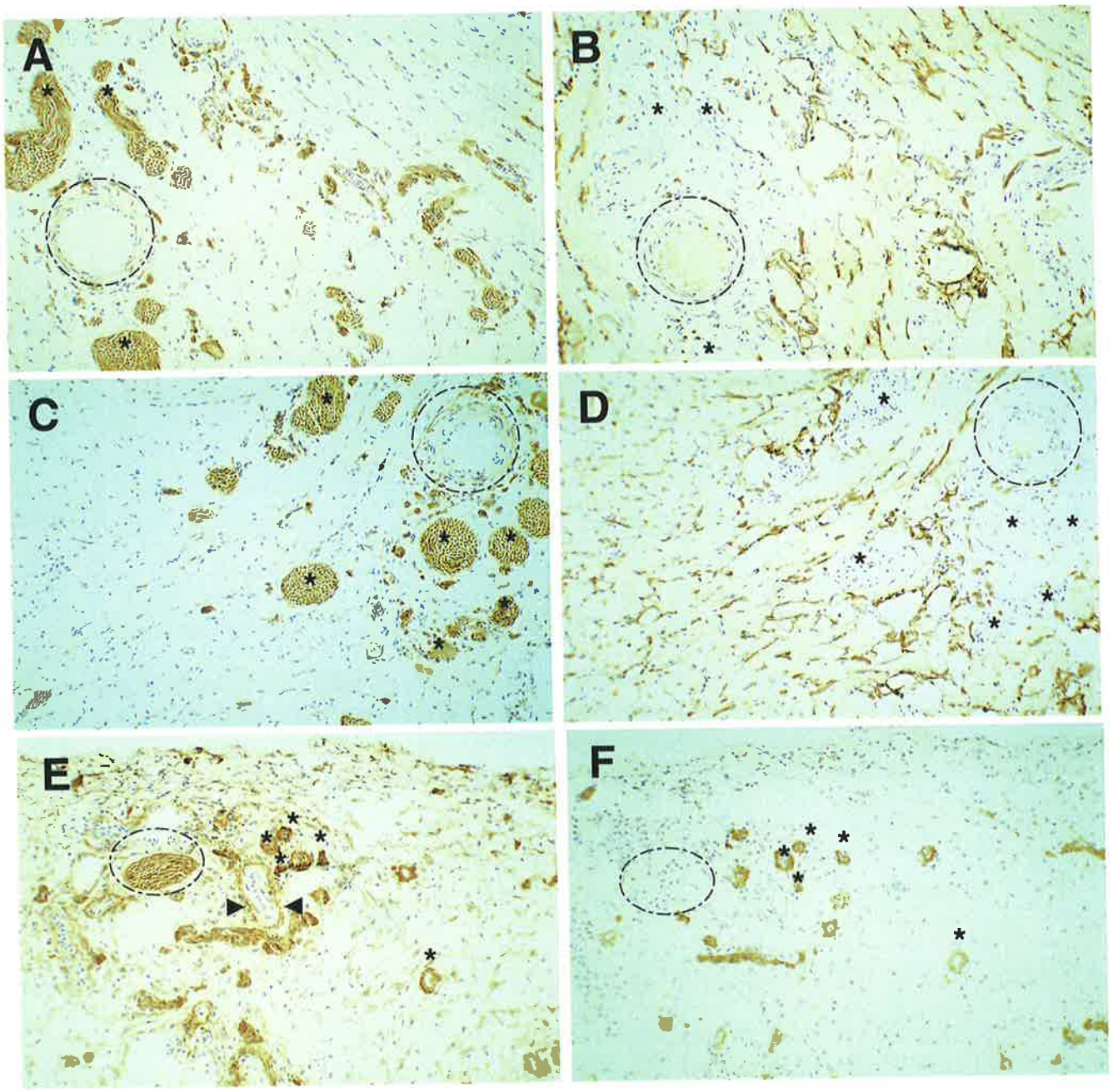
also been reported to be expressed by FB (Mason and Williams, 1980). Accordingly, an immunohistochemical investigation was undertaken of the reactivity of the mAb OX7 anti-rat Thy-1.1 (CD90) in rat tissue. Tissues examined included normal salivary gland, liver, lymph node, small intestine, large intestine, kidney, heart, and mammary gland from normal rats, as well as tongue and synovium from both normal and arthritic (day 13 post induction with CFA) rats. The tissues were harvested, snap frozen and cryostat sections were prepared as described in Section 2.4.2. Indirect immunoperoxidase staining was carried out on ethanol-fixed fresh frozen sections as described in Section 2.4.3. Thymocytes in the thymus and peripheral nerve fibres in small and large intestine, sub-Achilles tendon region and sub-synovium served as positive controls for the activity of the antibody. The mAb UA009 (anti-CD36) was used as a control antibody and to help identify staining of microvascular endothelium in paired serial sections. The sections stained with mAb OX7 were directly compared with those stained with UA009 (Figure 3.11 A-F). This comparison assisted with identification of endothelium from small vessels.

In tissues from normal and arthritic rats, pericytes associated with blood vessels (Figure 3.11E) and other sites, such as the ureters, renal tubules, Bowmans capsule in the kidney, mammary ducts, endomysium (smooth, skeletal and cardiac muscle), salivary gland acini and ducts, and HEV in Peyers patches were all stained by mAb OX7. Fibroblasts were found to be stained in all tissues, including the lamina propria of bronchus, small and large intestine, the skin, and sub-intima of synovium or sub-synovium. Tongue from normal (Figure 3.11A) and arthritic rats (3.11C) were chosen as a representative tissues to demonstrate characteristic staining of Thy-1.1. As expected, nerve fibres in all tissues investigated were stained by mAb OX7 (Figure 3.11) and thymocytes were also found to be stained.

Within tissues harvested from the sub-Achilles region from arthritic rats, synovial intimal cells were not stained. Endothelial cells lining small and medium-sized vessels were stained strongly by mAb OX7, although endothelium from arteries was unstained (Figure 3.11E). Endothelium stained by mAb OX7 was only observed in inflamed

Figure 3.11 Distribution of Thy-1.1 antigen in inflamed synovial tissue, normal and tongue from normal and arthritic rats.

The left and right images (A & B, C & D, E & F) are paired serial sections. The images A,C & E are stained with mAb OX7 (Thy-1.1) and for comparison, images B, D & F are stained with mAb UA009 (CD43). A & B show images of normal tongue, stained with OX7 and UA009 respectively (original magnification 100x). The endothelium lining the artery, enclosed by the dashed circle, is unstained both by either mAb. Peripheral nerve fibres that are stained by mAb OX7 are seen in image A (indicated by a single asterisk) are unstained in image B (indicated by a single asterisk). Small vessels situated between myocytes of skeletal muscle are stained by mAb UA009 in image B, (upper right region) but are unstained by mAb OX7 in image A. C and D show serial images of tongue from an arthritic rat (original magnification 100x). As in A and B, mAb OX7 has stained nerve fibres (*) but not endothelium lining small (left) or large vessels (dashed circle), while mAb UA009 does not stain peripheral nerves (*) but stains small vessels within the endomysium (left) strongly. E and F show serial images of arthritic synovium (original magnification 100x). MAb OX7 (E) stains FB (seen throughout the section), peripheral nerve fibres (enclosed by dashed oval), pericytes surrounding a small artery (arrow heads) and endothelium lining a number of vessels (each indicated by an adjacent asterisk *). MAb UA009 did not stain the peripheral nerve (enclosed within a dashed oval), FB or pericytes. However, endothelium lining small vessels is stained strongly by mAb UA009. The vessels stained by both mAb OX7 and UA009 are indicated in both micrographs by adjacent asterisks (*).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synovial tissue (Figure 3.11.C). Pericytes were stained strongly by mAb OX7, as were peripheral nerves and the staining of FB was obvious (Figure 3.11E).

3.4 Discussion

A major aim of the project (reported in a subsequent chapter) has been to develop a method for isolation of EC from the microvasculature of inflamed synovium from DA rats with AA. Histological assessment of hind paws of arthritic rats was carried out to select a suitable site from which to harvest synovium involved in the pathology of AA. The criteria used to select the site, were that the chosen tissue should be representative of inflamed synovium and that it should be large enough to yield sufficient cells for cell separation and selection procedures. This analysis was necessary, because previous reports have described inter-strain variability in the anatomical regions of the rat hind-paw that are involved in AA (Billingham, 1983). For example in Long-Evans rats, the tarsal joints, heel and metatarsal joints were involved, whereas, in the Wistar strain, there was relatively more swelling associated with metatarso-phalangeal joints, with little in the heel region. In addition, tissue was sought which contained a sufficient microvascular bed from which EC could be isolated.

AA was first induced in rats almost 50 years ago, using a Freund-like adjuvant (Pearson and Wood, 1956). This followed an observation by Stoerk *et al.,* (1954) that a polyarthritis could be induced by immunisation with spleen homogenised in this adjuvant. The pathology of AA has been reported over a time period of up to 350 days post-inoculation (Pearson and Wood, 1962). The visible onset of arthritis was seen from day 10 to 14 post-inoculation. Arthritis develops in DA rats during the same period after inoculation with CFA. At day 13-14 after inoculation, the ankle region showed marked swelling around the calcaneum, the posterior aspect of the ankle joint superior to the calcaneum, the tibio-tarsal joint and the medial and lateral aspects of the ankle region. These features identified the region adjacent to the ankle as a potentially suitable site from which to obtain a substantial amount of synovial tissue.

An histological survey of the entire hind paw identified a suitable area of inflamed synovium and sub-synovium. The targeted area of tissue was bordered anteriorly by the tibia, had the Achilles tendon at its posterior margin and was bounded by the calcaneum at its inferior margin. It extends superiorly up to the musculo-tendinous junction of the soleus muscle and the Achilles tendon. This region contains inflamed synovial intimal tissue and sub-synovial tissue associated with the joints forming the ankle and contains abundant small blood vessels. From a loosely arranged connective tissue with abundant adipocytes it has been transformed by inflammation into highly cellular tissue composed mainly of cells that have infiltrated from the peripheral circulation as part of the inflammatory process.

The mAbs TLD-3A12, RECA-1, OX43, UA009, UA011, UA015 and UA016 were assessed for their reactivity with microvascular endothelium in the selected parcel of synovial tissue from arthritic rats. They were also assessed for their selectivity for endothelium and their reactivity with other components of synovium. Once a suitable area of synovium was chosen, it was planned to dissociate that tissue into a single cell suspension and to use an EC-specific antibody to purify synovial microvascular EC for subsequent use in an adhesion assay. It was envisaged that an EC-specific antibody would become available from this panel of mAbs and that a method could then be developed to positively select EC using immuno-magnetic beads.

The immunohistochemical study using these antibodies revealed that the tissue distribution of mAbs UA009, UA011 and OX43 were essentially identical, suggesting that they were directed against the same antigen. Work done subsequently, by Dr Xingqi Zhang in this laboratory has confirmed that the monoclonal antibodies UA009, UA011 and OX43 are indeed directed against the same antigen (Zhang, Ph.D thesis, 2001).

With the exception of pericardium, staining with mAbs UA015 and UA016 was limited to EC lining small and medium sized vessels. These two antibodies showed similar patterns of staining to each other and to mAb TLD-3A12 (PECAM-1). Like PECAM-1, the antigen

detected by these antibodies, appeared to be expressed mainly in association with the junctions between EC.

The mAbs examined, therefore, fall into 3 clusters. One, containing mAbs UA009, UA011 and OX43, is now known to detect the rat orthologue of CD36. This molecule has a range of functions, including fatty acid translocase activity (Abumrad *et al.,* 1983) and activity as a scavenger receptor that allows binding of oxidised low density lipoproteins (Endemann *et al.,* 1993) and anionic phospholipids (Rigotti *et al.,* 1995). The molecule also has affinities for high-density lipoproteins, thrombospondin and collagen (MacGregor *et al.*, 1989). These activities explain the distribution of the molecule on a range of cells including vascular endothelium, macrophages, adipocytes and cardiomyocytes. Nevertheless, within synovium, antibodies in this group have potential as selective agents for EC, provided that cells of haematopoietic origin can be removed first by other methods. Adipocytes are not expected to present a significant problem. These antibodies have the advantage that the antigen is expressed strongly on microvascular endothelium and less strongly (or absent) on vessels of larger calibre, especially in arterial circulation. Importantly, the antibodies do not react with FB.

The mAbs in the group including UA015, UA016 and TLD-3A12 appear to recognise antigens that are expressed exclusively by EC. They are, therefore, good candidates with which to purify EC, although, they do not stain microvascular endothelium with intensity equal to mAbs UA009, UA011 or OX43. The RECA-1 antibody has a staining pattern distinct from the other 2 groups of mAbs. While it appears to stain only EC, it does not stain microvascular endothelium strongly and this may be a disadvantage in its use to purify synovial EC.

Fibroblasts are a major problem when attempting to obtain pure cultures of EC (Jackson *et al.,* 1990). Characteristically, both EC and FB are adherent to culture sub-strata such as gelatin or fibronectin. Relatively small numbers of contaminating FB can proliferate rapidly to outgrow and overgrow EC populations. The mAb OX7 against the surface

molecule Thy-1.1 was investigated for its specificity for FB within inflamed synovial tissue. If a mAb with selectivity for FB could be identified in this tissue, it could be used to eradicate FB from preparations of EC by negative selection, utilising immuno-magnetic beads. This strategy was attractive, because it would avoid the necessity to positively select EC. Positive selection exposes the population of interest to antibodies that could alter their function. While mAb OX7 did not stain EC in normal tissues, including synovium, however, during a preliminary immunohistochemical survey, strong expression of Thy-1.1 was observed on the EC lining small and medium sized vessels in synovium from the hind paws of rats with AA.

Soon after this work was completed, it came to our attention that Thy-1 was induced on endothelium in rats after subcutaneous injection of CFA to induce dermatitis (Ishizu *et al.*, 1995). It has also been reported that EC *in vitro*, but not *in vivo,* from the inferior vena cava from rats express Thy-1.1, (Ishizu *et al.,* 1997), while ligation of Thy-1 by antibodies has been shown to induce changes to vascular permeability and cause rearrangement of vimentin filaments within cultured retinal EC (Ishizu *et al.,* 1995). The strong expression of Thy-1.1 by EC in arthritic synovium meant that this mAb would not be suitable for selective removal of EC from synovial cell suspensions. .

In summary, the aims relating to this part of the study have been largely fulfilled. The sub-Achilles region of the arthritic hind paw was identified as a suitable region from which to harvest inflamed synovial tissue reproducibly. The volume for harvest appeared sufficient to obtain a substantial amount of microvascular tissue. Monoclonal antibodies have been identified that have potential for use in isolation and purification of EC. However, mAb OX7 (anti-Thy-1.1) was found to react with EC in inflamed synovium, prohibiting its use to deplete FB from suspensions of cells from synovium.

Chapter 4

Development of a Method for Isolation and Short-Term culture of Endothelial Cells from Synovial Tissue from the Sub-Achilles Region of DA Rats with Severe Adjuvant Arthritis.

4.1 Introduction

In Chapter 3, a histological survey was carried out of hind paws from rats assessed to have severe or moderate adjuvant arthritis. This identified an area of synovium-rich tissue from which ECs from the microvasculature of inflamed synovium could be isolated and purified. These ECs could be used subsequently as an investigative tool with which to study *in vitro* interactions between ECs and lymphocytes. An investigation of such interactions was the major long-term goal of this project.

Several new mAbs that reacted with EC antigens were identified and described in Chapter 3. An immunohistochemical survey investigating the specificity's of mAbs UA009, UA011, UA015 and UA016 in a range of tissues, identified mAbs UA009 (CD36) and UA015 (PECAM-1-like distribution) as potential tools for isolations of EC and for subsequent identification of EC in culture. Monoclonal Ab UA009 has since, been shown to be specific for the rat homologue of CD36 (Zhang *et al.*, 2003).

Various researchers have investigated the mechanisms leading to adhesion of lymphocytes to ECs and their subsequent transmigration to sites of inflammation. The majority of these *in vitro* investigations, however, have utilised ECs isolated from easily accessible large vessels like human umbilical veins (HUVECS) (Haskard *et al.*, 1996) or rat aorta (Issekutz *et al.*, 1993). The isolation of EC from large vessels, particularly HUVECs, has become a routine procedure. However, the predominant vascular cells involved in inflammation are EC of microvascular origin and few have used cells from small vessels for *in vitro* studies. Issekutz and co-workers have investigated aspects of lymphocyte adherence using microvascular endothelium from rat heart (Issekutz, 1990; Wykretowicz and Issekutz,

1993).

Cells from large vessels have provided a useful and well-characterised model system and their use has helped to expand the vascular biology knowledge base immensely. However, it is widely accepted, that there exists significant heterogeneity and functional differences between EC derived from different sized vessels and those from different vascular beds (Kumar *et al.,* 1987; Page *et al.,* 1992; Kvietys and Granger, 1997; Gerritsen *et al.,* 1993). A better alternative is to investigate *in vitro* interactions between leucocytes and ECs isolated from the microvasculature of the specific tissue of interest. Furthermore, because EC in culture, rapidly lose specific differentiated characteristics, it is preferable to study vascular regional differences in freshly isolated cells.

The aims of this project were to isolate EC from the microvasculature of the periarticular tissues from inflamed synovial joints in DA rats with severe AA. These would be used subsequently to develop an in vitro adhesion assay in which interactions between EC and lymphocytes could be studied. The culture of microvascular EC is problematic because it is difficult to isolate the cells free from contamination of FBs. In addition, yields were expected to be relatively low. Although the tissue site selected for harvest of EC is richly vascular, it is not large and microvascular EC are a small proportion relative to connective tissue and inflammatory cells present.

The small number of EC that could be isolated, together with the need to screen large numbers of antibodies for their ability to block adhesion of lymphocytes, made it necessary to develop a system of micro-culture. The initial direction of this study was to establish a method for the isolation of these cells, their maintenance once in culture and culturing them in a micro-culture system that could be utilised in the adhesion assay. Terasaki plates, commonly used for HLA tissue typing, were investigated for use in a microculture system.

EC from microvascular beds are very difficult to isolate and grow as pure cultures. They make up a relatively small proportion of the total cells, are difficult to release from their

vessels and to separate from FBs. Fibroblasts are a problem because like EC, they are adherent. Furthermore, they are usually more numerous and when they contaminate monolayers, they proliferate, tending to rapidly overgrow the EC. Over the years, numerous methods have been described to purify or enrich EC. Methods such as manual weeding of FBs (Davidson *et al.,* 1980), the manipulation of culture media by amino acid substitution (Gilbert and Migeon, 1975; Picciano *et al.,* 1984), fluorescent activated cell sorting using uptake of fluorescently labelled (1,1'-dioctadecyl–1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate) acetylated low-density lipoprotein (a-LDL) by EC (Voyta *et al.*, 1984) and density gradient centrifugation (Greenwood and Calder, 1982; Bowman *et al.,* 1981) have all been used to isolate EC from mixed cell cultures or mixed cell suspensions. These methods give limited success, are time consuming and do not give a reproducible yield of early passage endothelial cells" (Jackson *et al.,* 1989). Jackson *et al.,* (1990) developed a relatively quick method to isolate pure populations of microvascular endothelium from human tissues. They used tosyl-activated Dynal beads coated with the lectin from *Ulex europaeus.*

The aim of this project was to use freshly isolated microvascular EC from the inflamed synovium-rich tissue so that endothelial markers and adhesion molecules upregulated by inflammation would be present during *in vitro* adhesion assays. A number of methods for isolation of vascular endothelium were studied and compared before positive selection by EC-specific mAbs and immunomagnetic beads was chosen.

4.2 Hypotheses and aims

4.2.1 Hypotheses:

- The sub-Achilles region in arthritic rats is a site from which synovium rich tissue can be harvested reproducibly.

- Enzymatic techniques can be adapted to produce sterile cell suspensions from rat synovial tissues.

- Microvascular ECs can be purified from a mixed synovial cell suspension.

- EC specific monoclonal antibody(s) can be used in the identification and selection of EC for subsequent *in vitro* investigations.

- ECs can be maintained in short term culture for use in lymphocyte – EC adhesion assays.

- ECs can be established in microculture, so that large numbers of antibodies can be screened for their ability to block adhesion between ECs and lymphocytes.

4.2.2 Aims:

- To establish a method to dissociate synovial tissue into a mixed single cell suspension.

- To establish a method to select/purify ECs from the mixed synovial cell suspension.

- To establish a micro-culture system suitable for examining the interaction of recently isolated microvascular ECs with lymphocytes.

4.3 Aseptic harvest of inflamed synovial tissue.

4.3.1 Preparation of the rat

Severe AA is established in DA rats 13 to 14 days after induction with a single injection of 0.1ml CFA into the tail base (Spargo *et al.*, 1996; 2001). The targeted area of tissue (Section 2.13.1), is excised anteriorly along the tibia, posteriorly along the Achilles tendon, inferiorly at the calcaneum and superiorly at the insertion point of the Achilles tendon into the gastrocnemius muscle (as described in Section 2.13.2).

All procedures were carried out in a sterile lamina flow hood. After euthanasia (see Section 2.3), the rat was saturated in 70% ethanol. It was then firmly secured in the supine position to a corkboard with pins in each paw. The board was anchored to the working surface with a screw clamp.

4.3.2. Harvest of Tissue

Initially the anterior surface of the rats' hind limb and foot was scored with a sterile #11 scalpel blade, just penetrating the epidermis and dermis. This incision was made in one long sweeping cut, at a consistent depth to avoid contamination of the subcutaneous tissues. Following this, the skin was incised with scissors superior to the patellar, so as to 'ring-bark' the skin of the hind limb well above the area of interest. With two pairs of large tooth forceps, the skin was then pulled distally toward the foot, so that it could be removed cleanly without contaminating the underlying tissue. At the heel, the dermis tended to adhere to the underlying tissue and required use of a sterile scalpel blade for its release. The skin was then peeled further until it passed over the distal phalanges. A large artery forceps was then attached at the distal phalanges and the handle of the forceps anchored to the cork board near the head of the rat. This flexed the limb at the hip joint and held it so that the skinned lower limb did not contact any surfaces during work on the sub-Achilles region, which could be accessed easily.

A medium sized curved artery clamp was attached to the musculotendinous junction of the Achilles tendon and the soleus muscle. The Achilles tendon and the tissue deep to this tendon were cut transversely through to the posterior surface of the tibia. The scalpel blade was then turned through 90° and used to excise tissue from the posterior tibial surface, reaching distally to the superior surface of the calcaneum. At this point, the tissue was also carved from around the lateral and medial malleoli of the ankle joint and surrounding the calcaneum. After dissecting the distal insertion of the Achilles tendon, the excised tissue, in one piece, was placed in a sterile container and weighed. Following this the tissue was immersed in pre-warmed (37°C) RPMI-1640 with antibiotics, L-glutamine and 2% FCS

until the tissue from both hindpaws of 6 rats had been collected.

4.3.1 Tissue yield

The weight of sub-Achilles tissue that was harvested varied according to the extent of arthritis. Younger rats aged 6 weeks, tended to show less severe arthritis than that observed in 8 to 10 week old rats. It was also found that unusually small for age rats developed less severe arthritis compared with age matched but heavier rats. To ensure consistency of cell harvest numbers, rats with a minimum weight of 170g, at 6 weeks old were selected for inoculation with CFA for subsequent collection of arthritic tissue. The mean weight of tissue collected from two paws of individual rats was 0.54 g ± 0.28 SD (n=64, range 0.12-1.73g).

4.4 Enzymatic dissociation of synovial tissue

4.4.1 Tissue preparation

Prior to enzyme digestion, the tissue was diced into cubes. Preliminary experiments showed that a small dice size was important to obtain maximal cell yields from the harvested tissue. This was achieved by coarse chopping with two large #22 sterile scalpel blades followed by fine dicing with a pair of small curved scissors. Enzyme digestion carried out on tissues diced at 0.5-1mm^3, produced an average 56% increase in yield of mixed synovial cells, compared with tissue chopped into 3mm cubes.

4.4.2 Preliminary Experiments-Enzyme Digestion Protocol and Assessment

Several enzymes were evaluated for their efficiency in dissociating cells from the diced synovial tissue and a number of existing methods were assessed for their use on the sub-Achilles tissue. The enzymes selected were collagenase/dispase, trypsin/pronase and collagenase class 1 (CLS-1). In each case, the synovial tissue was diced and resuspended in pre-warmed enzyme dissolved in RPMI-1640 at 37°C and incubated with continuous, gentle agitation. The process of digestion was monitored by macroscopic observation of cloudiness, as well as the appearance of the tissue clumps and counts of cell numbers in the supernatant, using phase contrast microscopy.

4.4.2.1 Collagenase / Dispase

The procedure used was adapted from the method of Greenwood and Calder (1982). These authors digested microvessel fragments from rat retinae by incubation for 60 minutes in 0.1% collagenase / 0.1% dispase at 37°C.

The diced synovial tissue was resuspended in 0.1% collagenase/ 0.1% dispase and incubated at 37°C for periods of 30, 60, 90, 120, 180 minutes and overnight (15 hours). In all samples (30mins to overnight incubation), macroscopic and microscopic assessment showed that digestion was incomplete. Even after overnight digestion, relatively large pieces of tissue remained undigested. Counts of mixed synovial cells were carried out immediately following incubation (Table 4.1). Cell viability, less than 50%, assessed by Trypan blue exclusion, was poor after long periods of incubation and there were few viable cells present after overnight digestion with the enzyme mixture.

4.4.2.2 Trypsin / Pronase digestion

A mixture of 0.6% trypsin and 0.4% pronase liberated very few cells from sub-Achilles tissue after incubation with agitation for 15, 30, 40 and 60 minutes and 15 hour (overnight) at 37°C (Table 4.2). At all sample times, tissue digestion was incomplete. The viability of cells that were liberated after the shorter incubations was assessed as poor and very few viable cells were present in the supernatant after overnight digestion.

4.4.2.3 Collagenase type 1 (CLS1) digestion

Collagenase type 1 was evaluated using a 0.2% solution of the enzyme in RPMI-1640 with 1% FCS, with continuous gentle agitation at 37°C. Preliminary experiments were carried out and monitored by microscopic assessment with cell counts and macroscopic observation after incubation times of 20, 40 and 80 minutes. After 20 minutes and 40 minutes incubation, tissue clumps were relatively abundant, compared with the final incubation time of 80 minutes. After 80

Table 4.1 Digestion of diced sub-Achilles tissues from arthritic rats, with enzyme mixture containing 0.1% collagenase/ 0.1% dispase. Shows mixed cell counts from crude synovial digests after various periods of digestion at 37°C with agitation.

Time (min)	Cell yield ($x10^7$)
30	0.0015
60	0.0012
90	0.0090
120	0.0080
180	0.0220
Overnight (15h)	0.020

Table 4.2 Digestion of sub-Achilles tissue from arthritic rats with 0.6% Trypsin and 0.4% Pronase. Mixed cell counts and the macroscopic / microscopic appearance of crude synovial digests are recorded after various periods of digestion in enzyme at 37°C with agitation.

Digestion time (min)	Cell count ($x10^7$)	Appearance
15	0.0050	Formed large clump
30	0.0054	Few single cells, undigested pieces
60	0.008	Some undigested pieces & fibrous CT visible
15 hours	0.080	undigested pieces present

minutes incubation in enzyme few tissue clumps were present, there were some undigested collagen strands and many single cells. Cell viability was assessed as good (greater than 90% viability). These preliminary results are summarised below in Table 4.3.

4.4.3 Refinement of the collagenase digestion method.

4.4.3.1. Collagenase Class 1 digestion time

Collagenase class 1 was selected as the most promising primary enzyme for digestion of the sub-Achilles tissue. Extension of the digestion time to 90 or 120 minutes at 37°C increased the yield of cells markedly (Table 4.4) and cell viability was good. Once the optimal digestion time with this enzyme was ascertained, further work in the development of the technique in order to maximise the cell yield was carried out.

4.4.3.2 Physical disaggregation

After incubation in the enzyme solution, tissue fragments and cell clumps remained. The suspension was further disaggregated by gentle manual homogenisation in a sterile glass homogeniser tube with loose fitting plunger. Homogenisation disrupted the tissue clumps leaving only undigested strands of collagen from tendon remnants.

4.4.3.3 Coarse filtration

Immediately following the homogenisation step, the digest solution was filtered through stainless steel mesh sieves, to remove undigested pieces of tendon from the suspension. This was carried out in two stages, initially using a coarse sieve with a pore size of 570μm diameter followed by a finer sieve with a 100μm pore size. The fine sieve was flushed 3 times with RPMI-1640 with 1% FCS (washing medium), to recover single cells trapped amongst the fragments. Sieving removed large debris, such as fragments of tendon and ligament, while allowing recovery of single cells and microvascular segments.

Table 4.3 Preliminary results of enzyme digestion of sub-Achilles tissue from arthritic rats with collagenase CLS-1. Cell counts after incubation for 20, 40 or 80 minutes at 37°C with continuous gentle agitation.

Time (mins)	Cell count ($x10^7$)	Appearance
20	0.004	Undigested clumps
40	0.25	Single cells and clumps
80	0.35	Single cells, few clumps, dense CT strands

4.4.3.4 Addition of secondary enzymes -Hyaluronidase and DNase

To further improve the yield, additional enzymes were added to the mixture. Hyaluronidase was added to assist with the digestion of the viscous extracellular ground substance in which connective tissue cells and fibres are embedded. Hyaluronidase was used at a final concentration of 50U/ml.

DNase was included because it had been noted that the cell suspension produced by collagenase digestion was viscous in nature and that the viscosity tended to clog the pores of the coarse sieve during filtration. The viscosity could trap cells, potentially reducing the yield. DNase reduced the viscosity, without damaging intact cells.

4.4.4 The final tissue digestion procedure

Each piece of tissue was cut into a large dice approximately $5mm^3$, then minced finely with a pair of small curved scissors into 0.5-1.0mm^3 size pieces. For enzyme digestion and further disaggregation, the tissue from 6 rats (12 ankles) was divided into two separate aliquots or samples, each containing the diced tissue from 3 rats, each of which was suspended in 20ml of enzyme mixture.

Enzyme digestion was carried out in a disposable, sterile, polystyrene 70ml container for two hours at 37°C on a Ratek oscilating platform rotator, operating at approximately 100rpm. The enzyme mixture consisted of 250U/ml of collagenase type 1 (CLS-1) (Worthington Biochemical Corporation, NJ, USA), 50U/ml of Hyaluronidase and Deoxyribonuclease 10μg/ml (200μg/100μl).

After incubation, the digest was gently disaggregated manually with a loose fitting glass homogeniser. Large particles and tendon fragments were removed from the digested tissue by filtration through stainless steel mesh sieves, the first with a 250μm pore size and then second with a 100μm pore size. This process produced a suspension that consisted mainly of single cells and

Table 4.4 Counts of mixed synovial cells from sub-Achilles tissue after collagenase type 1 digestion for 30, 40, 60, 80 or 120 minutes at 37°C. Tissue was routinely harvested from three or six rats in each experiment. Cell counts from a number of combined experiments are expressed as yield per single rat (x 10^7).

Digestion time (min)	Mean synovial cell count per rat (x 10^7)	± SE	Replicates (n)
30	0.012	0.001	3
40	0.48	0.12	20
50	0.42	0.15	8
80	0.67	0.35	13
90	0.96	0.16	6
120	1.05	0.25	9

Table 4.5 Comparison of cell counts of mixed synovial cells obtained by digestion of sub-Achilles tissue with either collagenase type 1 alone or a mixed enzyme cocktail of collagenase, hyaluronidase and DNase. Digestion at 37°C for 90 or 120 minutes.

Time (mins)	Collagenase alone (CLS-1) (x 10^7)	CLS-1 + hyaluronidase + Dnase (x 10^7)	Relative % ↑ yield
90	0.96	1.64	70.8
120	1.05	1.5	42.8

relatively small cell aggregates. The cells were a mixed population, including inflammatory cells of haemopoietic origin, red blood cells, FBs and EC. A cell count and assessment of viability was carried out using a haemocytometer and Trypan blue exclusion.

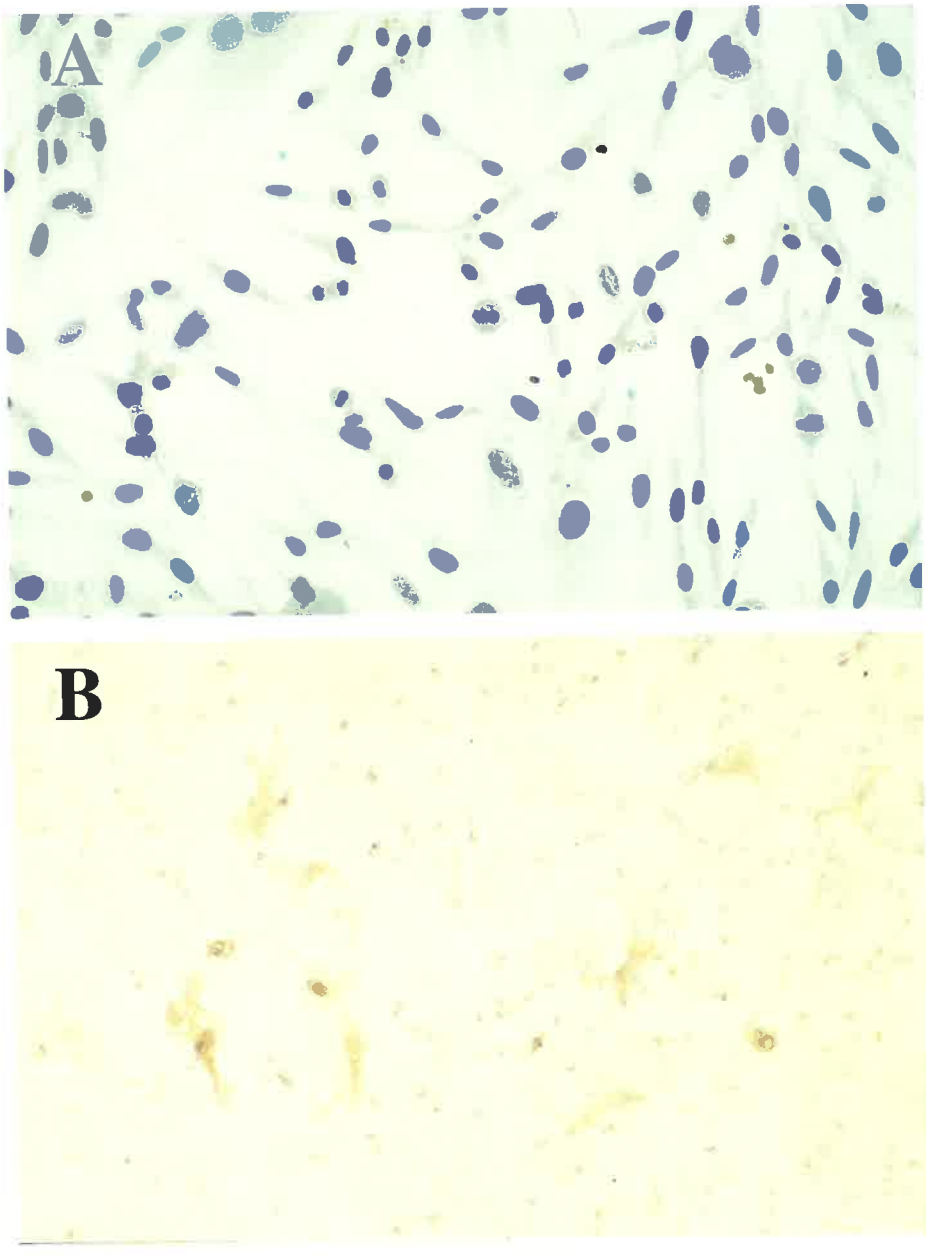
The sequential improvements led to a significant increase in the cell yield (Table 4.5). The total mixed synovial cell yield was increased by 70%, compared with the yield following collagenase digestion alone. Using this method it could be expected that sub-Achilles tissue harvested from 6 arthritic rats would yield approximately $1x10^8$ total cells, plus many microvascular segments. The viability of cells in the suspension was greater than 85%.

4.5 Monolayers cultured from mixed synovial cell suspensions.

Cells from the collagenase digest were cultured to establish suitable growth conditions. Adhesive cells were allowed to attach to a gelatin matrix and cultured to establish the viability of the cells and their growth. Conditions for culture were adapted from a method used for the long-term culture of HUVECS by the Vascular laboratory of the Hanson Cancer Research Centre (personal communication Dr J. Gamble) as described in Section 2.6.5a. Briefly, the cells from the collagenase digest were washed 4 times, resuspended in RPMI-1640 with 20% FCS (EC growth media) (see Section 2.6.5.1) and seeded onto the gelatin coated tissue culture plastic (Section 2.6.5.3) at a density of $2\text{-}5x10^7$ cells per T75 flask (area $75cm^2$). The cells were allowed to adhere to the gelatin matrix for 15 hours at 37°C in a 5% CO_2 atmosphere before the non-adherent cells were removed. Non-adherent cells were separated from adherent cells in the flask, by washing with EC growth media followed by 2 changes of 10ml pre-warmed (37°C) RPMI-1640 with 1% FCS (wash media). Growth medium was then replaced with fresh medium.

Figure 4.1 Monolayers of mixed populations of adherent synovial cells.

(A) Monolayer of cells from the mixed synovial population cultured on gelatin and stained with haematoxylin after fixation. Growth is indicated by presence of at least 8 cells within this field undergoing mitotic division. Note the different cell morphologies present, ranging from spindle shaped FB-like cells to relatively large, rounded cells with processes. These cells are representative of an unseparated population and are probably a mixture of FBs, EC from large and small vessels and macrophages.(original magnification 400x) (B) Micrograph of a monolayer of mixed synovial cells, stained with mAb UA009 using indirect immunoperoxidase technique. A relatively small number of cells were stained, indicating that only a relatively small proportion of mixed synovial cells are EC of microvascular origin. (original magnification 200x)


A
B

A monolayer of cells with varied morphology was observed adherent to the gelatin matrix. The monolayer was composed of cells with morphologies that varied from fusiform or spindle shape to larger pear shaped cells with processes. With continued culture in the absence of EC supplement, the cells reached confluence within 4 days of seeding. Cells within the monolayer appeared to be healthy and proliferating rapidly. Fixed monolayers stained with Giemsa stain contained numerous mitotic figures (Figure 4.1.A). Cells with the morphology of unhealthy or stressed ECs were not observed. When stressed, ECs exhibit extensive vacuolation, elongation of the cell body and the appearance of unusually long stringy, 'dendrite-like' processes that can reach 5 to 10 times the original length of the cell.

Cells of adhesive character that attach to the gelatin matrix are likely to include FBs, ECs and some macrophages. The former two cell types have similar morphology when observed using phase contrast microscopy, and it was not possible to differentiate between them with confidence. Endothelial cells derived from small vessels (the microvasculature) often have morphology similar to that of FBs in culture (Kumar *et al.*, 1987). In contrast, ECs from large vessels (eg HUVECs), tend to possess a 'cobblestone-like' morphology and they have been differentiated from FBs by their dimensions; using the criterion that the length of FBs is usually at least 2 times the width of the cell (Kumar *et al.*, 1987).

The results indicated that the cells prepared from the sub-Achilles tissue by collagenase digestion had long-term viability and growth potential. They grew quickly in EC growth medium and did not require the addition of EC growth factors (ECGF) to maintain health and achieve proliferation of the cultures for periods of up to four days.

4.6 Identification of endothelial cells in suspensions of mixed synovial cells and in monolayers of adherent cells.

4.6.1 Immunohistochemical investigation of EC in adherent monolayers of mixed synovial cells.

EC within the tissue had been observed previously in tissue sections, using either routine histological (H&E) staining (Section 3.3.1.2) or immunohistochemical staining with a number of EC specific mAbs (Section 3.3.2). Endothelial cells are far out-numbered in the tissue by FBs

Monolayers of adhesive cells from the mixed synovial cell suspension were prepared in eight well chamber slides. Firstly, the cells were washed, then resuspended in EC growth medium (Section 2.6.5), seeded into the culture wells and allowed to attach overnight (15 hours). The following day, non-adherent cells were washed away and the remaining cells were stained with the mAb UA009 (anti-CD36), using a modified indirect immunoperoxidase technique (described in Section 2.4.3). To maintain adhesion of the cells through the washing steps, it was found that glass or Permanox (plastic) culture surfaces required pre-coating with fibronectin or gelatin respectively (Section 2.6.5.2).

Microscopic examination of the stained monolayers (summarised in Table 4.6), revealed that only about 8% of adherent cells within the monolayers were stained by mAb UA009 (Figure 4.1.B). The stained cells varied in their morphology, from FB-like cells with spindle shape, to broader elongated cells with short cytoplasmic processes (Figure 4.1.B). An additional population of smaller, round cells was stained by mAb UA009 and these cells appeared likely to be macrophages which are known to express CD36. Staining with mAb TDL-3A12 (anti-PECAM-1) revealed a larger population of stained cells, with approximately 18% of the cells in the monolayers assessed as PECAM-1 positive (not shown).

The earlier immunohistochemical studies on tissue sections (Section 3.3.2.4) had shown

that mAb UA009 strongly labelled microvascular ECs and macrophages. However, EC from larger vessels were either unstained or weakly stained by this antibody. PECAM-1, however, is expressed constituently by EC in both large and small blood vessels. Therefore, staining with mAb TDL-3A12 is likely to reveal the entire EC population, whereas mAb UA009 will only reveal EC from the microvasculature.

These experiments indicate that only 10-20% of the adherent cells from the mixed population released from synovium by collagenase are EC. Before monolayers could be used for lymphocyte adherence studies, it was essential to develop a method to purify the EC from contaminating FBs and macrophages.

4.6.2 Flow cytometric analysis of endothelial cells within the mixed synovial cell suspension.

Single colour, flow cytometric analysis was carried out on a suspension of freshly isolated cells prepared from the sub-Achilles tissue from an arthritic rat. Monoclonal antibodies, UA015 (Figure 4.2B), UA009 (Figure 4.2D) and UA016 were utilised to identify EC within the whole population of viable cells (Figure 4.2.A). As a means of identifying ECs amongst the mixed cell population, cells labelled with mAb UA015 were backgated and their forward and side scatter of light was plotted electronically (Figure 4.2.C).

Monoclonal antibodies UA015 and UA016 (both believed to be directed against rat PECAM-1), stained approximately 5.5 to 6% of the total cell population (including non-adhering cells). And were therefore, identified as endothelial (Figure 4.2.B). Cells that were stained by these mAbs would include ECs from both small and large vessels, while unstained cells would include FBs and the large numbers of cells of haematopoietic origin that are present in inflamed synovium.

Flow cytometry revealed a much larger population of cells that were stained by mAb UA009.

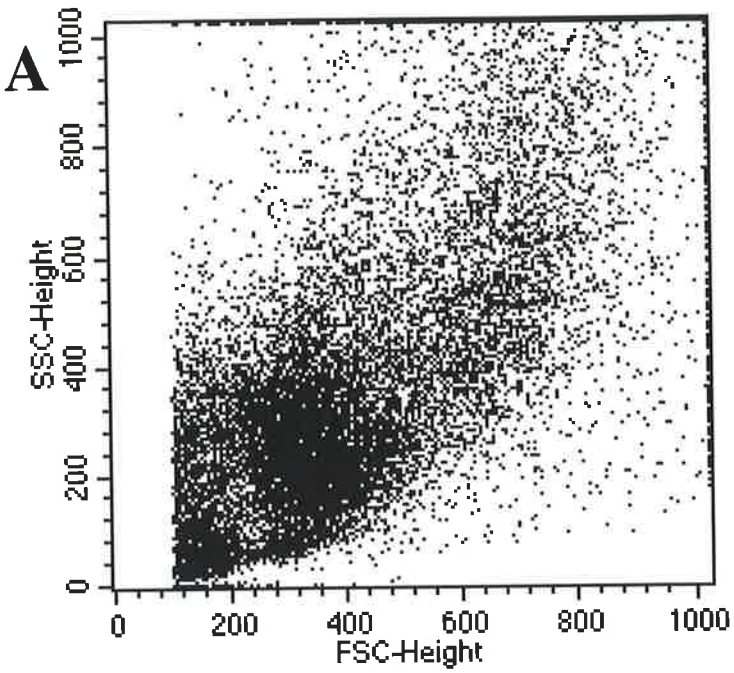
Table 4.6 The proportion of cells in adherent monolayers of mixed adherent cells prepared from synovium that express PECAM-1 (mAb TDL-3A12) or CD36 (mAb UA009) *.

Antibody	% positive	EC origin
TDL-3A12	16.5-18	Large & small vessels
UA009	7-7.5	Small vessels (microvascular)

* Cells with presumed macrophage morphology were excluded in this estimate.

Figure 4.2 Flow cytometric analysis of cells isolated from tissue from the sub-Achilles region of arthritic rats.

Dot plot and histograms showing analysis of crude single cell suspension prepared from inflamed synovium from rats with AA. (A) Plot of forward light scatter (indicative of cell size) and side scatter (indicative of cell complexity), constructed from the total viable cell population. (B) Fluorescence of the cells labelled with mAb UA015 (PECAM-1-like) by the indirect immunofluoresence technique (green). Gate M1 identifies ECs present within the mixed population, (comprising 5.5-6.6% the total cells present). Binding of isotype control mAb 1B5 shown in black. (C) Dot plot showing relative position of cells labelled by mAb UA015 within the mixed population, derived by backgating. (D) Fluorescence of the cells labelled with mAb UA009 by indirect immunofluoresence technique (green). The histogram shows 2 peaks indicating different populations of cells stained by this antibody. The brightest peak is thought to contain the relatively large population of macrophages found within inflamed tissue. The shoulder of cells on the dimmer peak, with fluorescence above background (mAb 1B5, black) is thought to include ECs from the microvasculature.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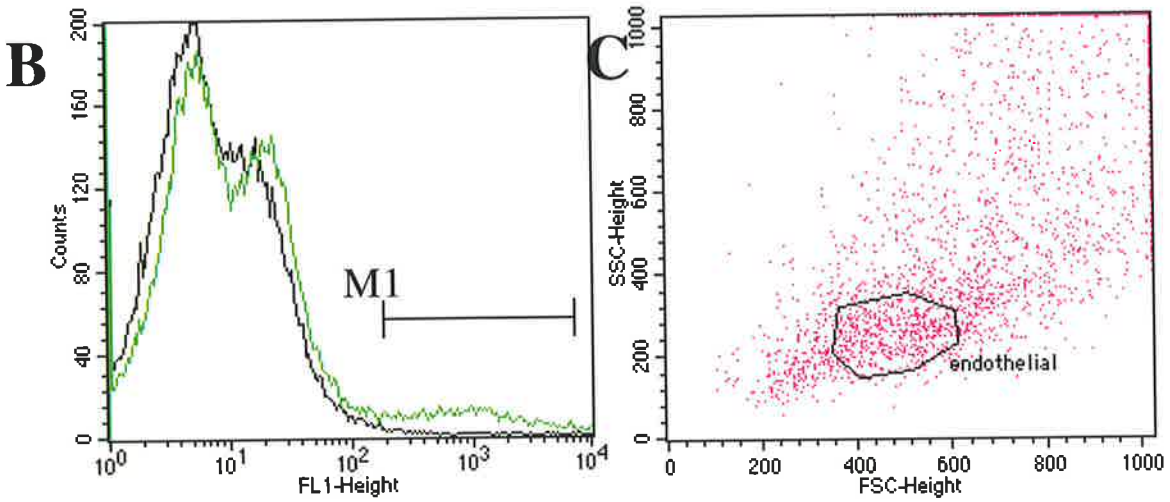
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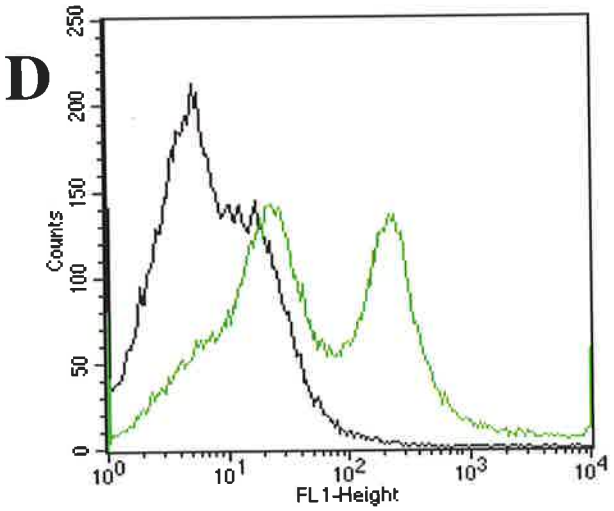
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Counts
FL1-Height

The UA009 antigen (CD36) was expressed by between 35-40% of cells within the total mixed population (Figure 4.2.D). In addition to ECs, this population is likely to have included a number of (non-endothelial) cell types that express CD36, including macrophages and non-adherent cells (erythrocytes and polymorphonuclear cells).

4.7 Comparison of methods used for isolation of endothelial cells from the mixed cell population.

Many different methods have been used to isolate and purify EC. These sources of EC include vessels of different sizes and vessels from different vascular beds, organs and species. A number of these have been investigated in an attempt to adapt them for use in purifying EC from the microvasculature of the synovium from the arthritic rat. Existing methods, such as differential adherence times, Percoll density gradient centrifugation, collection of vascular segments and the use of immunomagnetic beads, were examined. The more successful methods were then selected and attempts were made to adapt them to suit the conditions and requirements of this project. The main objective was to obtain a primary EC culture, free from contamination with FBs.

4.7.1 Adherence of endothelial cell and fibroblasts to different matrices and differential adherence times.

Differential adherence of EC compared with FBs was explored, using different adherence times and adherence to different culture matrices. In these experiments, mixed suspensions of cells derived from arthritic sub-Achilles tissue were allowed to adhere for varying lengths of time, to either glass or plastic culture surfaces (Tissue Culture Chamber Slides). In the initial studies, the culture surfaces were either uncoated or coated with gelatin. Cell adherence to the culture surfaces was examined after incubation periods of 30, 60, 120 minutes, 5 hours and 14 hours. The cultures were set up in pre-warmed RPMI-1640 containing glutamine, antibiotics and 20% FCS, and after gentle washing to remove non-adherent cells, the cells were observed using phase contrast microscopy. Adherent cells

could not be identified as either EC or FB, according to their morphology. EC from the microvasculature were differentiated from FBs by modified indirect immunohistochemistry, utilising the mAb UA009. Rounded shaped adherent cells that were stained by mAb UA009 were judged to be macrophages, although EC undergoing mitotic division could also have rounded morphology. Later experiments substantiated the presence of macrophages, where CD45-positive cells were depleted or where macrophages were identified by staining with mAb WT5 (anti-Mac1).

Phase contrast microscopy revealed that few cells adhered to either uncoated or gelatin-coated glass. A number of mAb UA009 negative, fusiform cells were observed to be adhered to the plastic culture surface. However, most cells that appeared to be attached to this surface became detached from the uncoated plastic surface during washing. As a consequence, few adherent cells remained attached and available for identification by immunohistochemistry. It was concluded that differential adherence to any of these attachment matrices was unsuitable for purification of synovial microvascular EC. However, better adherence of cells was observed in plastic culture chambers that had been coated with gelatin (Table 4.7).

4.7.2 Purification of endothelial cells by density gradient centrifugation

Density gradient centrifugation using Percoll, was explored as a method to isolate a pure population of ECs from the mixed synovial cell population. Aliquots of the mixed crude cell suspension were separated by centrifugation through Percoll gradients, assessed by phase contrast microscopy and the identity of the adherent cells was investigated with indirect immunohistochemistry using mAbs UA009 and UA015 (anti-PECAM). The mAb UA009 was found to be the most useful for this purpose, because it indicated the presence of EC that were specifically of microvascular origin. As mentioned, discontinuous gradients (see Section 2.10) comprised of 20, 30, 40, 50 and 60% concentration steps. Cells were recovered from each interface and cultured for several hours on a gelatin matrix in a plastic (Permanox™), Tissue Culture Chamber Slide.

Table 4.7 Summary of observations on adherent cells that expressed either FB or EC morphology on glass or plastic culture surfaces (Labtech slides), either gelatin coated or uncoated. Putative FB or EC were distinguished by their morphology and staining with mAb UA009, utilising a modified indirect immunoperoxidase technique identified microvascular ECs.

Culture surface	**Time (hr)**	**Description of Adherent cells**	**UA009+ve with EC or FB morphology**
Glass	0.5-14	Very few adhered cells	None present
Glass + gelatin	0.5-14	Few cells, all fusiform	None present
Plastic	0.5-14	Mainly long thin fusiform, cells washed off easily	None present
Plastic + gelatin	0.5	Very few cells adhered	None present
	1	++ cells, but lost in wash	None present
	2	+++ cells, mixed morphology	Scattered cells & many –ve cells †
	3	Fusiform, cobblestone	Scattered cells & many –ve cells †
	14	(same for 2-14h inclusive)	Scattered cells & many –ve cells †

† The cells that were not stained by mAb UA009 were probably FBs, but some could be EC from larger vessels. EC from large vessels and FBs are not stained by mAb UA009 (Figure 4.3.C).

After density gradient centrifugation, cells that formed a band at the 30% Percoll step interface were found to be enriched for EC of microvascular origin, as indicated by morphology and staining of monolayers with mAb UA009 (Figure 4.3.C). Cultures from this fraction were enriched and contained approximately 30% of adherent cells with 'EC-like' morphology (Table 4.8). Staining of smears of cells from the 30% interface with the antibodies UA009 or WT5 (Mac-1) supported these results (not shown), showing that the majority of the UA009-positive cells were not macrophages. Although density gradient centrifugation led to enrichment of the microvascular EC over the 30% Percoll step, it was clear that many FBs were also collected in the same fraction.

An alternative strategy to obtain EC was to collect the vascular segments manually, using a drawn out pipette. Vascular segments were collected from the mixed cell suspension using a dissecting microscope and put into plastic culture wells coated with fibronectin in RPMI-1640 with 20% FCS (Figure 4.3.A). Cells were observed to begin migration onto the matrix after only 2 hours, as the tips of cell projections became visible at each end of the segment. After 16 hours, a few cells were visible surrounding the original vascular segment, after 24 hours the vascular segments began to lose form and after 48 hours a relatively large number of EC had migrated and proliferated to form groups (Figure 4.3.B). Collection of vascular segments appeared to offer promise as a source of microvascular EC.

4.7.3 Isolation of vascular segments, as a potential source of endothelial cells

A shorter period of enzyme digestion followed by physical homogenisation of the tissue produced a relatively larger yield of blood vessel segments than the protocol described above, which was designed to obtain a single cell suspension. Several methods were explored for the isolation of the vascular segments. These included selection of segments using a mouth suction pipette, filtration through large pore diameter pre-separation filters and density gradient centrifugation.

Figure 4.3 Enrichment and purification of endothelial cells.

(A) Phase contrast micrograph of vascular segments, isolated manually by modified suction pipette (original magnification 200x). (B). Vascular segments after 15 hours in culture; showing disaggregation of the cellular components of the individual segments. Evidence of cell outgrowth is visible as single cells attached to the culture matrix (Phase contrast, original magnification 200x). (C). Monolayer of EC and FBs stained with mAb UA009. Percoll density gradient has enriched the UA009+ve EC from 5% up to approximately 30%. (original magnification 100x). (D). EC isolated with Dynabeads employing mAb UA009 as the primary antibody. After 24hrs in culture, note the relatively large population of EC with Dynabeads (small brown spheres) attached. (original magnification 200x). (E). EC with Dynabeads attached, after 48hrs in culture. Note the reduction in the relative numbers of cells covered with attached magnetic beads. The reduced numbers of beads per cell could be due to detachment by capping of the antigen or to replication of the cells. (original magnification 100x). (F). Micrograph of ethanol fixed, toluidene blue-stained cells. EC isolated with mAb UA009 and detachable CELLection kit Dynabeads and cultured as a monolayer. Note the absence of attached Dynabeads on individual cells.

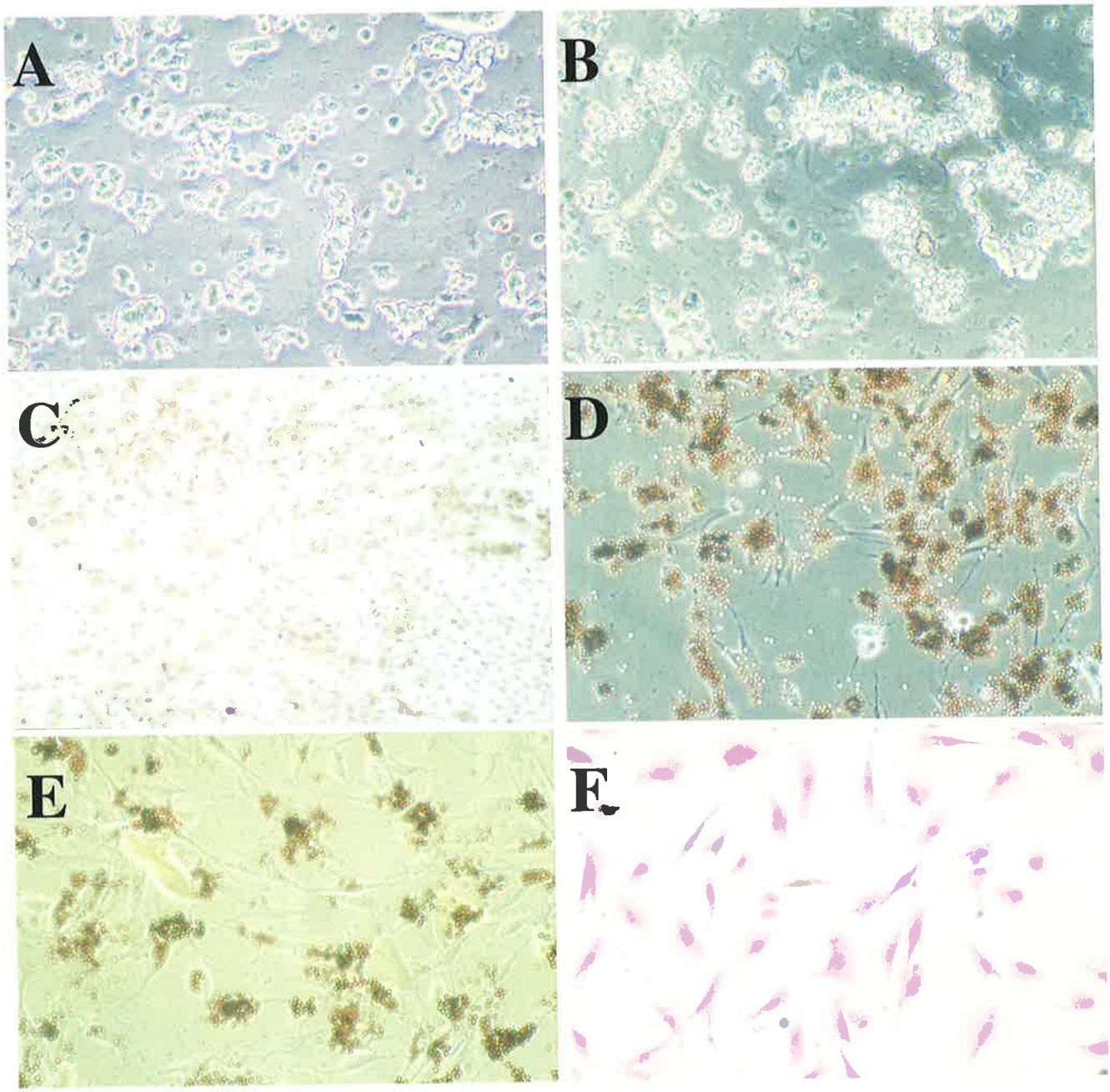

A
B
C
D
E
F

Table 4.8 Subfractionation of synovial cells by density gradient centrifugation. Synovial cells were harvested from arthritic rats 13 days after inoculation of CFA, disassociated into a single cell suspension and centrifuged through a discontinuous Percoll density step-gradient (20-60%). Fractions overlaying the density steps were examined microscopically in suspension, and ECs were identified by indirect immunohistochemistry of cultured monolayers from each interface.

Fraction/ interface	% UA009 +ve with EC morphology (monolayers)	Cell description/ Morphology (in culture)
control mixed cells	5.8%	
20	3%	† Segments, single cells
30	30%	Many single cells
40	<1%	† Segments, single cells
50	<1%	† Segments, FB-like
60	-	PMN, plasma cell

† Vascular segments- intact fragments or segments of the microvasculature.

PMN- polymorphonuclear leucocytes

4.7.3.1 Mouth suction pipette

After washing the suspension, vascular segments were collected by mouth suction using an apparatus similar to that commonly used to collect ova for manipulation in reproductive biology. The apparatus was constructed from a drawn-out glass Pasteur pipette, with silicone tubing attached at one end of the pipette and at the other a mouthpiece. As a safety measure a 0.22μm Millipore filter (Millex, Millipore) filters was inserted between the mouth-piece and the pipette. Vascular segments were collected by suction and deposited into a sterile vessel. Segments were identified using phase contrast microscopy.

Vascular segments obtained using this method (Figure 4.3A) were seeded onto gelatin or fibronectin and cultured to assess the feasability of obtaining EC monolayers by outgrowth from the segments (Section 4.3) in Tissue Culture Chamber Slides. Assessment of the cultures was carried out 1 hour, 2 hours, 3 hours, 5 hours and 15 hours (Figure 4.3.B) post-seeding. The morphology of cells growing out of the segments was observed at various time points with phase contrast microscopy.

After 1 hour, the segments were observed to begin to flatten and lose their rounded appearance. Cells were observed to begin migration onto the matrix after only 2 hours, as the tips of cell projections became visible at each end of the segment and adhered to the culture surface (Figure 4.3). After 15 hours, a few cells were visible surrounding the original vascular segment, after 24 hours the vascular segments began to lose its form and after 48 hours a relatively large number of cells had migrated and proliferated to form groups. The known capacity of FBs to replicate and grow more rapidly than EC, means that any contaminating FBs from the periphery of the vessel would be a major problem for EC culture purity. In order to obtain pure populations of EC, using this method, further purification steps were required. Isolation of segments by this method did prove relatively successful, but was extremely labour intensive and several steps were required to separate the segments from all the contaminating single cells.

4.7.3.2 Pre-separation filtration (30μm diam pore)

A different method explored for the collection of segments was the use of a 30μm pre-separation filter, supplied with Max beads (Quiagen Pty Ltd, Australia). Using this filter, single cells and smaller segments passed through the pores of the pre-separation filter, but longer segments of vessels were retained. This method was not pursued further because it was difficult to recover the segments from the surface mesh of the filter. Furthermore, other non-vascular debris was also trapped with the vascular segments.

4.7.3.3 Density gradient centrifugation

Discontinuous Percoll density gradients were used in an attempt to separate vascular segments from single cells, on the basis of density. Percoll gradients comprised of 20, 30, 40 and 50% concentration steps.

After centrifugation, cellular material was recovered from each interface and assessed using phase contrast microscopy. Smears of material from each interface were stained as described in Section 2.4.5. Examination of the smears revealed that vascular segments were not confined to any particular density interface. This was attributed to the random sizes of the segments, and possible also to varying amounts of perivascular extracellular matrix associated with them. Due to these differences in density, at their isopycnic position (equilibrium), segments were distributed through several density interfaces.

4.7.4 Unit gravity differential sedimentation of segments versus single cells

The rate of sedimentation is a function of its size. Because the vascular segments are large, differential sedimentation of a particle at unit gravity was investigated as a means of isolating them from single cells. This was carried out at room temperature, using standard tissue culture media or preformed linear density gradients. After phase contrast microscopic examination of the gradient fractions, it was found that most vascular segments remained trapped in the mixed cell layer at the top of the Percoll gradient. This method was not pursued further.

4.7.6 Positive selection of endothelial cells using immunomagnetic dynabeads

4.7.6.1 Tosyl-activated Dynabeads coated with the lectin, *Griffonia simplicifolia* I

In human tissues, ECs are known to express glycoproteins containing α-L-fucosyl residues. The lectin, *Ulex europeaus* I, which binds selectively to this sugar, stains endothelium of vessels of all sizes in human tissues as well as cultured HUVECS (Holthofer *et al.,* 1982). This lectin, when covalently coupled to magnetic polydisperse polymer particles (or immunomagnetic beads), Dynabeads™ was used successfully to isolate microvascular ECs from human neonatal foreskin and synovium (Jackson *et al.,* 1990).

The lectin *Griffonia simplicifolia* I (GSA-I) was identified as a potential means by which EC could be selected using Tosyl-activated Dynabeads because it is known to stain capillary endothelium in normal rat tissues (Bankston *et al.,* 1991; Laitinen 1987; Porter *et al.,* 1990). GSA-1 binds with high affinity to the α-D-galactosyl residues N-acetyl-galactosamine and α-D-galactose. The binding pattern of GSA-1 to cryostat sections of arthritic synovial tissue was investigated using peroxidase-conjugated lectin. In synovial tissue, capillary EC was stained by GSA-1, but at all concentrations that stained endothelium there was also a relatively high level of generalised background staining. It appeared that connective tissue matrix components as well as endothelium were stained by GSA-1 in inflamed synovium (not shown). The affinity of the lectin for non-endothelial components of inflamed sub-Achilles tissue excluded it from further use in the purification of EC by positive selection using GSI-1-coated, Tosyl-activated Dynal beads.

4.7.6.2 Positive selection of endothelial cells using monoclonal antibodies specific for EC and immunomagnetic beads coated with anti-Ig.

Immunomagnetic beads used in conjunction with specific mAbs are used routinely to purify specific populations of cells from a mixed cell suspension, either by positive selection or by depletion of non-target cell type(s) (negative selection). The process involves exposing the cells to specific primary mouse mAb(s), rosetting the labelled cells with anti-mouse-IgG-coated immunomagnetic beads and capturing the resultant complexes with a magnetic

device. It would be preferable to purify EC by negative selection, to avoid binding of antibody and magnetic beads to the cells. However, no antibody was available that labelled FBs selectively.

It was decided, therefore, to purify EC from the mixed cell suspension by positive selection, using either mAbs, UA015 and UA016 (EC specific) or UA009 (specific for microvascular EC but also binding to macrophages and red blood cells) as primary antibodies in combination with anti-Ig-coated Dynabeads, were explored. Preliminary results using positive selection were promising.

4.7.6.3 Positive selection of endothelial cells using mAb UA009

Using the mAb UA009, per experiment, a mean EC population totalling $2.13x10^6$ ±0.28 was obtained (6 pairs of feet per experiment; n=20 experiments) from a mixed starting population containing between 0.8-$1x10^8$ cells (mean= $0.96x10^8$ ±0.09). The isolated cells were viable, adhered to the culture matrix surface and replicated well in the conditions supplied. Using this mAb, EC from the microvasculature were selected and those from larger vessels were avoided. However, a large macrophage population present in this tissue was also selected. Although macrophages could be differentiated visually from EC with phase contrast microscopy, adherent macrophages would compromise the interpretation of lymphocyte adhesion to monolayers. To isolate pure microvasculature EC from mixed synovial cells using mAb UA009 requires pre-depletion of macrophages.

An additional problem was the retention of Dynabeads to the EC for long periods of time. The beads were retained on the surface of adhered EC in culture, despite attachment of the cells to the culture matrix (Figure 4.3.D & E). The cells were cultured for up to two weeks, with daily observations. While the number of attached Dynabeads reduced during this period, they were still observed in significant numbers throughout this time. Varying the concentration of FCS in the culture media and incubation with trypsin failed to remove the Dynabeads. The presence of relatively large numbers of Dynabeads attached to the EC surface was unsatisfactory for monolayers to be used for adhesion assays.

4.7.6.4 Endothelial cell selection with mAbs UA015 and UA016

Earlier immmunohistochemical investigations indicated that expression of the UA015 and UA016 antigens was restricted to the vascular endothelium, as reported in Section 3.3.2.2. The antigen showed similarities to PECAM-1 in its distribution, with distribution restricted to junctions between EC. Although the antigens were expressed by both large vessel and microvascular endothelium these mAbs have the advantage that they do not bind to macrophages. It was also hoped that Dynabeads attached via mAbs UA015 or UA016, might be released from EC after they attached *in vitro*. It was found that EC could be selected positively by the use of mAb UA015. Although the numbers of beads were fewer than observed with UA009 was used, they were retained on the surfaces of the cells after attachment in tissue culture.

In general terms, the results of EC isolation using immunomagnetic beads were encouraging. However, the problem related to the retention of Dynabeads and co-selection of macrophages by mAb UA009 required resolution.

4.7.6.5 Detachable immunomagnetic beads

During the course of the project, a detachable variety of Dynabead (CELLection™) was developed by Dynal and made commercially available. This innovative immunomagnetic bead had sheep anti-mouse Ab (pan mouse IgG) attached to its surface via a DNA linker. In principle, the addition of DNAse should cleave the DNA fragment, thus releasing Dynabeads attached to positively selected cells.

In practice, when the manufacturers recommended procedure was followed, DNase treatment was only partially successful. Approximately 70% of the selected cells retained their immunomagnetic beads after treatment with the enzyme. A small change to the protocol, involving a three-fold increase in the length of the DNase incubation time (from a recommended 15 min up to 45 min), resulted in a substantial improvement. Following the modified procedure, up to 90% of the selected cells had released their retained Dynabeads.

4.7.6.6 Removal of contaminating macrophages from EC populations.

Monoclonal antibody UA009 detects CD36 expressed by both microvascular EC and macrophages. In order to avoid the co-isolation of macrophages, an additional step was inserted prior to selection of EC. This involved removal of cells of haematopoietic from the mixed cell suspension by a preliminary depletion step using a mAb (OX1) directed against CD45 (described in Section 2.13.5.1).

Using this step, macrophages were removed successfully from the mixed synovial cell population along with other cells of haematopoietic origin ("leucocytes"). This had the additional benefit of reducing the total number of cells by approximately two thirds, ie only approximately 33% of the original mixed population remained. This led to a significant enrichment in the relative numbers of EC in the total mixed population. Other cell types that would remain include FBs, smooth muscle cells, vascular pericytes, red blood cells and possibly a few skeletal muscle cells. Reducing the total number of cells and improving the proportion of EC led to an improved efficiency of capture by the immunomagnetic beads. The result was a 4.4 fold increase in EC yield (see below). A mixed cell suspension using mAb OX1, mAb UA009 yielded approximately $2.4x10^6$ cells (a 4.4 fold increase). Compared to use of mAb UA009 without pre-depletion of leucocytes.

4.7.6.7 Endothelial cell yield

Sub-Achilles tissues harvested from 6 rats inoculated with CFA 14 days earlier yielded approximately $1x10^8$ mixed synovial cells. Following pre-depletion of leucocytes from the (the average yield was approximately $5.52x10^5 \pm 0.74$). The purified microvascular EC (Figure 4.3.F) comprised approximately 2 to 2.4% of the original total mixed synovial cell suspension (Table 4.9).

Table 4.9 Summary of the percentage of cells remaining after depletion of CD45+ve cells and the mean EC yield from mixed synovial populations with or without haematopoietic cell pre-depletion. Note the mean 18% increase in EC yield from cell suspensions that had been pre-depleted of OX1 +ve cells (haematopoietic cells).

	Mixed synovial cell preparation	**Single step positive selection†**	**1st step of two step selection***	**Final purification**
		Yield of $CD36^+$cells	**Yield of $CD45^-$ cells**	**Yield of $CD36^+$cells**
# **Cell number**	9.83×10^7	2.0×10^6	3.2×10^7	2.4×10^6
% of original cells	100	2	33	2.4
% of $CD45^-$ cells	-	-	100	7.4

† Positive selection of CD36+ cells, using mAb UA009 and anti-Ig armed Dynabeads

* Negative selection of CD45+ cells, using mAb OX1 (step 1), Followed by positive selection of CD36+ cells from the CD45- fraction.

Mean number of viable cells recovered by collagenase digestion of sub-Achilles tissue obtained from the hind paws of 6 rats , 14 days post-inoculation of CFA. Data 20 individual estimations.

4.8 Identification of EC in culture

It was difficult to distinguish between EC and FBs in culture by morphology (Figure 4.4.A). EC from different vascular beds and from different sized vessels are reported to be heterogeneous in morphology when compared to the classical cobblestone monolayer appearance of HUVECs (Kumar *et al.,* 1997). EC and FBs are both adherent cell types. Some authors have identified FBs morphologically by their fusiform shape (Figure 4.4.C), however, in my experience, some EC also exhibit this morphology. Cobblestone and more elongated morphologies have been described for EC from different types of vessels (Kumar *et al.,* 1987). EC tend also to have this morphology when they are cultured at sparse or sub-confluent density. Because FBs present a major contamination problem in EC cultures, it is important to verify the purity of the isolated cells.

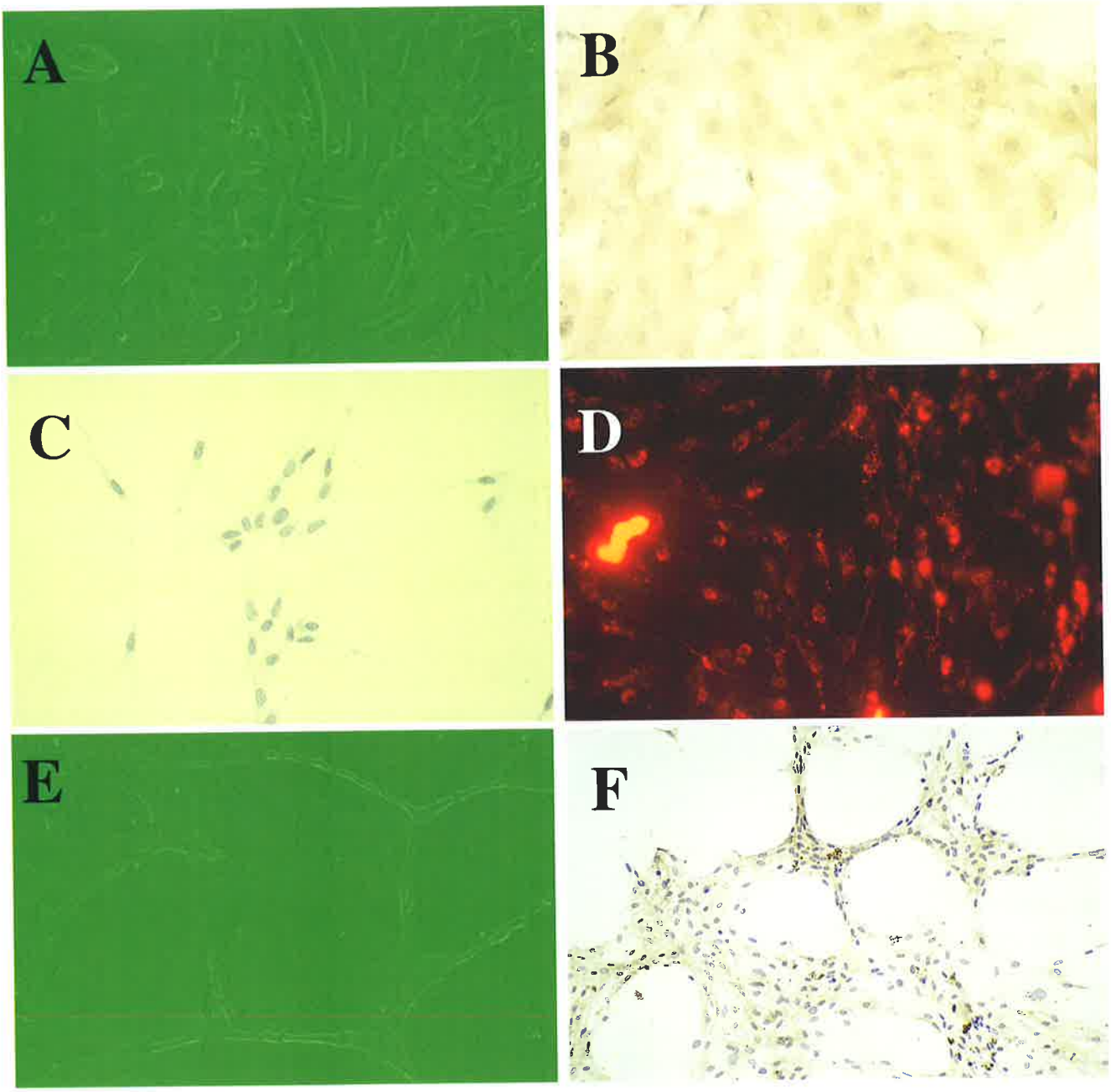
Cells were identified as endothelial by several methods. EC were identified in culture monolayers by immunohistochemistry with mAbs UA009 (Figure 4.4.B), UA015 and TDL-3A12. Cells isolated by the process described above (Section 4.7.6) scavenged fluorescently conjugated, acetylated-low-density lipoprotein (see Figure 4.4.D and E) (Section 2.13.7.1) (Voyta *et al.* 1984). They showed some variability of fluorescence intensity because EC derived from rat differ from those obtained from human tissues in uptake of LDL, as do those from different vascular beds (Kumar *et al.*, 1987; Gumkowski *et al.,* 1987). A non-quantitative means of positively identifying EC was by observing their ability to form tube-like structures. In the presence of excess fibronectin matrix, capillary tube –like structures were visible in the cultures, confirming their identity as EC (Figure 4.4E).

4.9 Discussion

The aim of investigations described in this chapter was to develop a method for obtaining highly enriched microvascular endothelium from rat synovial tissues and to culture the cells as adhesive

Figure 4.4 Identification of EC isolated by mAb UA009 using Dynal CELLection kit

(A) Phase contrast micrograph, showing the morphology of purified microvascular ECs, (in culture) isolated from arthritic tissue (original magnification 100x). (B) Microvascular ECs stained with mAb UA009 by indirect immunoperoxidase technique (original magnification 200x). (C) Fibroblasts cultured from cells remaining after depletion of CD45-positive cells and removal of UA009 positive cells from the mixed cell suspension. The cells show typical FB-like fusiform morphology. Fixed, stained with toluidine blue (original magnification 100x). (D) Identification of ECs in culture using characteristic scavenging of DiI-Acetylated-LDL by these cells. NB: although EC and macrophages both take up the label, macrophages have been predepleted from the suspension and any contaminating macrophages are easily differentiated by morphology. Fluorescence micrograph, (original magnification 100x). (E) & (F) Identification of ECs by their abilty to exhibit capillary-like tube formation when cultured on fibronectin. Phase contrast micrograph. (E) and light micrograph of ethanol fixed cells stained by indirect immunoperoxidase technique with haematoxylin counterstain, (original magnification 100x).



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monolayers. The resulting monolayers would be used to investigate adhesive interactions between the freshly isolated ECs with lymphocytes. The first step was to establish a method to harvest EC reproducibly from the selected area of sub-Achilles, synovium rich tissue, using sterile technique.

4.9.1 Production of a mixed synovial cell suspension

Several enzymes were investigated to establish an enzymatic digestion protocol that would reproducibly yield a mixed single cell suspension. Collagenase class 1 alone was found to produce a suspension of cells, but addition of other enzymes increased the yield of cells. Inclusion of the additional enzymes, hyaluronidase and DNAse, combined with gentle mechanical dissociation by homogenisation, improved the yield by 71% in comparison with digestion with collagenase class 1 alone.

The final enzyme cocktail included the three enzymes, collagenase class 1, hyaluronidase and DNase. Collagenase was the most important component, probably due to the high collagen component of the connective tissue, that is the major constituent of synovium. The collagen remained intact after trypsin digestion and the long incubation times required with this enzyme lead to cell damage and low recovery of viable cells. Hyaluronidase was included to improve the efficiency of digestion because it is known to cleave endo-N-acetylhexosaminic bonds in hyaluronic acid and chrondroitin sulphate bonds. These glycosaminoglycans are found in high concentrations within the extracellular matrix of connective tissue. The third enzyme, DNase was included to reduce viscosity during filtration. The viscosity was likely to be caused by the release of DNA from damaged cells, leading to trapping of cells on the filter. When cultured, mixed monolayers were observed consisting of mainly FB-like and endothelial-like cells. From an immunohistochemical examination of cultured cells from the mixed single cell suspension using mAbs UA009 and TLD3A12, it was apparent that ECs from the microvasculature were present, although in relatively low numbers.

4.9.2 Culture of dissociated cells

The cells from the synovial digests were able to adhere to an artificial matrix (gelatin or fibronectin) and proliferate under conditions that were adapted from those used for growing and maintaining HUVECS. Addition of ECGF was found to be unnecessary for short-term cultures. After a period of culture, the cells formed monolayers, but EC could not be confidently differentiated from FBs by observation with phase contrast microscopy. The use of the mAbs UA009 and TLD-3A12 in indirect immunohistochemistry and the uptake of fluorescently conjugated Ac-LDL, allowed confident identification of ECs could be differentiated from macrophages in culture, which also stain with mAb UA009 and take up Ac-LDL, by their morphology. Endothelial cells were identified also by their engagement in capillary-like tube formation in monolayers of cells that were seeded into fibronectin-coated wells. Means of identifying EC were important, in order to determine the efficiency of subsequent methods developed to isolate and purify EC.

4.9.3 Endothelial cell isolation methods

A number of methods to purify or enrich EC were tried and found to be unsatisfactory. These included differential adhesion, growth from vascular segments and the use of density gradient centrifugation. There appeared to be no selective adhesion of EC on the various culture surfaces that were tested. Manual selection of vessel segments showed some potential as a means for growing out EC. However, it was not possible to exclude outgrowth of contaminating FBs, meaning that a further purification procedure would be necessary.

The use of discontinuous Percoll density gradients, achieved an enrichment of EC purity of up to 30%, as demonstrated by immunohistochemistry with mAb UA009. The cells not stained by mAb UA009 had FB or EC-like morphology and they were not stained by mAb WT5 (against Mac-1 found on macrophages). They were presumed to be FBs or CD36 negative EC derived from large vessels. Fibroblast contamination, even in small numbers is undesirable in cultures because these cells rapidly over-grow EC. It was concluded that isolation of EC by Percoll density gradient centrifugation alone was not a method that

would be suitable for the purification of EC originating from the microvasculature.

It was decided, therefore, to purify EC by positive selection, employing either a lectin or mAb that identify EC and immunomagnetic beads. Immunomagnetic Dynabeads are super-magnetic polystyrene beads with a ferrous core. They can be used in conjunction with lectins or mAbs to positively or negatively select cells. Jackson *et al.*, (1990) utilised Dynal beads with the lectin *Ulex europaeus* successfully to positively select EC from human synovium. The ligand for the lectin *Ulex europaeus* is not expressed in rodent cells, but in the rat, endothelium from small vessels express a ligand for the lectin *Griffonia simplicifola* (Roussel and Dalion 1988). Because oligosaccharides that bind GSA-1 have a wide tissue distribution, the potential of this method was discounted.

Success was achieved with the use of mAb UA009 (anti-rat-CD36), in conjunction with Dynal beads). This allowed positive selection of microvascular EC from mixed cell suspensions. Contaminated only with macrophages and erythrocytes. Erythrocytes were removed easily because they are non-adherent, but macrophages were a problem because they adhere to plastic, gelatin or fibronectin culture surfaces and survive for several days in culture. A step was incorporated to deplete macrophages, prior to positive selection of the EC. The mAb OX1 (anti-rat CD45) was used to predeplete all nucleated cells of haematopoietic origin. This step was successful not only in removing macrophages from the cell suspension, but also selected out other leucocytes that were abundant in inflamed synovium. Removal of all leucocytes enriched the EC population, leading to a significant improvement of the yield of EC. Positive selection with mAb UA009 following macrophage depletion, yielded a pure population of cells that was composed of EC originating from cells of the microvasculature.

A remaining problem was the retention of large numbers of Dynabeads bound to the selected cells, due to persistent expression of mAb UA009 to CD36 expressed on the cell surface. Attempts to remove these beads were unsuccessful initially and it appeared that long-term

culture might be the only alternative. This was an undesirable option, because the aim of these experiments was to use freshly isolated EC that retained their differentiated characteristics.

Positive selection of EC was also attempted using mAb UA015, as it appeared that less Dynabeads remained adhered to the EC compared with mAb UA009. The purified EC had fewer beads attached to them, probably because this antigen is less abundant on the cell surface. However, mAbs UA015 and UA016 had been found previously to bind to both microvascular EC and EC in large blood vessels (described in Section 3.3.2.2) and were, therefore, not suitable for specific selection of microvascular EC. A further disadvantage of these mAbs is that they appear to be specific for the cell adhesion molecule PECAM-1. Because the objective was to use the purified EC for lymphocyte adhesion assays, it was desirable to avoid using cells that had been exposed to a mAb against an adhesion molecule.

In general terms, the results of EC isolation using immunomagnetic beads were encouraging. The problem of retained dynabeads was resolved, when a detachable Dynabead product became available. The immunomagnetic beads incorporated in the Dynal CELLection kit were unique. The secondary antibody was attached to the bead via a fragment of DNA, thus allowing the beads to be detached by cleavage of DNA with DNase. Using this method, in conjunction with mAb UA009 a pure population of EC could be isolated reproducibly and freed of attached beads before seeding the cells into culture.

4.9.4 Identification of endothelial cells in mixed and purified populations

The identity of EC in culture was confirmed by immunoperoxidase staining for CD36 (mAb UA009) and by uptake of fluorescently labelled Ac-LDL. The pattern of uptake of Ac-LDL allowed easy distinction of EC and macrophages by microscopy. The identification of EC could be confirmed by immunohistochemical staining with mAbs UA015, TDL-3A12 (anti-PECAM-1), and by observation of capillary-like tube formation on a fibronectin matrix. In cultures prepared by the definitive method described above, the adherent cells were stained

by mAb UA009 and are therefore, confirmed to be EC of microvascular in origin. Microscopic examination of immunoperoxidase-stained monolayers revealed that essentially all of the adherent population consisted of EC.

4.9.5 Conclusion

In summary, an efficient and reproducible technique was developed for the isolation, identification and culture of microvascular EC from inflamed synovial tissue from rats. To collect the tissue from 6 rats and obtain a pure population of EC, occupies one individual for approximately 12 hours. Once the EC are purified, and form monolayers, they can be used within hours of seeding the culture wells, as passaging is not required to obtain a pure culture. The benefit of this method is that the isolated EC originate from small vessels of the inflamed synovial tissue and not from large vessels such as aorta, which is used commonly as a source of rat endothelium and that they do not require extensive culture *in vitro* prior to use. The latter is an important consideration, as there is extensive evidence that EC lose differentiated characteristics after prolonged tissue culture (Jackson *et al.*, 1990).

Chapter 5

Flow Cytometric Analysis of Lymphocytes from Inflamed Synovial Tissue of Rats with Severe Adjuvant Arthritis.

5.1 Introduction

The presence of significant numbers of lymphocytes in synovium from rats with AA has long been recognised (Pearson, 1964) and the disease was transferred adoptively by TD lymphocytes (Whitehouse *et al.,* 1969). Numerous studies have implicated T lymphocytes in the pathogenesis of AA (Taurog *et al.,* 1983, 1984; Holoshitz *et al.,* 1983, 1984; Larsson *et al.,* 1985; Yoshino and Cleland,1992; Spargo *et al.,* 1996). AA can be prevented by depletion of T lymphocytes *in vivo* (Billingham *et al.,* 1990; Yoshino and Cleland,1992; Pelegri *et al.,* 1996a) and the disease can be transferred adoptively by TD lymphocytes (Spargo *et al.,* 2001) and by lymphocyte clones (Holoshitz *et al.,* 1983; 1984).

A number of studies have shown that the CD4+ T cell subset specifically plays a pivotal role in the pathogenesis of AA. Treatment with non-depleting anti-CD4 mAb can prevent development of the disease (Pelegri *et al.,* 1995b). It has been demonstrated that the cells of the CD4+ subset (Pelegri *et al.,* 1996a; Hoffman *et al.,* 1997; Spargo *et al.,* 2001), but not the CD8+ subset (Larsson *et al.,* 1985; Hoffman *et al.,* 1997; Spargo *et al.,* 2001) are pivotal in the pathogenesis of AA. Furthermore, lymphoblasts obtained from the TD lymph of rats during the prodrome of AA, are recruited in significant numbers to normal and inflamed synovium after adoptive transfer (Spargo *et al.,* 1996). Recently, Spargo *et al.,* (2001) showed that AA can be transferred adoptively with TD lymphocytes from donors in the prodrome of active disease. In this model, *ex vivo* stimulation of the cells is not necessary, thus allowing the specific subsets of arthritogenic lymphocytes to be identified. Spargo *et al.,* (2001) showed that the effector cells responsible for transferring disease were CD4+ T cells that expressed markers of recent activation. These markers include MHC class II, CD25, CD71 (transferrin receptor) and CD134 (OX40 antigen).

Lymphocyte subsets have been characterised according to expression of antigens that are expressed differentially by naïve, effector and memory cells. Markers follow the transition from naive to effector or memory cells, so that the level of expression of a range of markers can be used to assess the functional state of a T cell (reviewed by Sprent, 1997; Swain *et al.,* 1996; Westermann and Pabst, 1996; Mackay, 1991). Isolated lymphocytes can be assessed by single and multiple fluorochrome flow cytometric analysis, using a panel of markers expressed at different levels on naïve, effector or memory cells and the pattern of expression can be used to assess the functional state of the cell (Reviewed by Sprent, 1997; Swain *et al.,* 1996; Mackay, 1991).

Prior to activation, small resting or naïve T cells in the G_0 state express low levels of certain activation markers and adhesion molecules. After leaving the thymus, CD4+ and CD8+ T cells enter the circulation as resting cells in the G_0 stage of the cell cycle. These naïve T cells have not yet encountered antigen and characteristically they express low levels of the adhesion molecules CD44 (hyaluronate receptor), CD11a (LFA-1), CD54 (ICAM-1), CD49d (VLA-4) and the IL-2 receptor (IL2R), CD25. In contrast, naïve T cells express a high level of the lymphocyte homing receptor, CD62L (L-selectin). The expression of this molecule allows naïve T cells to bind to a mucin-like vascular addressin, that is expressed by HEV in the peripheral lymph nodes. This is the first step that allows the cells to extravasate and migrate through the EC layer and into the node (Bradley *et al.,* 1994; Tedder *et al.,* 1995).

Naïve T cells continue to recirculate between blood and lymph, as part of the recirculating lymphocyte pool, until they encounter and recognise an antigen-MHC complex presented by an antigen presenting cell (APC), or target cell (Butcher and Picker, 1996). This interaction will lead to activation of the lymphocyte. Associated with activation, the cells increase in size and proliferation as blasts in the lymph node (Garside *et al.,* 1998; Mackay, 1993a), there is down-regulation of the expression of L-selectin and upregulation of the expression of adhesion molecules CD44, CD11a, CD54 and CD49d (or $\alpha4\beta7$ integrin in mucosal lymphoid tissues) (Swain *et al.,* 1996). Five to 7 days post-activation, effector

cells that exhibit increased expression of these cell adhesion molecules are released from the lymph node (Kelly, 1994). The upregulated cell-adhesion molecules are believed to function in several ways, firstly keeping activated cells at the site of activation and later assisting in recruitment to sites of inflammation and retention of the cells at these sites (Sfikakis and Tsokos, 1995). Effector cells are short-lived, compared with the relatively long life-span of memory cells (Mackay, 1993b).

Memory T cells are also generated from effector cells or from the naïve cell population of T cells (Kelly, 1994). These cells have longer life-spans and express many of the membrane molecules found on effector cells. Like naïve cells, the majority are in the G_0 stage of the cell cycle and they can recirculate in the blood and lymph. They can confer protection, and upon secondary challenge mount a rapid response (Dutton, 1998). Like effector cells they have low levels of L-selectin and they express relatively high levels of other adhesion molecules.

Naïve and activated / memory CD4+ cells can be distinguished from each other by the isoforms of CD45 that they express. Naïve T cells express a high molecular weight isoform of CD45, designated CD45RC in rats, CD45RA in humans and CD45RB in mice. These isoforms of CD45, named according to the terminal exon, are involved in regulation of activation through the tyrosine phosphatase activity of CD45 molecules (Mackay, 1993a). The CD45 isoform differs between species, for example, in humans, activated CD4+ T cells express the low molecular weight CD45RO+ isoform and in rats, activated cells are assessed by their loss of CD45RC expression (Powrie and Mason, 1989). Other activation markers include CD71, the transferrin receptor that is expressed by dividing cells due to the requirement for iron by dividing cells (Jefferies *et al.*, 1986; Trowbridge and Omary, 1981) and CD25, the IL-2R α-chain. CD25 is part of the high affinity IL-2R complex expressed by activated T cells (Iannone *et al.*, 1994; Reizis *et al.*, 1994), although it is also expressed by a subset of CD4 T cells (Barclay *et al.*, 1997). Expression of activation markers, adhesion molecules and CD45 isoforms, are therefore, tools commonly used to assess the functional state of T cells.

Memory cells (CD45RO+) can be further divided into two functionally different subsets by the expression or absence of CCR7, a chemokine receptor ($CD45RA^-CCR7^+$ or $CD45RA^-CCR7^-$). Memory cells that are $CCR7^+$ have been named central memory (T_{CM}) and those that are $CCR7^-$ effector memory cells (T_{EM}) (Sallusto *et al.,* 1999). The T_{CM} subsets possess LN homing receptors (coexpression of CD62L) and recirculate through the 2° lymphoid tissues). However, these cells are not involved in immediate effector function (lack inflammatory and cytotoxic function) but with the stimulation of DCs and can differentiate into T_{EM} cells. The other subpopulation of T_{EM} ($CCR7^-$) cells, express receptors for migration into inflamed tissues (3° lymphoid tissues) and function directly as effector cells (Sallusto *et al.,* 1999).

The memory effector cells do not recirculate through lymph nodes via HEVs. Instead they tend to migrate to non-lymphoid tissues, like lamina propria of the gastrointestinal tract and epithelial surfaces of the lung and skin (Picker and Butcher, 1992; Picker1992; Mackay, 1993b; Shimizu, 1992). Generally, sites in which the cells are originally stimulated determine the pattern of memory T cell migration and recirculation through tertiary lymphoid tissues. For this reason, memory cells are often the major inflammatory cell type that are found in chronic inflammatory lesions (Mackay, 1993b).

There are few studies that have looked at lymphocyte populations in inflamed synovium from rats with AA. Furthermore, most of these have been carried out using immunohistochemistry on tissue sections for example, from the knee (Pelegri *et al.,* 1995a; Carol *et al.,* 2000), or they have looked at lymphocyte populations found in peripheral blood (Franch *et al.,* 1994). Issekutz and Issekutz (1991), have observed migration of T lymphocytes by accumulation of radiolabelled lymphocytes in the joints of arthritic rats. The presence of some dispersed CD5+ cells (T lymphocytes) in synovial tissue from the knees of arthritic rats was reported in tissue sections, but few lymphocytes were detected by flow cytometry on cells from enzyme digests of the tissue (Pelegri *et al.,* 1995a).

The aim of this part of the study was to define the composition of the lymphocyte subsets obtained from inflamed synovial tissue of rats with severe adjuvant arthritis and to investigate the activation markers and adhesion molecules expressed by these cells. TD lymphocytes from AA rats were analysed, using flow cytometry to compare the activation markers and adhesion molecules expressed by the various subsets with those expressed by the cells that accumulate in inflamed synovium.

Cells were liberated by enzyme digestion of inflamed sub-Achilles tissue from DA rats with severe adjuvant arthritis. These cells were utilised to assess lymphocyte sub-populations by the use of flow cytometry. The method used to dissociate the tissue into a single cell suspension is described in Section 2.13.3. Aliquots of cells were labelled for single or dual fluorochrome analysis with mAbs, as described in Section 2.11.1 or 2.11.2. Mouse anti-rat monoclonal antibodies used in this study are listed in Table 2.1 and flow cytometry was performed, as described in Section 2.12.

5.2 Hypothesis and Aims

5.2.1 Hypotheses:

- Cells in enzymatic digests of the sub-Achilles region in arthritic rats can be used to capture and identify T cells that accumulate in inflamed synovium.

- Flow cytometric analysis can be utilised to characterise the cell surface phenotype of the lymphocyte sub-populations found in inflamed synovial tissue.

- T cells that accumulate in the synovial tissues in rats with AA will express markers of activation, increased levels of adhesion molecules associated with transmigration through inflamed vascular endothelium and adhesion molecules involved in tissue retention of the cells.

5.2.2 Aims:

- To establish a means of capturing in single cell suspensions lymphocytes from within arthritic synovial tissue from rats with severe AA.

- To characterise the cell surface phenotypes of the lymphocyte sub-populations found in inflamed synovial tissue by flow cytometry.

- To identify activation markers and adhesion molecules expressed by the α/β TCR+ cells present in the inflamed synovium.

- To provide a guide to the phenotype of activated T cells appropriate for use in a prospective system for studying adhesive interactions between T cells and synovial microvascular endothelium.

5.3 Results

5.3.1 Analysis of lymphocyte populations from arthritic hind paws and TDL

Cells were prepared by enzymatic digestion of sub-Achilles tissue from 6 rats, 14 days after inoculation with CFA. In these crude digests, yields ranged from 4.2 – 8.5 x 10^7 viable cells (n=5). Flow cytometric analysis revealed that of the total mixed population of cells in the digest, approximately 2.5% were α/βTCR+ cells. After enrichment of the mononuclear leucocyte fragment by centrifugation over Lymphoprep to remove most red blood cells, neutrophils and large fraction, approximately 40% of cells remained. The forward and side scatter of light by the

cells in the Lymphoprep-enriched population is shown in Figure 5.1, which shows the position of α/β TCR+ lymphocytes within the small "lymphocyte gate" and also "large" lymphocytes within the large "lymphocyte gate". Of these cells, 85% were identified as leucocytes by staining with

Figure 5.1 Flow cytometric analysis of cells from arthritic synovium.

Dotplot of forward and side scatter from flow cytometric analysis of cells obtained from arthritic synovium 14 days after innoculation with CFA. Cell suspensions were prepared by enzymatic digestion of sub-Achilles tissue of arthritic rats. The cells were centrifuged over the density gradient Lymphoprep, to enrich the mononuclear leucocyte population. The cells in the small gate (indicated by the pink shaded area) were identified by back gating of cells positive for the mAb R73 (α/β TRC+) and classified as small lymphocytes. The larger gate included cells classified as large lymphocytes.

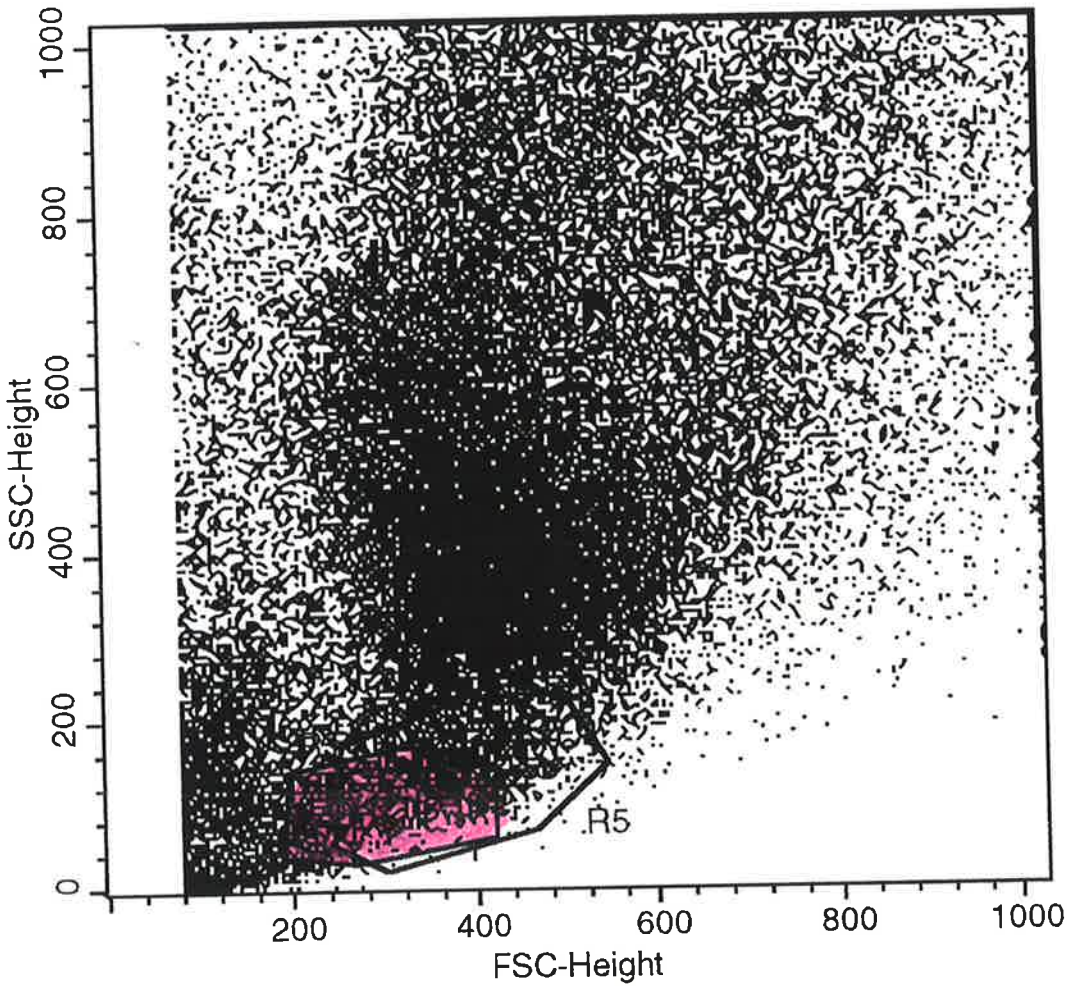
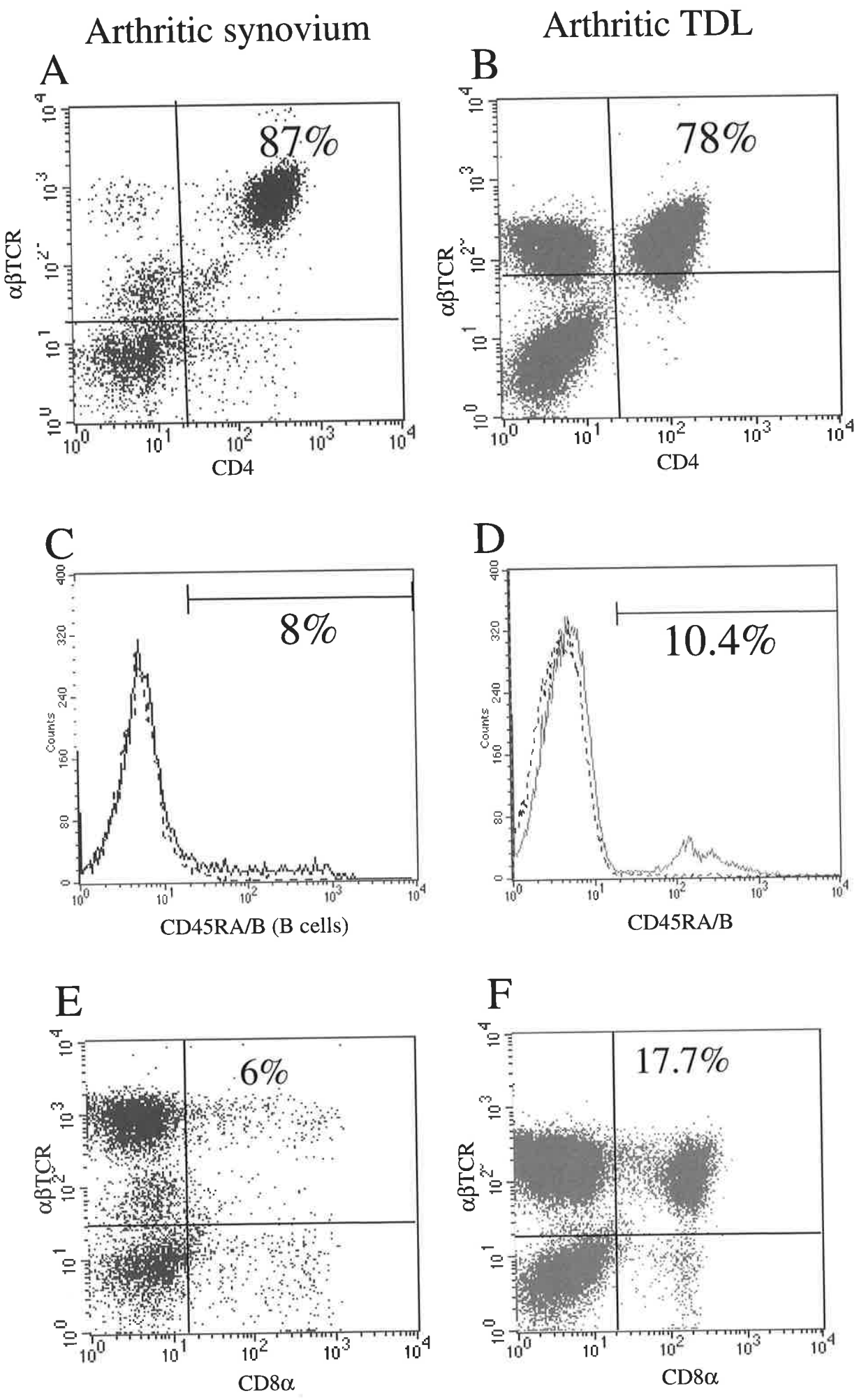

SSC-Height
0
200
400
600
800
1000
FSC-Height
200
400
600
800
1000
R5

Figure 5.2 Detection of lymphocyte sub-populations from AA synovium and TD lymph.

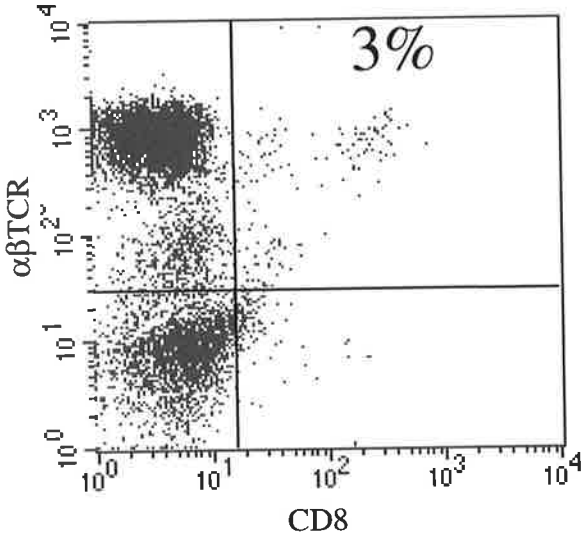
Suspensions of cells from AA synovium (left-side panel) and TD lymph (right-side panel) were labelled for single or dual colour flow cytometric analysis. For single colour analysis the cells were labelled by the indirect immunofluorescence technique with mAb OX33 (anti-CD45RA; FITC) (Fig. 5.2C & D) or mAb V65 (anti-γ/δTCR) (Fig. 5.2K). For dual colour analysis the cells were labelled first by the indirect immunofluorescence technique, labelling with mAbs W3/25 (anti-CD4; PE) (Fig. 5.2 A & B), OX8 (anti-CD8α; PE) (Fig. 5.2E & F) , 341 (anti-CD8β; PE) (Fig. 5.2G & H) or 10/78 (anti-CD161; FITC) (Fig. 5.2I & J). This was followed by direct immunofluorescence with mAb R73 (anti-α/βTCR; PE). In the case of single colour (C,D & K), the broken line represents the auto-fluorescence of lymphocytes incubated with the negative control antibody 1B5. The staining profile of cells incubated with mAb OX33 (anti-CD45RA; FITC) or mAb V65 (anti-γ/δTCR) is superimposed as an unbroken line. The percentage of labelled cells is indicated below the marker in those panels. In the case of dual colour, the percentage of the α/βTCR population that was double positive for each marker is indicated in large font size.


Arthritic synovium
Arthritic TDL
A
B
87%
78%
αβTCR
CD4
C
D
8%
10.4%
Counts
CD45RA/B (B cells)
CD45RA/B
E
F
6%
17.7%
αβTCR
CD8α

Arthritic synovium

Arthritic TDL

G



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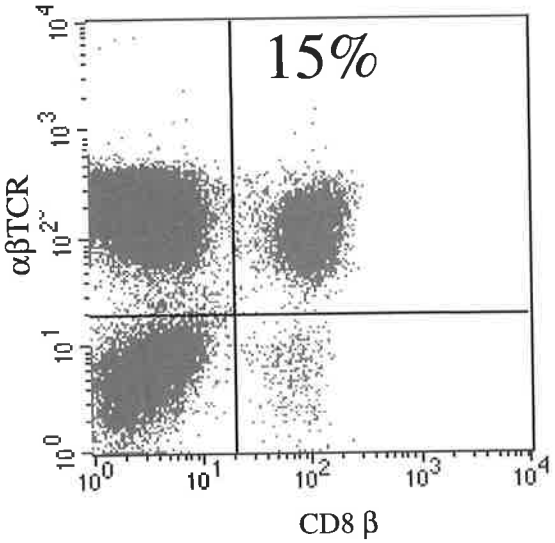

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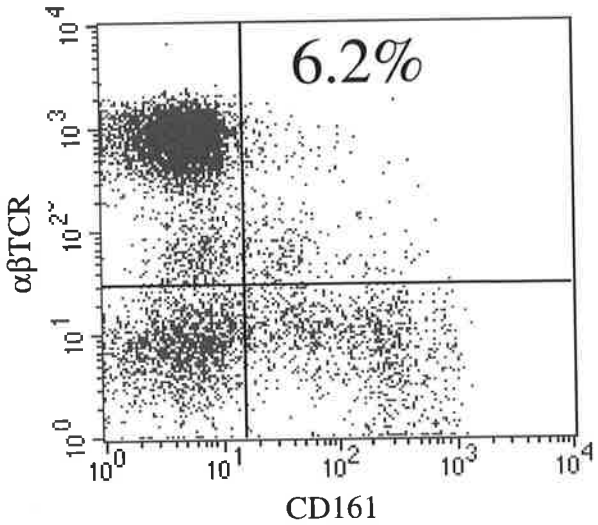

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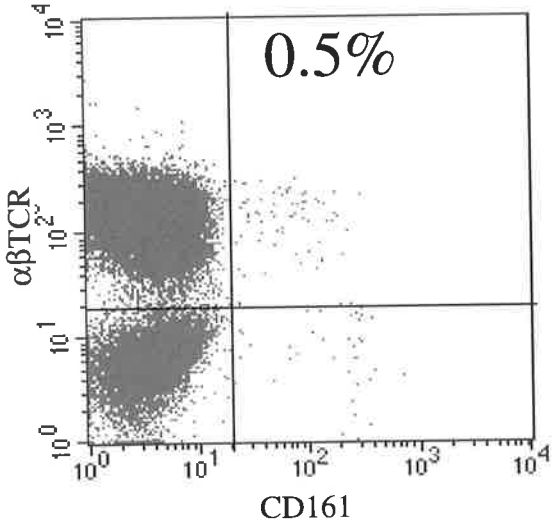

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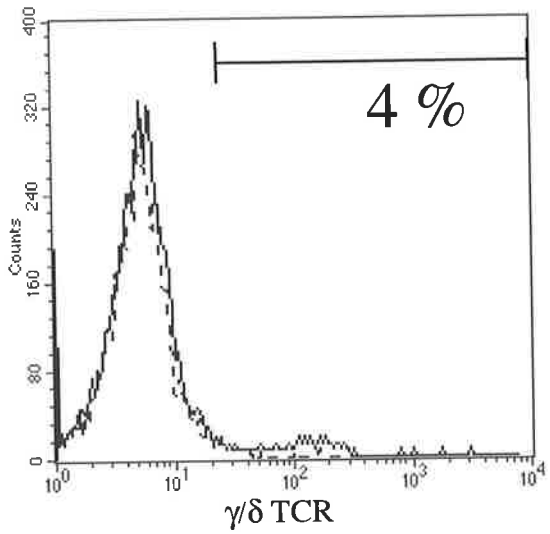


Table 5.1 Summary of lymphocyte sub-populations from synovium and thoracic duct lymph. Analysis of cells was performed by flow cytometric analysis of cells that fell within the lymphocyte gate defined in Figure 5.1, using either single fluorochrome indirect immunofluorescence (mAbs R73, OX33, V65 or dual immunofluorescence (mAbs R73 and W3/25, OX8, 341 or 10/78). For TD lymphocytes, all cells were included in the analysis. Synovial cells were obtained from rats 14 days after inoculation of CFA. TD lymph was obtained from rats 9 days after inoculation with CFA.

Antibodies	Lymphocyte sub-population	% +ve cells	
		AA Synovium	AA TD lymph
R73	α/β TCR (single col)	80	81
R73 + W3/25	α/β TCR+ / CD4+	87	78
OX33	CD45RA or A/B (single col)	8	10
R73 + OX8	α/β TCR+ / CD8α chain	6	18
R73 + 341	α/β TCR+ / CD8β–chain	3	15
R73 + 10/78	α/β TCR+ / CD161+	6.2	0.5
V65	γ/δ TCR (single col)	4	0.6

mAb OX1 (CD45). Approximately 80% of the CD45 population were α/βTCR+ cells.

Analysis of the cells within the lymphocyte gate revealed that approximately 80% were α/βTCR+ cells. This was similar to the proportion of α/βTCR+ cells in TD lymph from rats 9 days after inoculation of CFA (Table 5.1). Further investigation showed that approximately 87% of the α/βTCR+ cells in the lymphocyte gate were of the CD4+ subset (Figure 5.2A), compared with approximately 78% in TD lymph from the arthritic donor (Figure 5.2B). Only about 8% of synovial cells within the lymphocyte gate were stained by mAb OX33+ (Figure 5.2C). Most of these CD45RA/B+ cells were relatively large in size and they probably represent B cell blasts. Approximately 10% of cells in TD lymph were stained by OX33 (Figure 5.2D). CD8α+ (Figure 5.2E) and CD8β+ cells (Figure 5.2G) represent only 6% and 3 % respectively of the cells in the lymphocyte gate of synovial cells, while these cells comprised 18% (Figure 5.2F) and 15% (Figure 5.2H) of the cells in TD lymph. In synovium and TD lymph, essentially all α/βTCR+ cells were unstained by mAb 10/78 (anti-CD161) (Figure 5.2I and Figure 5.2J).

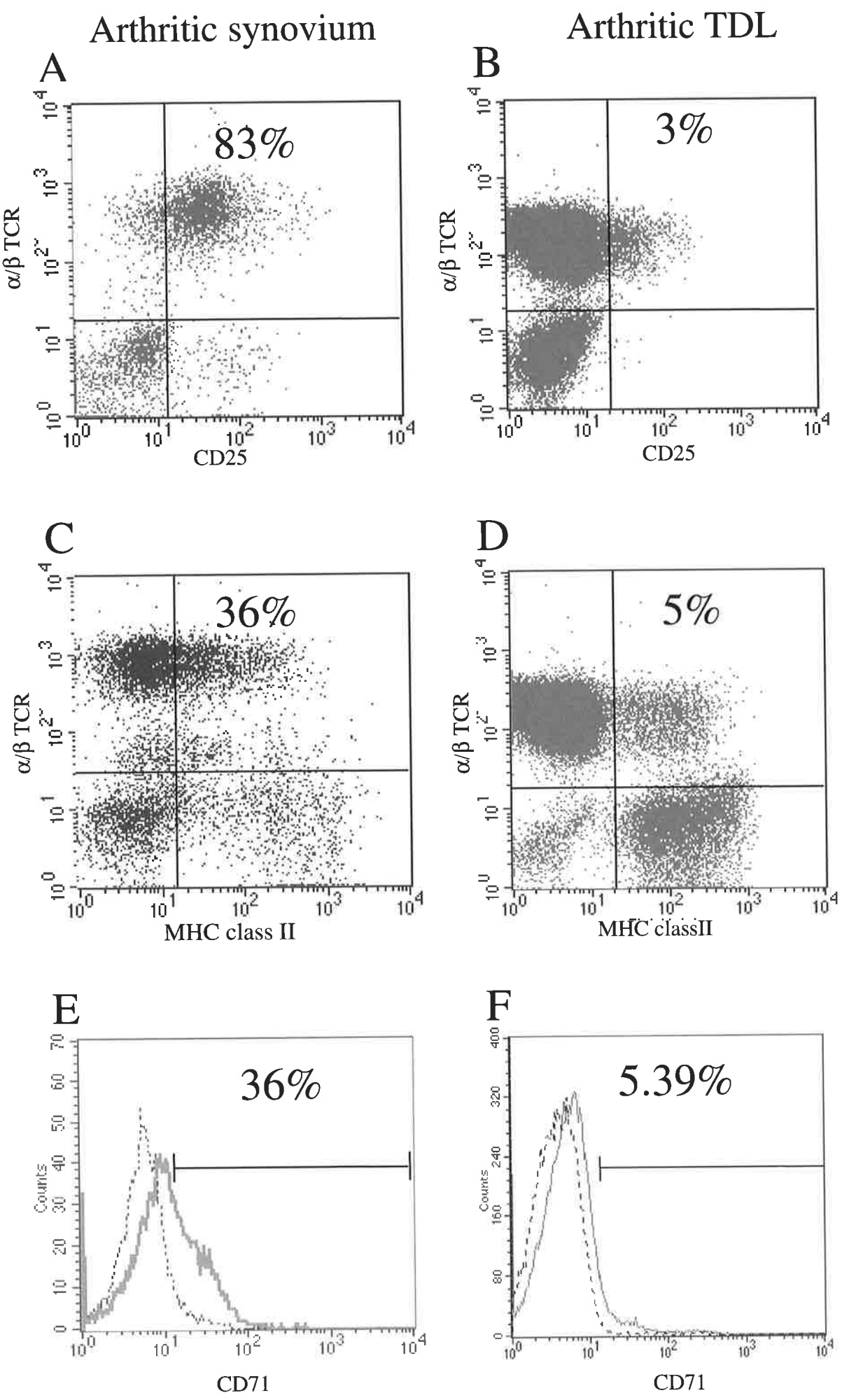
Other work in the laboratory (L. Spargo, personal communication) has shown that the weak staining of cells by R73-PE conjugate is non-specific. Some of these cells are also CD161+ but are unlikely to be NK T cells. NK T cells were notably absent from the TD lymph population. Other subsets detected in synovium were α/βTCR- $CD161^{bright}$ and α/βTCR- $CD161^{dim}$. These are likely to be NK cells and cells of the macrophage/ monocyte lineage (Figure 5.2I lower right quadrant). At least 4% the cells in the synovial sample were γ/δ T cells (Figure 5.2K), whereas in TD lymph, less than 1% of the lymphocytes were γ/δ T cells (plot not shown).

5.3.2 Activation markers

A selection of markers was chosen to examine whether the synovial T cells were activated recently (Figure 5.3). The results are summarised in Table 5.2. Eighty-three percent of the synovial α/βTCR+ cells expressed CD25 (IL-2R) (Figure 5.3A), while only 3% of the α/β T cells in TD lymph expressed this marker (Figure 5.3B).

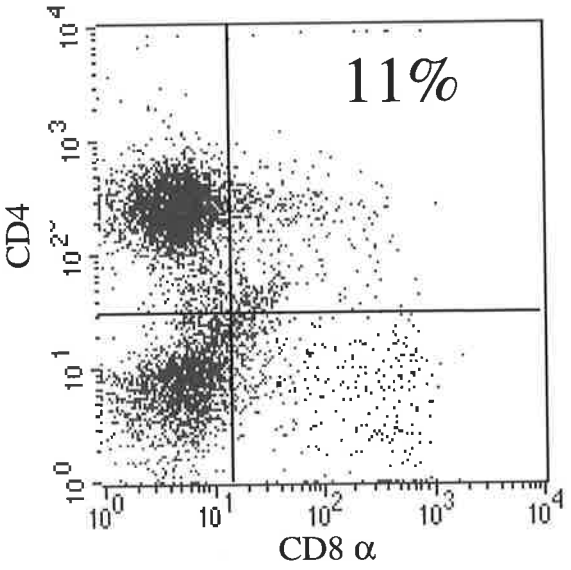
Figure 5.3 Detection of activation markers on lymphocytes from AA synovium and TDL.

Suspensions of cells from AA synovium (left-side panel) and TD lymph (right-side panel) were labelled for single or dual colour flow cytometric analysis. For single colour analysis the cells were labelled by the indirect immunofluorescence technique with mAb OX26 (anti-CD71; FITC). For dual colour analysis the cells were labelled first by the indirect immunofluorescence technique with OX39 (anti-CD25; FITC) (Fig. 5.3A & B), OX6 (anti-MHC Class II; FITC) (Fig 5.3C & D), OX8 (anti-CD8α; FITC) (Fig. 5.3G & H) or OX22 (anti-CD45RC; FITC) (Fig. 5.3I & J) followed by direct labelling with mAb R73 (anti-α/β TCR; PE) or mAb W3/25 (anti-CD4α; PE). In the case of single colour, the broken line represents the autofluoresecence of lymphocytes incubated with the negative control mAb 1B5. The staining profile of cells incubated with mAb OX27 (anti-CD71; FITC) is superimposed, on the fluorescence seen with mAb 1B5 with the percentage of labelled cells indicated adjacent the marker in these panels (Fig. 5.3E & F). In the case of dual colour, the percentage of the α/β TCR population that were double positive for each marker is indicated.


Arthritic synovium
Arthritic TDL
A
B
83%
3%
α/β TCR
CD25
C
D
36%
5%
MHC class II
MHC classII
E
F
36%
5.39%
Counts
CD71

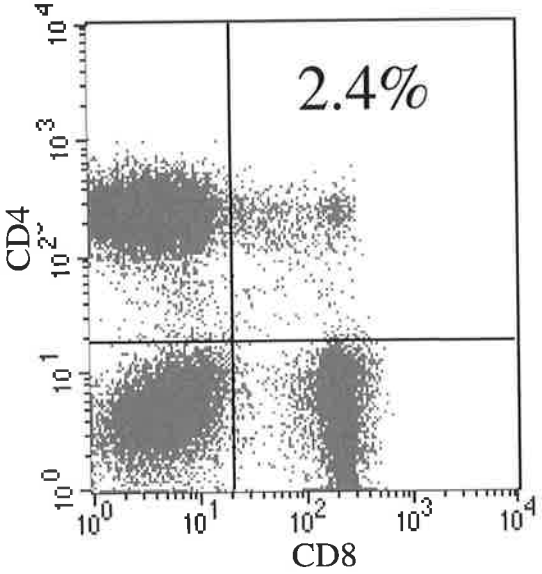
Arthritic synovium

G



Arthritic TDL

H



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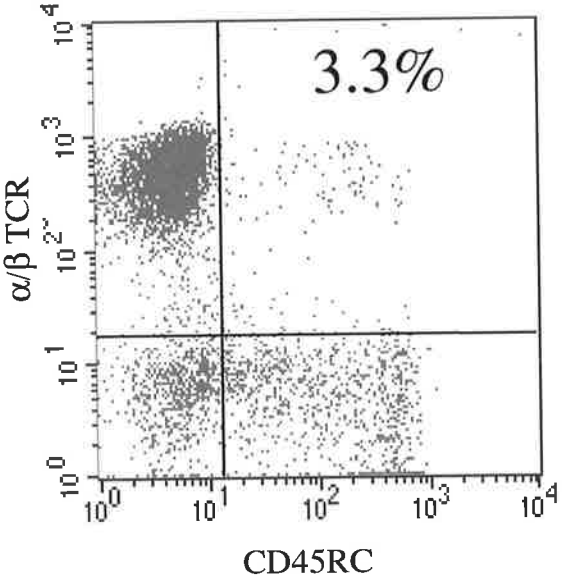

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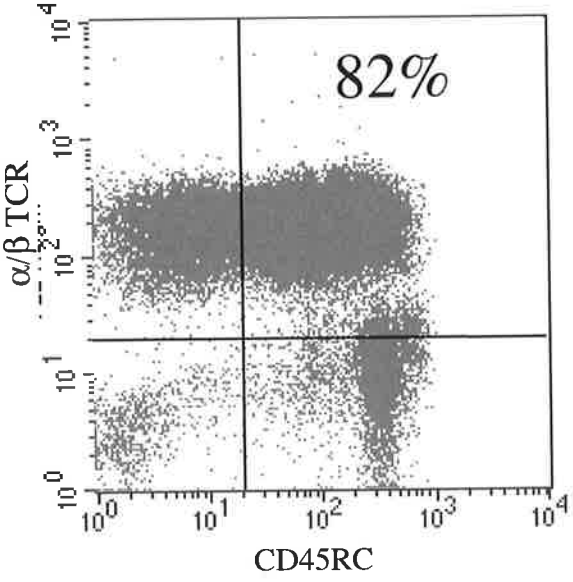


Table 5.2 Expression of activation markers by lymphocyte subpopulation from synovium and thoracic duct lymph.

Analysis of cells was performed by flow cytometric analysis of cells that fell within the lymphocyte gate defined in Figure 5.1, using either single fluorochrome indirect immunofluorescence (mAb OX26) or dual immunofluorescence(mAbs R73 with OX39, OX6, OX22, IA29 or WT1 and W3/25 with OX8). For TD lymphocytes, all cells were included in the analysis. Synovial cells were obtained from rats 14 days after inoculation of CFA. TD lymph was obtained from rats 9 days after inoculation with CFA.

Antibodies	**Activation Marker or Adhesion molecule**	**% +ve cells**	**% +ve cells**
		AA synovium	AA TDL
R73 + OX39	α/β TCR + CD25+	83	3
R73 + OX6	α/β TCR + MHC Class II	36	5
OX26	CD71 (single colour)	36	5.4
W3/25 + OX8	CD4+ CD8α	11	2.4
R73 + OX22	α/β TCR + CD45RC+	3.3	82

Thirty-six percent of synovial T cells expressed MHC class II (Figure 5.3C) in comparison with 5% in TD lymph (Figure 5.3D) while the respective percentages for cells in the lymphocyte gate expressing CD71 (transferin receptor) were 36% (Figure 5.3E) and 5% respectively (Figure 5.4F). Of synovial CD4+ cells, most of which are α/βTCR+ cells (Figure 5.2A), 11% also expressed CD8α (Figure 5.3G), compared with just 2.4% of the CD4+ cells in TD lymph (Figure 5.3H). In contrast, just over 3% of α/βTCR+ cells from synovium expressed CD45RC (mAb OX22) (Figure 5.3I), compared with 82% of α/βTCR+ cells in TD lymph (Figure 5.3J). The latter findings suggest that the majority of α/βTCR+ cells in the inflamed synovium are effector or memory cells, (whereas the majority of T cells in TD lymph appear to be naïve cells).

5.3.3 Adhesion molecule expression

The expression of adhesion molecules by T cells in synovium and TD lymph is shown in Figure 5.4 and summarised in Table 5.3. Seventy-five percent of the α/βTCR+ synovial cells (Figure 5.4A), but less that 4% in TD lymph cells, expressed CD54 (ICAM-1) (Figure 5.4.B). In cells from both sources, about 95% of α/βTCR+ cells expressed CD11a (LFA-1) (Figure 5.4C&D) and 95-98% expressed CD44 (Figure 5.4E&F). Importantly, the level of expression of CD54 was higher in cells from synovium than in T cells from TD lymph. In the case of CD49d (VLA-4), 51% of the α/βTCR+ cells in synovium expressed the molecule (Figure 5.4G), whereas just 5% of α/βTCR+ cells from TD lymph were positive (Figure 5.4H). L-selectin, which is down-regulated on activated T cells, was not detected on T cells from the synovium (Figure 5.4I) but it was expressed by 12% of T cells and most B cells from TD lymph (Figure 5.4J).

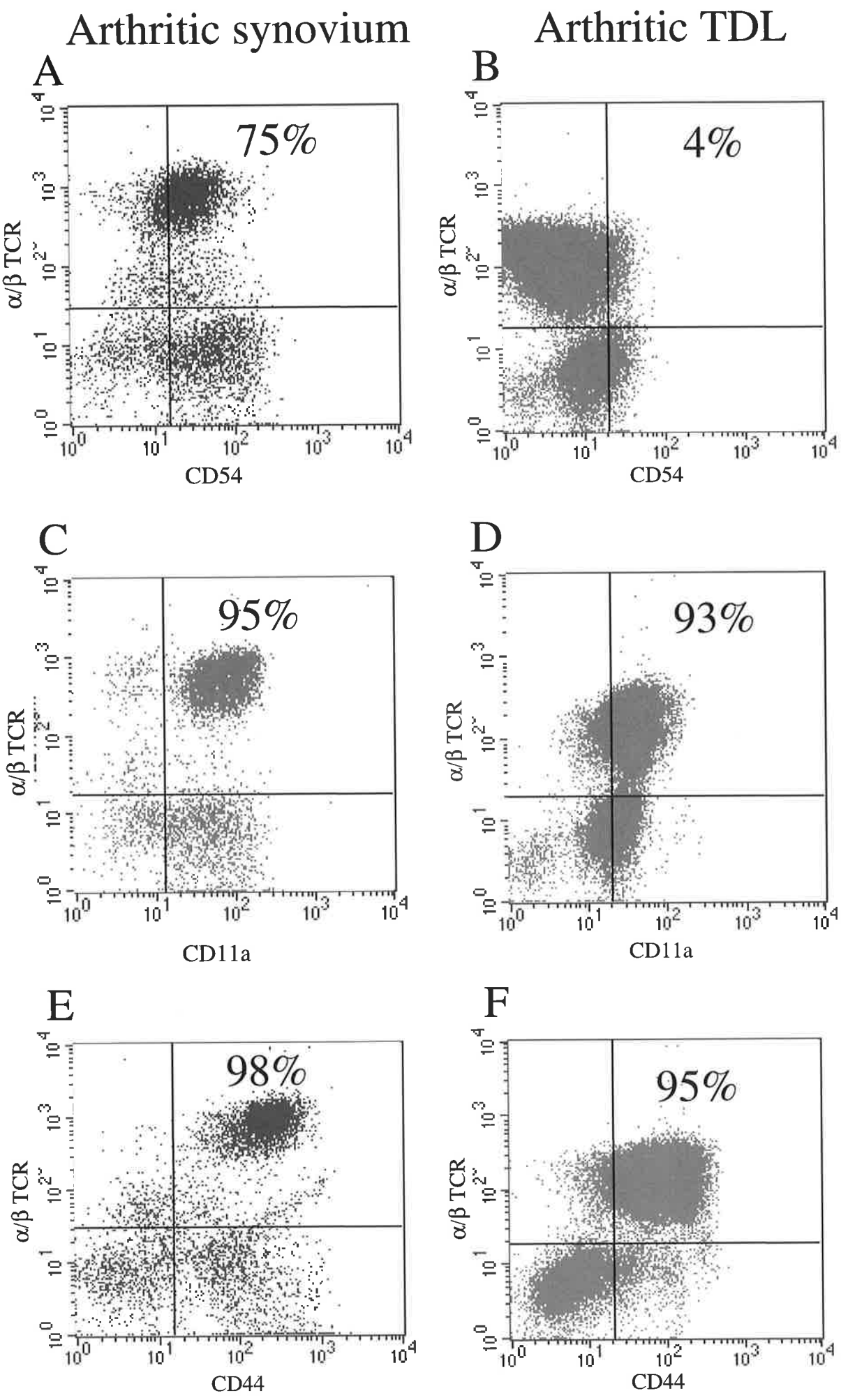
Of cells from synovium that fell within the lymphocyte gate, approximately 73% and 77% expressed CD11a and CD18 respectively (single colour), compared with approximately 95% of CD11a+ cells in TD lymph (plots not shown). In contrast, of the cells in synovium that fell within the lymphocyte gate, only 11% expressed CD11b. Cell numbers were insufficient to attempt dual colour analysis and the nature of the CD11b+ cells is uncertain, but they are likely to be macrophages and/or NK cells.

Table 5.3 Expression of adhesion molecules by lymphocyte subpopulation from synovium and thoracic duct lymph. Analysis of cells was performed by flow cytometric analysis of cells that fell within the large lymphocyte gate defined in Figure 5.1, using either single fluorochrome indirect immunofluorescence (mAb WT5, WT3) or dual immunofluorescence (mAbs R73 with IA29, WT1, OX50, Mrα41, OX85 and W3/25). For TD lymphocytes, all cells were included in the analysis. Synovial cells were obtained from rats 14 days after inoculation of CFA. TD lymph was obtained from rats 9 days after inoculation with CFA.

Antibodies	Adhesion molecule	% +ve cells	
		AA synovium	AA TDL
R73 +IA29	α/β TCR+ / CD54+	75	4
R73 +WT1	α/β TCR+ / CD11a	95	93
R73 + OX50	α/β TCR+ / CD44	98	95
R73 + Mrα4-1	α/β TCR+ / CD49d	51	5
R73 + OX85	α/β TCR+ / CD62L	0.5	12
WT3	CD18 (LFA-1 β_2 unit)	77	Not done
WT5	CD11b, subunit of MAC-1	11	0
R73 + OX62	α/β TCR+ / αE2	6	Not done

Figure 5.4 Detection of adhesion molecules on lymphocytes from AA synovium and TD lymph.

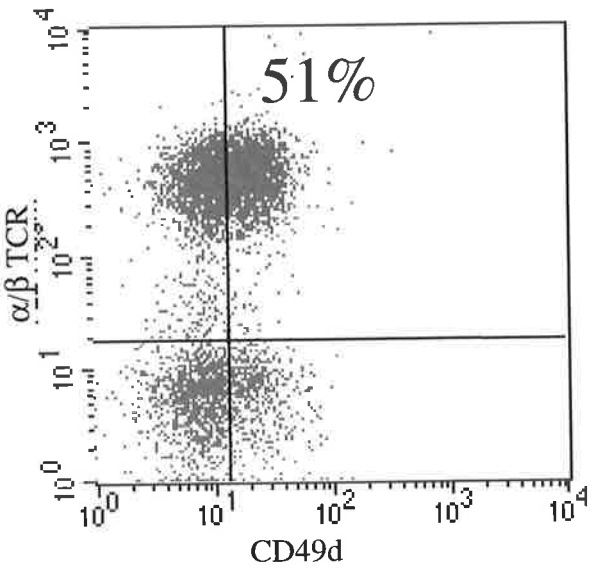
A suspension of cells from AA synovium and TD lymph were labelled for dual colour flow cytometric analysis. The cells were labelled first by the indirect immunofluorescence technique with IA29 (anti-CD54; FITC) (Fig. 5.4A & B), WT-1 (anti-CD11a; FITC) (Fig. 5.4C & D), OX50 (anti-CD44; FITC) (Fig. 5.4E & F), Mrα4-1 (anti-CD49d; FITC) (Fig. 5.4G & H) or OX85 (anti-CD62L; FITC) (Fig. 5.4I & J), followed by direct labelling with mAb R73 (anti-α / β TCR; PE). The percentages of the α / β TCR population that were double positive for each adhesion molecule is indicated.


Arthritic synovium
Arthritic TDL
A
75%
α/β TCR
CD54
B
4%
α/β TCR
CD54
C
95%
α/β TCR
CD11a
D
93%
α/β TCR
CD11a
E
98%
α/β TCR
CD44
F
95%
α/β TCR
CD44

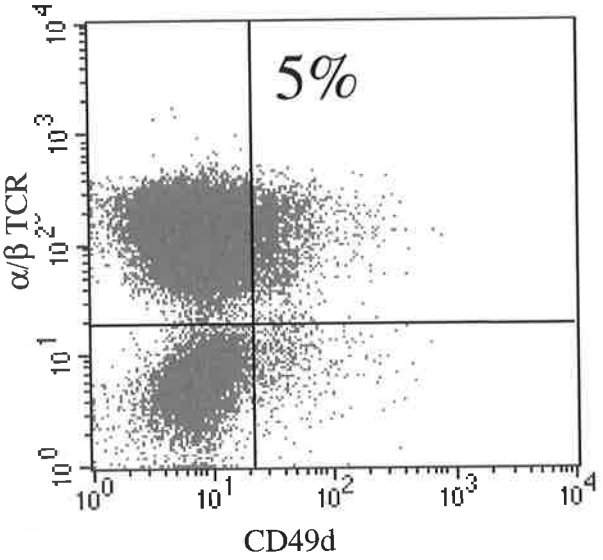
Arthritic synovium

Arthritic TDL

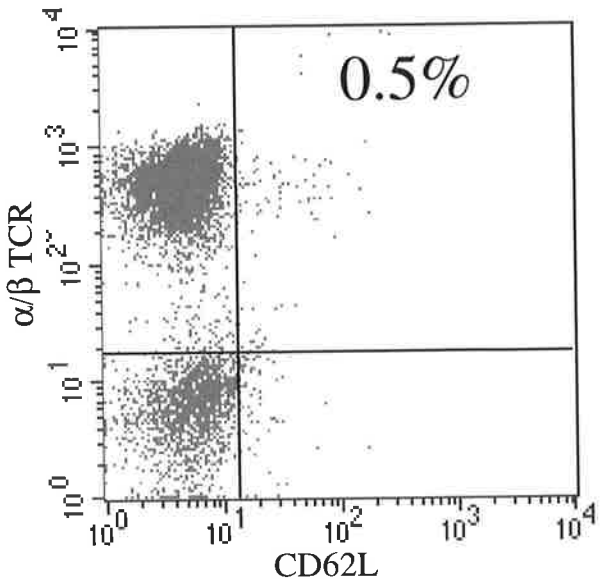
G



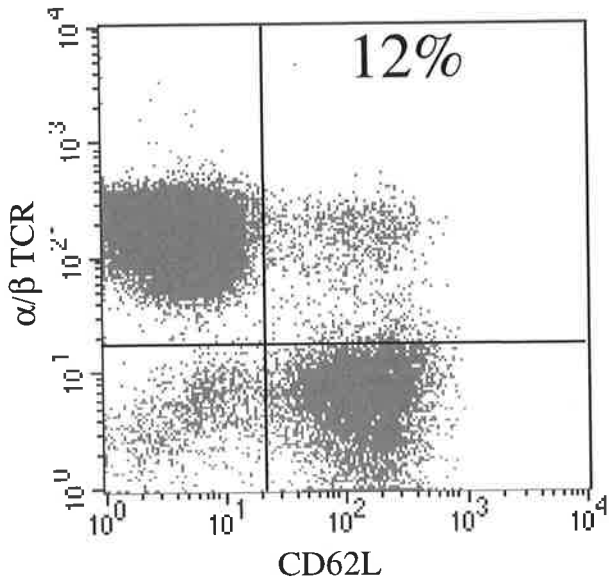
H



I



J



macrophages and/or NK cells. CD11b was not expressed by TD lymphocytes. Among synovial cells the αE2 integrin subunit was expressed by 6% of the α/βTCR+ cells in the lymphocyte gate. Although the percentages of positive cells were similar in synovium and TD lymph, all synovial T cells express high levels of CD44.

Relative to TD cells, synovial T cells expressed the adhesion molecules CD54 and CD49d in greater proportions. The contrasting low proportion of CD62L+ T cells in synovium, suggests that the majority of synovial T cells are activated and/or memory cells. The presence of a small proportion of the αE_2^+ cells suggests that a few of the synovial cells might arise from mucosal sites but that most probably originate from peripheral sites.

5.4 Discussion

Lymphocytes obtained from synovium-rich sub-Achilles tissue from rats with severe polyarthritis 13 to 14 days post-inoculation with CFA were analysed by flow cytometry, in order to characterise the subsets of T cells and their activation status. Lymphocytes were found to constitute 2.5% of the total leucocyte population. The majority of cells within the lymphocyte gate were of the CD4+ T cell subset. CD4+ T cells are believed to be of primary importance in the development, initiation and perpetuation of AA. The significant CD4+ lymphocyte infiltration in this tissue supports a central role of these cells in AA (Pelegri *et al.,* 1996a; Hoffman *et al.,* 1997). CD4+ T cells play a central role in RA, demonstrated by reduced symptoms in patients treated with anti-CD4 mAb (Herzog *et al.,* 1989) or in those who aquire diseases that deplete functionally competent CD4+ T cells (Espinoza *et al.,* 1989).

Only a small population of CD8+ cells were detected in the synovium. CD8+ cells are believed to play a minor role in the chronic inflammatory response (Larsson *et al.,* 1985; Pelegri *et al.,*; 1996b; Hoffman *et al.,* 1997). The minor population of B cells in the lymphocytes obtained from inflamed synovium supports earlier work that indicates B cells are not vital in the pathogenesis of AA (Smith *et al.,* 1980; Asherson *et al.,* 1972; Issekutz *et al.,* 1986). A recent study carried out in this laboratory demonstrated that adoptive

transfer of AA by TD lymphocytes from arthritic donors was mediated by the CD4+ subset of T cells, but not by the CD8+ subset. It was concluded that the arthritogenic subpopulation resides within the CD4+ subset of T cells (Spargo *et al.*, 2001).

The T lymphocyte antigen receptor γ/δ was detected on a small population (4%) of cells from synovium that fell within the lymphocyte gate. Almost 13% of γ/δ T cells were also positive for CD8. The minor proportion of γ/δ TCR+ T cells is elevated in peripheral blood, synovial fluid (Holoshitz *et al.*, 1989) and synovial membranes from patients with RA (Andreu *et al.*, 1991), but not in those with osteoarthritis. T cells expressing the γ/δ TCR are believed to contribute to the pathogenesis of murine collagen-induced arthritis (Peterman *et al.*, 1993) but α/β TCR+ cells appear to be sufficient to mediate the pathogenesis of AA (Pelegri *et al.*, 1996a; Spargo *et al.*, 1996; Broker *et al.*, 1991; Yoshino *et al.*, 1990). However, it has been suggested that γ/δ TCR+ cells have a protective role, where they may protect against joint damage during the pathogenesis of arthritis (Pelegri *et al.*, 1996b).

Approximately 6% of the α/β TCR+ cells within the lymphocyte gate expressed CD161. A few of these cells expressed the α/β TCR at high level but most were stained by mAb R73 at low intensity. The significance of the α/β TCR^{lo} CD161+ cells is uncertain. A feature of NK T cells in mice is that they express low levels of αβ TCR compared with classical T cells. They are referred to as TCR 'intermediate' cells (reviewed by Vicari and Zlotnic, 1996; MacDonald, 1995). It is possible, therefore, that the synovial cells stained weakly by mAb R73 could be NK T cells. However, further work is necessary to confirm this identification of the cells. Recent work in this laboratory has shown that the Serotec PE-conjugated mAb R73 stains a sub-population of synovial cells non-specifically. The small but substantial population of α/β TCR^- CD161+ cells in the synovial population (Figure 5.2I) raises the possibility that the weak staining of some CD161+ cells by mAb R73 could be artefactural.

NK T cells have been identified in humans (Porcelli *et al.*, 1993, Lanier *et al.*, 1994), mouse and rat (Brissette-Storkus *et al.*, 1994). The tissue distribution of these cells in mice as a percentage of total α/β T cells is 10-20% in thymus, 3% in spleen, 0.3% in lymph node, 20-30% in bone marrow and 30-50% in liver (Godfrey *et al.*, 2000). Some intraepithelial lymphocytes (Guy-Grand *et al.*, 1996) and lymphocytes of the small intestinal lamina propria also co-express CD161 and α/βTCR (Ohteki *et al.*, 1992).

NK T cells are believed to have an immunoregulatory function, e.g. they may protect vital organs, from damaging inflammatory immune responses (Godfrey *et al.*, 2000) and have a role in cytolytic responses to infected or stressed cells, through the recognition of damaged cells or unique bacterial antigens (Hashimoto, 1995; Vicari and Zlotnik, 1996). NK T cells have been found to be fewer in number in non-obese diabetic (NOD) mice (Godfrey *et al.*, 2000) and in humans with multiple sclerosis, RA, systemic sclerosis and type I diabetes. In patients with RA, their presence has been correlated with disease activity (Rodrigez-Palmero *et al.*, 1999).

In mice, expression of CD161 is induced by activation and can be subsequently lost (Chen *et al.*, 1997). It is possible that T cells identified within the inflamed rat tissue that express both an intermediate density of TCR and CD161, represent an activated sub-population of lymphocytes.

Most lymphocytes in synovial digests appeared to be either activated or memory T cells. Only about 3% of α/β T cells expressed CD45RC, a marker for naïve T cells. In addition, the expression of L selectin, which is known to become downregulated on activated and some memory T cells, was expressed by very few T cells.

The arthritogenic population of CD4+ T cells in TD lymph from arthritic donors expresses activation markers such as CD25, MHC Class II and CD71 (Spargo *et al.*, 2001). Over 80% of the α/β T cells in synovial digests expressed CD25 (IL-2R), while approximately one third expressed MHC class II. MHC class II expression in AA has previously been

reported in studies of rat limb joints (Pelegri *et al.,* 1995a; Halloran *et al.,* 1996). Cells expressing this marker are believed essential for the initiation of an immune response (Steinman *et al.,* 1991) by expression of fragments of the antigenic molecules on the cell surface to T lymphocytes via MHC Class II molecules (Crowley *et al.,* 1990). One third of the α/β TCR+ T cells expressed the transferin receptor (CD71). Because lymphoid cells express CD71 when they are in cell cycle, it is likely that such a substantial proportion of synovial T cells may be proliferating (Jefferies *et al.,* 1991). High levels of expression of the MHC Class II, CD25 and CD71 are all characteristics of activated T lymphocytes and effector cells.

The adhesion molecules CD54, CD11a and CD49d were expressed at elevated levels by α/β TCR+ cells from synovium, consistent with recent activation and trafficking of these cells. Clusters of T cells that express CD54 have been found in synovium of rats from day 14 post-induction of AA, suggesting an ICAM -1 dependent pathway in the pathogenesis of AA (Iigo *et al.,* 1991; Carol *et al.,* 2000). Expression of ICAM-1 on synovial fluid lymphocytes from human RA subjects is elevated compared with control lymphocytes (Takahashi *et al.,* 1992; Potocnik *et al.,* 1990, Gerritsen *et al.,* 1993; Sfikakis and Mavrikakis, 1999). Increased CD54 expression by synovial T cells may be involved in the engagement of these cells with DC during the induction of AA by CFA and also during interaction of effector T cells with DC locally in the synovium. Increased expression of CD11a (LFA-1) by the T cells may be involved in interactions with DC and also in LFA-1/ ICAM-1 mediated migration of cells into synovium.

CD49d also plays an important role in mediating T lymphocyte migration to arthritic joints in rats with AA (Issekutz and Issekutz, 1991). VLA-4, a member of the integrin family ($\alpha_4\beta_1$) is upregulated on peripheral T cells after activation (Albelda and Buck, 1990). It binds to the vascular cell adhesion molecule (VCAM-1), whose expression is increased on the surface of ECs at sites of inflammation (Elices *et al.,* 1990; Schwartz *et al.,* 1990), and in the case of RA this is well documented (Hemler *et al.,* 1986; Cush *et al.,* 1988; Hale *et al.,* 1989; Laffon *et al.,* 1989). Lymphocyte VLA-4 (CD49d) binding to endothelial

VCAM-1 is believed to be important in lymphocyte homing in RA (van Dinther-Jannssen *et al.,* 1991; Ruegg *et al.,*1992). CD49d, as a component of $\alpha_4\beta_7$ integrin can also bind to VCAM-1 but it also has a function in mucosal (gut) homing by binding to MadCAM (Berlin *et al.,* 1993; Hogg and Berlin, 1995; Imhof and Dunon, 1995). A relatively higher proportion of T cells from both synovial membrane and synovial fluid express increased levels of VLA-4, compared with peripheral T cells from the same RA patients (Postigo *et al.,* 1992; Takahashi *et al.,* 1992; Laffon *et al.,* 1991).

A small population of α/βTCR+ cells expressing the rat homologue of the α_{E2} subunit (CD103) of the human $\alpha_E\beta_7$ was also detected in the synovial sample. This corroborates the notion that some synovial T cells could have an origin in a mucosal site such as the gastrointestinal tract. In mice, rats and humans this CD103 is restricted mainly to mucosal T cells (intraepithelial lymphocytes and lamina propia lymphocytes) and it is expressed by few peripheral α/βTCR+ cells (Brennan and Rees, 1997). In addition, a small population of α/βTCR+ cells expressing this marker has been detected in TD lymph, lymph nodes and spleen from the nude rat (Murphy, PhD thesis, 1999).
T cells that express β_7 integrins have been detected in RA synovium as reported by Lazarovits and Karsh, (1993), who found both $\alpha_4\beta_7$ T cells and $\alpha E\beta_7$+ T cells therein. The expression of $\alpha_4\beta_7$ and $\alpha E\beta_7$+ together suggest a possible mucosal origin. Spargo *et al.*, (1996) found some homing of lymphoblasts from mesenteric duct lymph to inflamed (but not non-inflamed) synovium. Together the findings suggest a degree of mesenteric duct lymph cells trafficking to inflamed synovium. The significance of this is uncertain, although clinical associations between gut and joint inflammation are well recognised (Burmester *et al.,* 1995).

Levels of expression of CD44 on CD4+ lymphocytes from TD lymph from normal and arthritic rats were found to be similar (Spargo *et al.*, 1996). Expression of CD44 by PB lymphocytes from RA patients and normal controls has also been found to be similar (Bond *et al.,* 1997). However, only a small proportion of the total cells in TD lymph or blood would be expected to be involved in the pathogenesis of arthritis. It is, therefore,

interesting that cells isolated from the synovium exhibit high intensity of expression of CD44. Rather than playing a role in migration, this could indicate a role for this adhesion molecule in the retention of lymphocytes at an inflammatory site through adhesive interactions with components of the extra-cellular matrix.

In summary, this study identifies the phenotypes and activation status of T cells found within the severely arthritic joints of rats with AA. It is the first study to describe these populations and their sub-populations in any detail, using quantitative flow cytometric analysis on freshly isolated cells from the ankle joint. Other studies have looked at homing using 'in vitro' activated donor cells or have used immunohistochemical techniques. One group carried out flow cytometric analysis of AA tissue from the knee joint and reported insignificant numbers of lymphocytes (Pelegri *et al.,* 1995a). The present study shows that T lymphocytes from synovium in AA are mainly CD4+ cells that express activation markers and increased levels of adhesion molecules. These characteristics are consistent with effector cells that express low levels of CD45RC and L-selectin, accompanied by high expression of CD44, CD11a, CD54, CD49d, MHC class II, the transferin receptor, CD71, and the IL-2 receptor, CD25.

It is probable that they represent cells that were activated elsewhere and after migration to the joint have been preferentially retained there due to their surface phenotype. However, the expression of CD71 by approximately one third of the cells, suggests that some may be proliferating in response to local antigen. The findings are consistent with the selective recruitment of activated CD4+ T cells from the circulation (Spargo *et al.,* 1996; 2001). Most of these cells probably originate from peripheral secondary lymphoid tissues, but some may be recruited from mucosal sites. The nature of the synovial vascular adhesion molecules that mediate recruitment of activated T cells is, therefore, an important issue.

Chapter 6

Development of a Lymphocyte Adherence Assay: Preparation of Lymphocytes and Endothelial Cell Monolayers for the Assay.

6.1 Introduction

A lymphocyte adherence assay was required, in order to test a range of monoclonal antibodies for their ability to block adhesion of lymphocytes to the microvascular EC monolayers. Endothelial cells were purified as described in Section 2.13. To obtain the most information from the small number of synovial microvascular EC that were available, Terasaki microculture plates were utilised for the assay. To my knowledge, they have not been used previously for this purpose.

This chapter focuses on establishing optimal conditions for each stage of the assay of lymphocyte adherence in micro-culture wells. Aspects that required further development were the nature of lymphocytes used in the assay, the density of lymphocytes applied to the EC monolayers in each well, a method to remove unbound lymphocytes reproducibly, the effect of protein present in the medium during the assay and quantification of the bound lymphocytes.

The choice of the lymphocytes to be used as targets for adherence is important because they should be relevant to the AA disease model, where T cells are recruited into inflamed synovium. The CD4+ T cell subset is known to play a major role in the pathogenesis of AA (Pelegri *et al.,* 1996a; Hoffman *et al.,* 1974; Spargo *et al.,* 2001) and lymphocytes arising from TD drainage in arthritic rats are recruited in significant numbers to inflamed synovium (Spargo *et al.,* 1996). It was planned, therefore, to set up the adhesion assay using readily available Con A stimulated lymphocytes and to then use purified CD4+ T lymphocytes from the TD lymph of rats in the late prodromal phase of arthritis. Flow cytometric analysis of surface adhesion molecules and activation markers on these cells was carried out and the

results compared with the surface antigen markers present on lymphocytes that had migrated *in vivo* to the inflamed synovium (Section 5.3).

6.2 Hypotheses and aims

6.2.1 Hypotheses:

- Expression of activation markers and adhesion molecules by Con A stimulated normal lymph node cells will be similar to those expressed by activated lymphocytes obtained from TD lymph or that accumulate in the synovium of arthritic joints.

- Expression of markers of activation and adhesion molecules will change with increasing duration of Con A stimulation.

- Conditions can be established that will allow the measurement of lymphocyte adherence to microvascular EC monolayers and the effects of mAbs on adhesion.

- Conditions developed for measuring adhesion of Con A-activated T lymphoblasts to microvascular endothelium can be adapted also to the adherence of CD4+ TD lymphocytes that contain an arthritogenic subpopulation.

6.2.2 Aims:

- To establish a method to measure adherence of lymphocytes to EC monolayers that is sensitive to the effects of antibodies that block interactions between cognate adhesion molecules.

- To establish a method to enrich or isolate CD4+ lymphoblasts from TD obtained from donors during the late prodromal period of AA.

- To investigate the effect of foetal calf serum on adherence of lymphocytes to EC monolayers.

- To establish a washing procedure that does not incur the loss of EC from the monolayers in Terasaki plates.

- To establish a washing procedure that allows the efficient removal of free lymphocytes from Terasaki plates while retaining adherent lymphocytes.

- To establish a reproducible method for quantifying adherent lymphocytes.

- To compare the cell surface phenotypes found on Con A stimulated lymph node cells and lymphocytes in TD lymph from donors in the prodromal period of AA.

- To characterise the cell surface phenotypes found on CD4+ lymphoblasts in TD lymph from donors in the prodromal period of AA.

6.3 Results

6.3.1 Flow Cytometric Analysis of Con A Stimulated Lymphocytes.

Lymphocyte suspensions harvested from lymph nodes (LN) were prepared as described in Section 2.8.1. LN lymphocytes were stimulated with Con A as described in Section 2.8.2. Using flow cytometric analysis, the cell surface expression of adhesion molecules and activation markers were assessed after 0, 24, 48 or 72 hours of stimulation. The proportion of cells stained by mAb R73 (α/β TCR) was approximately 70% without stimulation and increased to 80-90% during the period of stimulation. The activation markers and adhesion molecules expressed by these LN cells stimulated *in vitro* can be compared with those expressed by lymphocytes in collagenase digests of synovium and T cells from TD lymph from arthritic rats (shown in Section 5.3, Tables 5.2 and 5.3).

6.3.1.1 Expression of activation markers by Con A stimulated lymphocytes

The expression of activation markers and adhesion molecules was identified by single

colour flow cytometry of the cells lying within the lymphocyte gate (Figure 5.1). After 24 hours of Con A stimulation, the proportion of cells expressing CD25 (IL2-R) had increased from approximately 15% to 75%. After 48 hours of stimulation, over 90% of cells expressed CD25 and a similar proportion were CD25+ at 72 hours (Table 6.2). Approximately 12% of cells expressed CD71 (transferin receptor) in the unstimulated population and this rose to about 50% after 24 hours of stimulation and reached approximately 90% by 48 hours and 72 hours. The proportion of cells expressing MHC class II increased two-fold (to 58%) after stimulation for 24 hours and increased further to 89% after 48 hours stimulation. The proportion of MHC class II+ cells decreased to 36% by 72 hours. As assessed by the expression of the Ki67 antigen, the proportion of dividing cells in the cultures were 15%, 55% and 93% after stimulation with Con A for 24, 48 hours and 72 hours respectively.

6.3.1.2 Expression of adhesion molecules by Con A stimulated lymphocytes

Adhesion molecule expression was also altered by Con A stimulation. ICAM-1 (CD54) increased from 36% to 66% after 24 hours and by 48-72 hours 96% of the cells expressed the molecule (Table 6.2). The CD11a+ and CD18+ cells increased in proportion by similar amounts, from approximately 20% in unstimulated cells to approximately 50% at 24 hours of stimulation and 90% in cells stimulated for 48-72 hours. As expected, the proportion of CD11b positive cells was less than 1% after stimulation. The proportion of cells expressing CD44 varied little during the course of stimulation when it was almost universally expressed. The proportion of cells expressing CD49d (β_1 integrin) was unchanged at 24hrs, but then it reached 65-70%, after 48 to 72 hours. L-selectin expression, which is known to be down regulated after activation of T cells, was expressed by approximately 70% of unstimulated cells. The proportions of CD62L+ cells decreased to 23% at 24 hours, to 14% and were stable at 12-14% at 48 to 72 hours. Expression of the CD62P-ligand was not detected on unstimulated cells or on cells stimulated for 24 hours. However, at 48 and 72 hours it was detected on 16% and 22% of the cells respectively.

Cells stimulated with Con A for 24 hours had comparable proportions of cells expressing

CD25, CD71, MHC class II and CD54. CD11a/CD18+ and CD49d were most similar to expression of these markers by synovial T cells, albeit with somewhat more cells expressing CD71 and MHC class II and fewer cell expressing CD11a/CD18 and CD49d (shown in Section 5.3, Tables 5.2 and 5.3).

After 48 hours *in vitro* stimulation, the proportions of CD25+, CD71+, MHC class II+, CD54+ and CD49d+ cells exceeded those found on lymphocytes from inflamed synovium. The proportions of CD11a/CD18+ cells were similar in stimulated LN cells and cells from arthritic synovium. Little difference was detected in the proportions of cells expressing activation markers or adhesion molecules between cells stimulated for either 48 or 72 hours. The exception was the proportions of cells expressing MHC class II, which had peaked at 48 hours stimulation.

Based on the above findings, LN lymphocytes stimulated for 24 hours were considered to be the most physiologically appropriate cells to use in developing the lymphocyte assay. However, LN cells stimulated with Con A for 48hours were also used in preliminary experiments as their greater adhesion molecule expression seemed likely to enhance prospects for displaying cell adhesion to monolayers of microvascular EC obtained from synovial tissue digests from arthritic rats.

6.3.2 Adherence of lymphocytes to endothelial cell monolayers.

6.3.2.1 Adhesion of untreated, 24 and 48hr Con A stimulated lymph node lymphocytes to endothelial cells monolayers.

To further refine the adherence assay protocol, an initial series of experiments was performed to assess lymphocyte adherence to the EC monolayers cultured *in vitro*. Once adherence of lymphocytes to EC monolayers was confirmed, methods for quantifying the numbers of adherent cells could be addressed.

EC monolayers were prepared as described in Section 2.13. At least 10 replicate wells were included for each treatment group. The adhesion of lymphocytes, either unstimulated LN

Table 6.2 Activation markers and adhesion molecules detected on normal and Con A stimulated lymph node lymphocytes. Single fluorchrome, flow cytometric analysis was used to determine the proportions of cells expressing adhesion molecules and activation markers in normal LN preparations and on cells stimulated for 24 hours, 48 hours or 72 hours with Con A. Results are expressed as the percentages of total with surface expression of these markers.

Activation markers / Adhesion molecules	Normal Lymphocytes	Con A 24hr stimulation	Con A 48hr stimulation	Con A 72hr stimulation
CD25	15	75	98	95
CD71	12	48	93	90
MHC CLII	23	58	89	36
KI67	0	15	55	93
CD54	36	66	96	96
CD11a	20	52	88	93
CD18	22	49	85	87
CD11b	1	0.9	0.9	0.3
CD49d	31	28	65	68
CD44	90	96	97	99
CD62L	69	23	14	12
CD62P-ligand	0	0	16	22

cells or cells stimulated with Con A for either 24 or 48 hours, was evaluated and compared. The lymphocytes were labelled with CFSE (Section 2.8.4) to allow easy identification. The mannose sugar, α-D-mannopyanoside was included in all assays involving Con A activated lymphocytes (Section 2.8.3). After removal of non-adherent lymphocytes by gentle washing, the monolayers and adherent cells were fixed lightly with glutaraldehyde and the wells were photographed using an inverted fluorescence microscope for quantitative analysis (described in Section 2.5). Adherent CSFE-labelled lymphocytes were counted manually from the photomicrographs.

The results of these preliminary experiments (Figure 6.1) revealed that both unstimulated and stimulated lymphocytes adhered to the EC monolayers. However, there was a clear difference between the adhesive interactions of stimulated and unstimulated LN lymphocytes, with a mean of 540 adhered unstimulated lymphocytes counted per well, compared with 3673 in the wells receiving 24 hour stimulated lymphocytes and 3992 in wells receiving lymphocytes stimulated for 48 hours. Thus, the use of lymphocytes stimulated for 24 hours with Con A led to a 6 to 6.8-fold increase in adherence, when compared to unstimulated cells. A 7.4 fold increase in adherence was observed using lymphocytes that had been stimulated for 48 hours. Statistical analysis of the data (Anova and post hoc, Tukeys test), showed highly significant differences between adherence of unstimulated and stimulated lymphocytes ($p=0.0001$). No significant difference in adherence was detected between 24 hour and 48 hour stimulated lymphocytes ($p=0.36$).

The aim of these experiments was to assess the feasibility of the assay. It was concluded that adhesive interactions between lymphocytes and the EC monolayers were present in this assay model and that these lymphocytes could be both detected and quantified. It was also possible to confirm from this experiment and the investigation of activation markers and adhesion molecules, that LN lymphocytes stimulated for 24 hours *in vitro* were suitable as a model for exploring lymphocyte adhesion to synovial microvascular EC monolayers.

6.3.2.2 Seeding density of lymphocytes

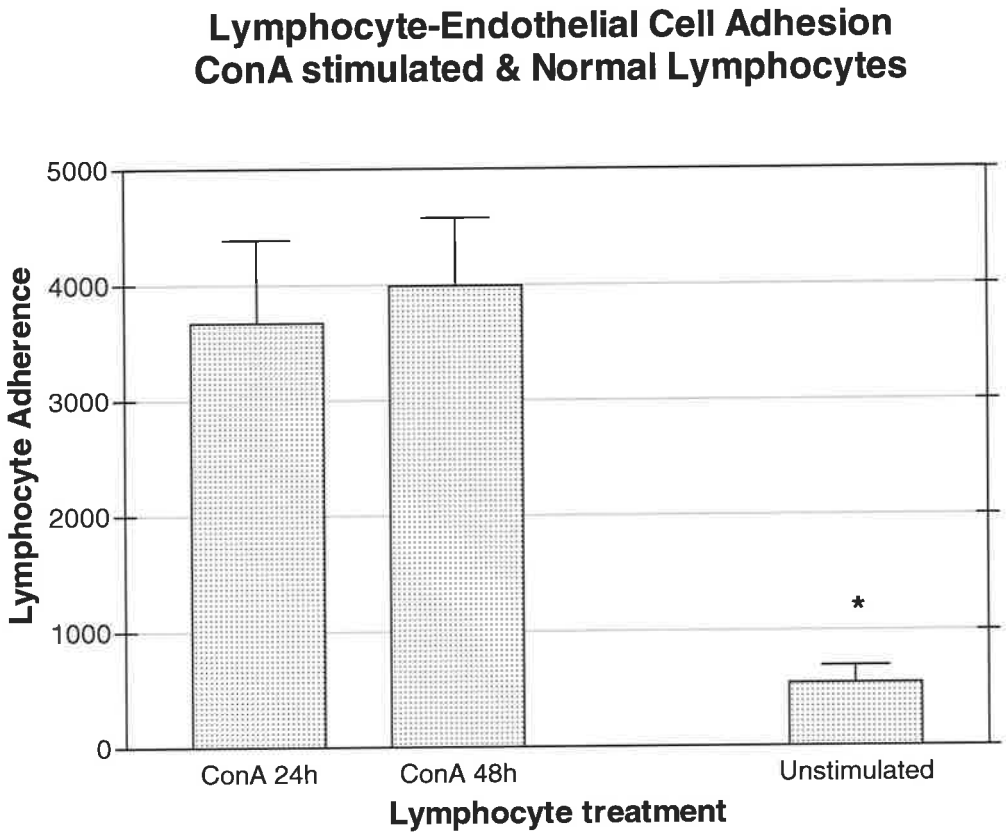
To allow efficient adherence of lymphocytes to EC interactions to occur, it was important to add an even layer of cells to the EC monolayer. Preliminary experiments revealed that overcrowding of the wells with lymphocytes created difficulties in removing lymphocytes. It was necessary therefore, to determine the optimal density of lymphocytes to be applied to each well. Essentially two criteria needed to be fulfilled. Firstly, the entire surface of the EC culture needed to be covered by an even layer of lymphocytes, thus allowing maximum contact with the EC and promoting adhesive interactions. Secondly, the addition of too many lymphocytes was undesirable, because overcrowding the wells with lymphocytes could favour homotypic interactions between lymphocytes to form aggregates. These aggregates of lymphocytes bind directly to EC. This made it difficult to quantify accurately lymphocyte - EC binding.

Lymphocyte suspensions were prepared at $1x10^7$/ml and $0.5x10^7$/ml and volumes of 5, 10, 15 or 20µl per well were aliquoted to 5 replicate wells for each resulting concentration of cells. The cells used were 24 hour Con A stimulated lymphocytes. These aliquots resulted in the following numbers of cells per well; $0.25x10^5$, $0.5x10^5$, $0.75x10^5$, $1x10^5$, $1.5x10^5$ and $2x10^5$. The lymphocytes were allowed to settle at unit gravity (10min) or centrifuged at low speed (200rpm) for 2 mins. The cells were then observed microscopically and the coverage of the culture plate surface by lymphocytes assessed. It was noted that some cells settled on the sloping sides of the wells and remained adherent there. This meant some cells were lost from the monolayer surface, but this could not be prevented. Cells on the sloping sides of the well were excluded from cell counts.

The most suitable cell density was observed with a total of $0.5x10^5$ Con A stimulated lymphocytes per well (5µl at $1x10^7$/ml). Cells applied at this density, and allowed to settle at unit gravity, formed a uniform blanket over the EC monolayers, with little or no accumulation of lymphocytes at the periphery of the wells.

Figure 6.1 Adherence of unstimulated and Con A stimulated LN lymphocytes to monolayers of synovial microvascular endothelial cells from arthritic rats.

The histogram shows adherence of lymphocytes to monolayers of microvascular EC. The plot shows a comparison of the adherence of unstimulated lymphocytes and lymphocytes that have been stimulated *in vitro* with Con A for either 24 hour or 48 hours. A count of adherent fluorescent lymphocytes was carried out from micrographs and results are expressed as mean cells per well (± SEM) (*significance). Each treatment group was replicated in 10 wells.


Lymphocyte-Endothelial Cell Adhesion
ConA stimulated & Normal Lymphocytes
Lymphocyte Adherence
5000
4000
3000
2000
1000
0
*
ConA 24h
ConA 48h
Unstimulated
Lymphocyte treatment

Centrifugation caused more lymphocytes to collect around the periphery of the wells.

6.3.2.3 Washing the monolayers to remove non-adherent cells.

For EC to adhere to the wells of the polystyrene Terasaki plates, the cells require a growth matrix. EC are commonly seeded onto wells coated with a gelatin matrix. In this study, the EC were found to adhere to this substrate. An efficient washing regime was necessary after seeding with purified EC to remove non-adherent cells from the wells without causing loss of adhered EC. In addition, following the lymphocyte incubation period, the washing procedure must also remove non-adherent lymphocytes only and allow accurate counting of adherent cells. During preliminary experiments, it was noticed that EC were lost from the central region of the wells, when the monolayers were washed to remove unbound lymphocytes. Loss such as this would lead to an uneven distribution of EC and lead to inaccuracy during adherence assays, where several washes are required. For these reasons, an optimal washing regime needed to be established.

6.3.2.4. Development of a washing protocol to remove non-adherent cells after seeding with purified EC.

EC were seeded in replicate wells onto two different growth substrates, gelatin or fibronectin (Section 2.6.5). The EC were allowed to adhere to the substrate, and 5 hours post-seeding, a number of different speeds of washing were tested. Washing was carried out using a modified disposable sterile saline drip infusion set. The sterile bag was filled with sterile warm (37°C) wash buffer (RPMI-1640 with 1% FCS). The bag was suspended 55cm above the bench surface and the flow standardised at 1, 2, 3 or 4 drops per second, or at a continuous flow. Washing was carried out with the Terasaki plate held vertically, in a standard retort stand. The wash buffer was introduced into the periphery of each well via a 19-gauge hypodermic needle. Each well was washed for a total of 20 seconds, the wash cycle being divided into 4 quadrants (5 seconds at each quadrant) to ensure even washing.

Both before and after washing, the culture surface of each well was examined for evenness of EC distribution, using phase microscopy. From preliminary experiments, it was noted

that any cell loss tended to be initially from the central region of the well, rather than from the periphery. Any unevenness in the distribution of EC post-washing was indicative of EC loss. Videoimage analysis of ethanol fixed, toluidine blue stained preparations of EC monolayers, was used to estimate the percent confluence of EC in the wells. Estimations were made on 5 replicate culture wells, comparing unwashed wells with each of the washing protocols. Three different areas were assessed and in each well a percentage area covered by EC was determined.

EC seeded onto the gelatin matrix were lost from the culture surface at washing speeds between 1 and 4 drops per second (Table 6.3.). Loss of EC from the gelatin matrix after washing ranged from 17% with 1 drop per second, over 60% of the EC at 4 drops per second, to nearly 90% loss with continuous flow of washing buffer. In the case of monolayers seeded onto fibronectin, no loss of EC was detected at wash speeds of between 1 and 3 drops per second. Loss of around 8% of EC was observed at a washing speed of 4 drops per second, increasing to approximately 13% with continuous washing. The EC remained firmly adhered to the fibronectin culture matrix throughout washing. It was concluded, therefore, that EC seeded onto fibronectin and washed at a speed of 2 drops per second for 20 seconds per well, was a suitable washing regimen for the establishing the EC monolayers.

6.3.2.5 Development of a washing protocol for removing non-adherent lymphocytes

Efficiency of washing for removal of excess CFSE labelled lymphocytes was assessed using inverted fluorescent microscopy. Peripheral and central regions of the well were compared for even distribution of single or small groups of fluorescent cells. Clumping, or retention of clusters of cells, was assessed as ineffective washing. It was not possible to carry out assessment of fluorescent cells with video-image analysis. Instead, a manual count was carried out from photomicrographs, as described in Section 2.15.2.

Washing in a continuous stream, or at drop rates of 4 per second resulted in loss of most lymphocytes from the central region of the well. A slower washing rate of 2-3 drops per

second resulted in retention of both EC and adherent lymphocytes. The majority of free lymphocytes appeared to be removed during washing at 2 drops per second, with just a few 'trapped' lymphocytes at the periphery of the culture well. These 'trapped' lymphocytes were observed as a higher density of retained fluorescent cells, compared with the rest of the culture well. It was assumed that these 'trapped' lymphocytes were not adhered to the EC, but had collected at the periphery.

The optimal washing speed of 2 drops per second, (with conditions as described in Section 6.3.2.4) was adopted for all subsequent experiments and assays. The retention of some lymphocytes at the periphery of the wells led to the exclusion of this region from calculations of adherent lymphocytes

6.3.2.6 The effect of protein concentration on adherence of lymphocytes to EC

A further series of preliminary experiments was carried out to investigate the effect of protein concentration on the adherence of lymphocytes to EC. The lymphocytes were incubated with EC monolayers in the presence of concentrations of FCS in the RPMI assay buffer of 0, 1, 10 and 20%. Differences in adherence were evaluated. Cell counts of adhered lymphocytes were made from photomicrographs (Figure 6.2). Statistical analysis of the data (Anova with post-hoc Tukeys test) revealed that no significant differences in the numbers of lymphocytes adherent to the EC monolayers in the absence of FCS. Consequently, FCS was included in the assays at a concentration of 1%.

6.3.3 Testing the assay: The blocking activity of a monoclonal antibody.

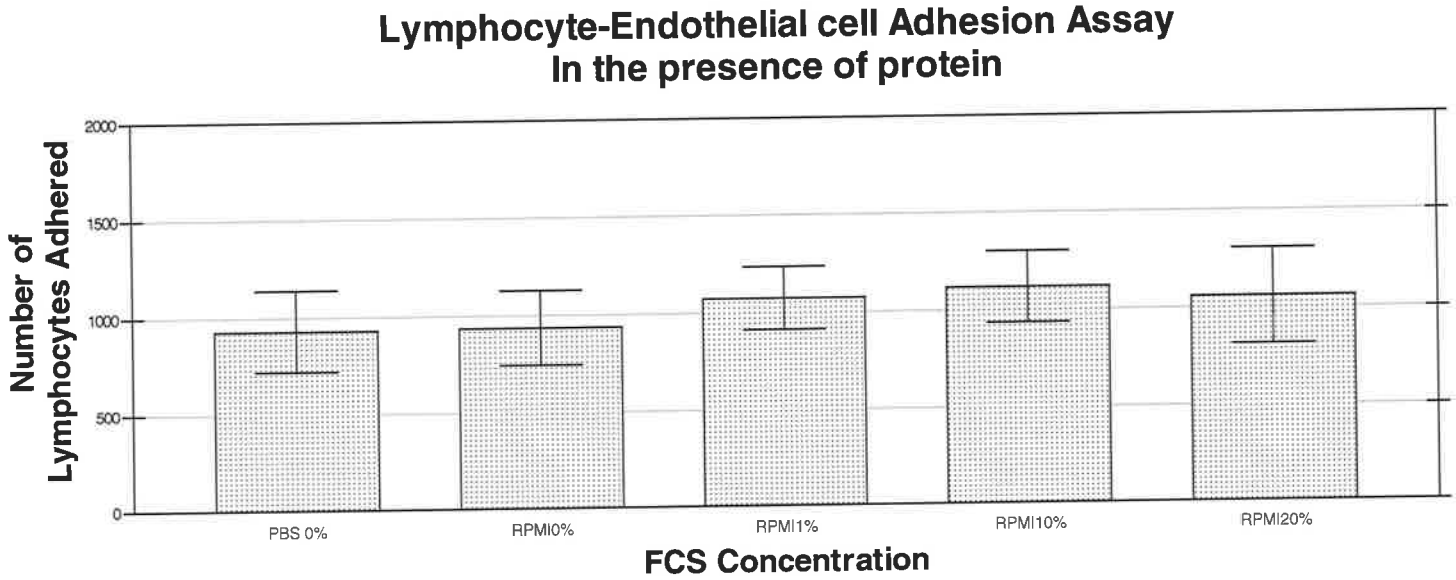
To distinguish whether the assay was a suitable tool to use for investigating the role of adhesion molecules in lymphocyte adhesion to synovial microvascular EC, experiments were carried out to investigate the blocking activity of mAb 1A29, (anti-ICAM-1, CD54). ICAM-1 binds the leukocyte β_2 integrin LFA-1 (CD11a/CD18) (Zimmerman *et al.,* 1992) and is known to be an important adhesion molecule in EC – T cell interactions (Shimizu, 1992). ICAM-1 has been shown to have a role in leukocyte migration to inflamed joints in rats with AA (Iigo *et al.,* 1991).

Table 6.3 Effects of matrix and washing rates on the retention of adherent EC in in Terasaki plates. Monolayers of EC were seeded into wells coated with gelatin or fibronectin matrices. Percent of the well surface covered with EC was determined using videoimage analysis. Five replicate cultures were seeded in EC growth medium and allowed to adhere (5 hours @ 37°C), then washed for 20 seconds at drop speeds of 1-4 drops per second or with a continuous flow. The results are expressed as the percent of the well base covered by EC relative to EC coverage without washing.

Percent Endothelial cell confluence (% retained)		
Drops/second	**Gelatin matrix**	**Fibronectin matrix**
0	100	100
1	83	100
2	58	100
3	33	100
4	33	92
Continuous flow	12	87

Figure 6.2 The effect of protein in the medium on lymphocyte adherence to EC.

Lymphocytes were incubated with monolayers of EC in either PBS or RPMI-1640 medium containing 0, 1 or 10% FCS. The results are expressed as the mean (±SE) adherent lymphocytes per well calculated from 10 replicate wells. No significant difference was detected in the number of adhered lymphocytes in the presence of 1, 10 or 20% FCS, when compared with RPMI-1640 or PBS alone.


Lymphocyte-Endothelial cell Adhesion Assay
In the presence of protein
Number of Lymphocytes Adhered
2000
1500
1000
500
0
PBS 0%
RPMI0%
RPMI1%
RPMI10%
RPMI20%
FCS Concentration

The adhesive interactions of unstimulated LN lymphocytes, and of LN lymphocytes stimulated for 24 hours with Con A, were investigated in the presence or absence of anti-ICAM-1, 1A29 mAb (50μg/ml). The assays were carried out under the conditions described in Section 2.15.2. CFSE labelled lymphocytes were prepared as described previously in Section 2.8. and added to the endothelial monolayer (preparation described in Section 2.13). Adherent cells were quantified (described in Section 2.15.3) and the results are shown in Figure 6.3.

In the presence of the mAb 1A29 (anti-CD54), the number of Con A stimulated lymphocytes that adhered to the EC monolayer was reduced by more than 50%, compared with adherence in wells without antibody. Statistical analysis of the data revealed a highly significant difference between these treatment groups ($p=0.00036$). In contrast, in the case of unstimulated lymphocytes, there was no significant difference in adherent lymphocytes in the presence or absence of the antibody ($p=0.977$) (Figure 6.3). Thus, the results indicated that the assay was sensitive to the blocking effects of a mAb against an adhesion molecule that is known to play an important role in the adhesion of lymphocytes to vascular endothelium.

6.3.4 Selection of CD4+ blast cells for use in assay

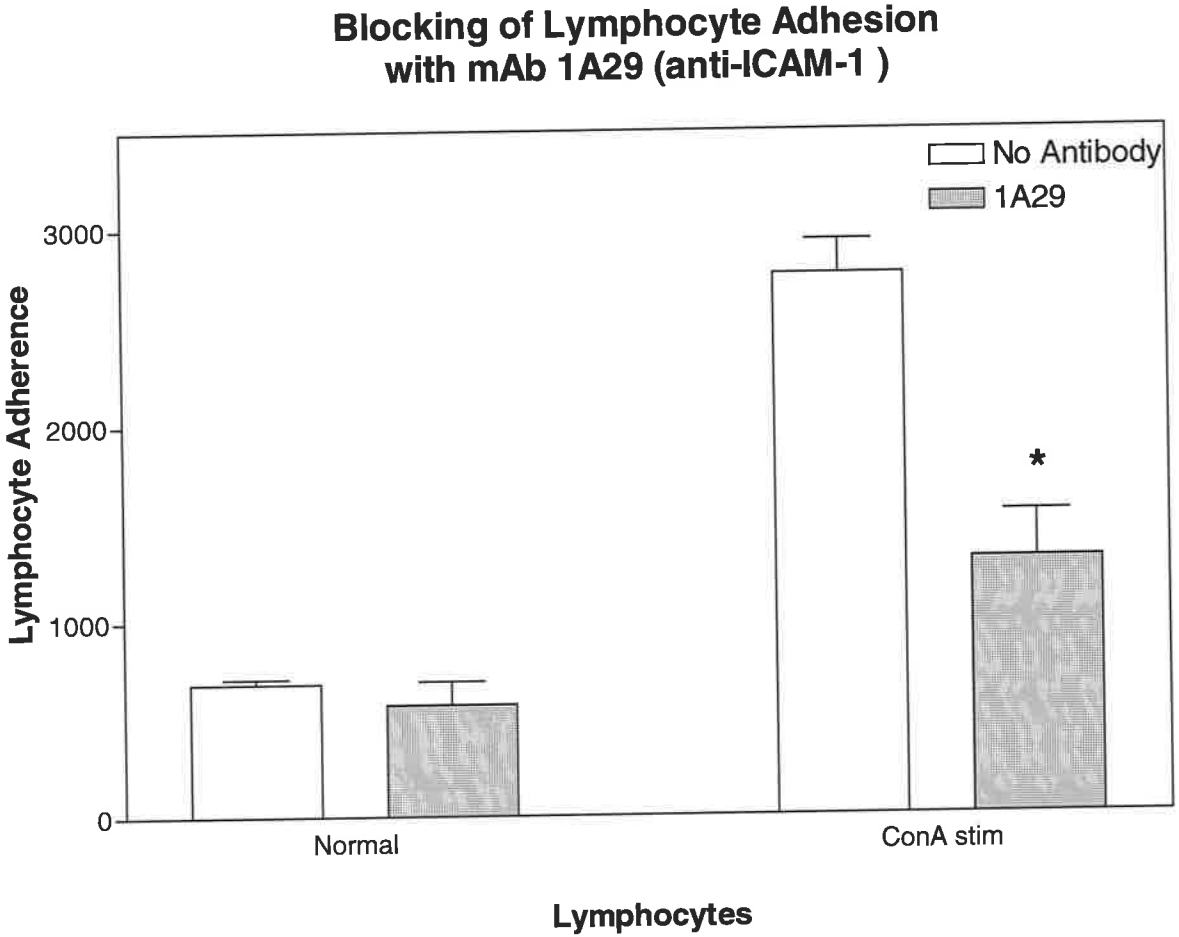
6.3.4.1 Enrichment of Lymphoblasts from TD lymph

The lymphocytes of key interest in the pathogenesis of AA belong to a population of activated CD4+ T cells that are present in the TD lymph of rats during the prodrome of AA (Spargo *et al.*, 2002). A method for the enrichment of CD4+ lymphoblasts was established and the surface expression of activation markers and adhesion molecules by these cells was analysed by flow cytometry.

Thoracic duct lymph was collected, as described in Section 2.9, from arthritic rats 9 days after inoculation with CFA. Blast cells were enriched using a discontinuous Percoll density gradient (as

Figure 6.3 Test of assay by blocking lymphocyte EC adherence with mAb 1A29

The effect of mAb 1A29, anti-ICAM-1 on the adherence of CFSE labelled normal LN cells and LN cells stimulated i*n vitro* for 24 hours with Con A to monolayers of microvascular EC. Lymphocytes (stimulated or unstimulated) were incubated with monolayers of EC in the presence or absence of the mAb 1A29 (50 μg/ml). Adherent cells were counted from photomicrographs of the cultures, prepared using an inverted fluorescence microscope. In the case of Con A stimulated lymphocytes, the presence of mAb 1A29 had a significant (*) effect on the number of lymphocytes adhering to EC ($p=0.00036$). There was no significant effect of mAb on adherence of normal LN lymphocytes.


Blocking of Lymphocyte Adhesion
with mAb 1A29 (anti-ICAM-1)
No Antibody
1A29
Lymphocyte Adherence
3000
2000
1000
0
*
Normal
ConA stim
Lymphocytes

described in Section 2.10.). Briefly, the gradient consisted of layers of 30, 40, 45, 50 and 55% isotonic Percoll. After the cells had settled to their isopycnic positions in the gradient during centrifugation, the cells at each interface were collected for analysis by flow cytometry to identify layers were enriched for blast cells. Blast cells were identified by their relatively large forward scatter and low to moderate side scatter of light (Figure 6.4).

Within the unseparated TD lymphocytes approximately 8% had the characteristic light scatter of activated lymphoblasts (Figure 6.4(a)). A similar percentage was found in the pellet, after centrifugation (Figure 6.4(b)). Analysis of cells found overlying the steps of 35, 40 and 45% Percoll revealed presence of 2-9% lymphoblasts (Figures 6.4(c), (d) and (e) respectively). Overlying the 50% and 55% steps, the proportion of blast cells had been enriched to 35% and 22% respectively (Figures 6.4(f) and (g) respectively). By pooling cells collected over the 50 and 55% steps, the proportion of lymphoblasts was approximately 34% (Figure 6.4(h)). The cells collected from above the 50% and 55% Percoll steps together totalled approximately 2 x 10^7 cells, representing the yield from TD lymph collected overnight from 4 donor rats in the prodromal phase of AA (Table 6.4).

6.3.4.2 Purifying a subpopulation of CD4+ lymphoblasts

The lymphocytes in TD lymph from rats 9 days after inoculation of CFA contained approximately 80% of cells that express α/β TCR+ (T cells) and of these approximately 87% were CD4+ T cells (see Section 5.3.1). To obtain a preparation enriched for CD4+ blasts prepared by Percoll density gradient centrifugation were depleted of B cells and $CD8^+$ cells by negative selection, using the Dynal Immunomagnetic bead system M450. The procedure is described in Section 2.15.1 with the exception that mAb OX1 was replaced with a cocktail of hybridoma supernatants containing mAbs OX8 (anti-CD8α) OX33 (anti-CD45, B cell specific isoform) and Mark-1 (anti-Kappa). This method removes B cells and CD8+ cells and it reliably produces CD4+ lymphocytes of 99% purity.

Evaluation of the expression of activation markers and adhesion molecules expressed by the purified CD4+ lymphoblasts was carried out by flow cytometry. The results are

summarised in Table 6.5. Almost one third of this population expressed KI67, the proliferation marker. Nearly all of the cells expressed adhesion molecule LFA-1 (CD11a and CD18) and approximately 10% expressed CD49d, few expressed CD11b. CD54 and CD44 were expressed by 25% and 99% of the CD4+ blasts, respectively. Approximately 14% expressed CD25 (IL-2R), compared with only 3% detected in unseparated TD lymphocyte (as a percent of the total TD lymph population). The proportion of cells expressing MHC class II (21%) was approximately 4 times higher than cells in unseparated TD lymph, (5%). Approximately 20% of cells expressed CD71.

6.3.4 Reproducibility of antibody blockade of adhesive interactions.

This assay was designed as a method to evaluate the ability of blocking antibodies to inhibit adherence to ECs by Con A stimulated lymphocyte and CD4+ lymphoblasts. The effects of the antibodies are expressed as the percentage of adhered lymphocytes in the presence of the antibody relative to that observed in the presence of assay buffer alone or an isotype matched negative control mAb.

Initially, counts of adherent cells were performed over an area equal to 80% of total surface area of the well. The counting technique was modified in the course of later studies by reducing the size of predefined area in which adherent cells were counted to an area equal to 40% of the well surface surface. This became necessary, because in the number of cells to be counted could range from 2000 to 3000 cells per well, requiring extremely long periods to complete the counting for each experiment. This change did not affect variation within experiments, although it understandably affected absolute counts of cells between experiments, in which areas counted were different.

Figure 6.4 Density gradient enrichment of lymphoblasts from TD lymph of rats in the prodromal phase of AA.

Thoracic duct lymph was collected overnight from rats, immunised 9 days earlier with CFA. The lymphocytes were pooled and centrifuged through density step gradients composed of 55%, 50%, 45%, 40% and 35% Percoll for 30 minutes at 1300rpm (400g). Cells overlying each density step and from the pellet were collected and analysed by flow cytometry, observing forward scatter (FSC) and side scatter (SSC) of light. Blasts were defined as cells with high FSC and low to moderate SSC. (A) unseparated or 'starting' population of TD lymphocytes. (B) Cells from the pellet. (C), (D) and (E), represent the cells collected overlying 35, 40 and 45% Percoll respectively. (F) and (G) cells overlying 50% and 55% density steps respectively. (H) pooled cells overlying the 50% and 55% density steps.

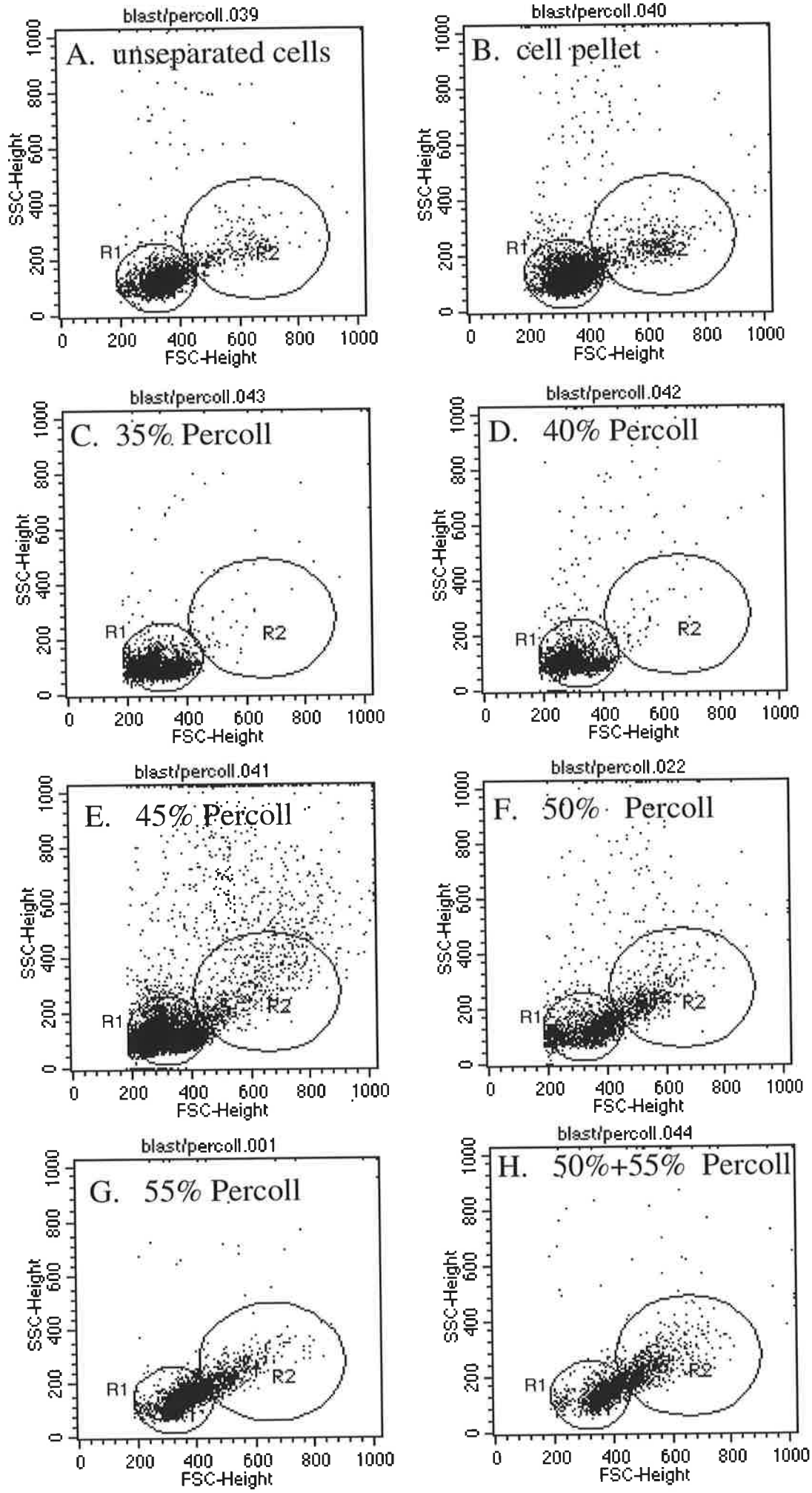

blast/percoll.039
A. unseparated cells
blast/percoll.040
B. cell pellet
blast/percoll.043
C. 35% Percoll
blast/percoll.042
D. 40% Percoll
blast/percoll.041
E. 45% Percoll
blast/percoll.022
F. 50% Percoll
blast/percoll.001
G. 55% Percoll
blast/percoll.044
H. 50%+55% Percoll
SSC-Height
FSC-Height
R1
R2
0
200
400
600
800
1000

Table 6.4 A summary of the recovery of lymphocyte and lymphoblast cell enrichment after discontinuous density gradient centrifugation of TD lymphocytes from rats in the prodrome of AA. TD lymph was collected overnight from 4 rats whose thoracic ducts were cannulated 9 days after inoculation of CFA. The washed lymphocytes were loaded on discontinuous Percoll gradients consisting of 35%, 40%, 45%, 50% and 55% Percoll and centrifuged (1300rpm, 400g) for 30 minutes. Cells over each density step were collected and analysed by flow cytometry. The percent of lymphoblasts was determined by analysis of forward and side scatter of light (see Figure 6.4).

Percoll Conc (%)	Cell count (x 10^7)	Lymphoblasts (%)
Total TDL	180.0 x 10^7	8
35	2.3 x 10^7	1.9
40	0.4 x 10^7	3.2
45	0.2 x 10^7	8.7
50	1.0 x 10^7	35
55	0.7 x 10^7	22
50 + 55	1.7 x 10^7	**34**
Pellet	100 x 10^7	8

By combining the methods for the Percoll enrichment of lymphoblasts with the negative selection of CD8+ and B cells, a suspension enriched for CD4+ T lymphoblasts was produced. These cells were then utilised in the assay to investigate their adhesive interactions with monolayers of freshly isolated EC from the microvasculature of arthritic rats.

Table 6.5 A summary of the results from flow cytometric assessment of activation markers and adhesion molecules, expressed on CD4+ lymphoblasts from TD lymph of arthritic rats. TD lymph harvested 9 day after inoculation of rats with CFA into the tail base. Blast cell enrichment was carried out using density gradient centrifugation followed by negative selection of CD8+ and B cells using Dynabeads. Comparison against the markers of proportionate expression specified can be made with unfractionated TD cells from day 9 AA rats and T cells from synovium of day 14 AA rats (Tables 5.2 and 5.3).

Adhesion molecule / Activation marker	TDL Percoll CD4+ blasts (%)
CD4	Approximately 100 %
CD25	14
KI67	31
CD71	20
MHC II	21
CD54	25
CD11a	89
CD18	100
CD11b	0.3
CD49d	12
CD44	99

6.3.4.1 Statistical Testing

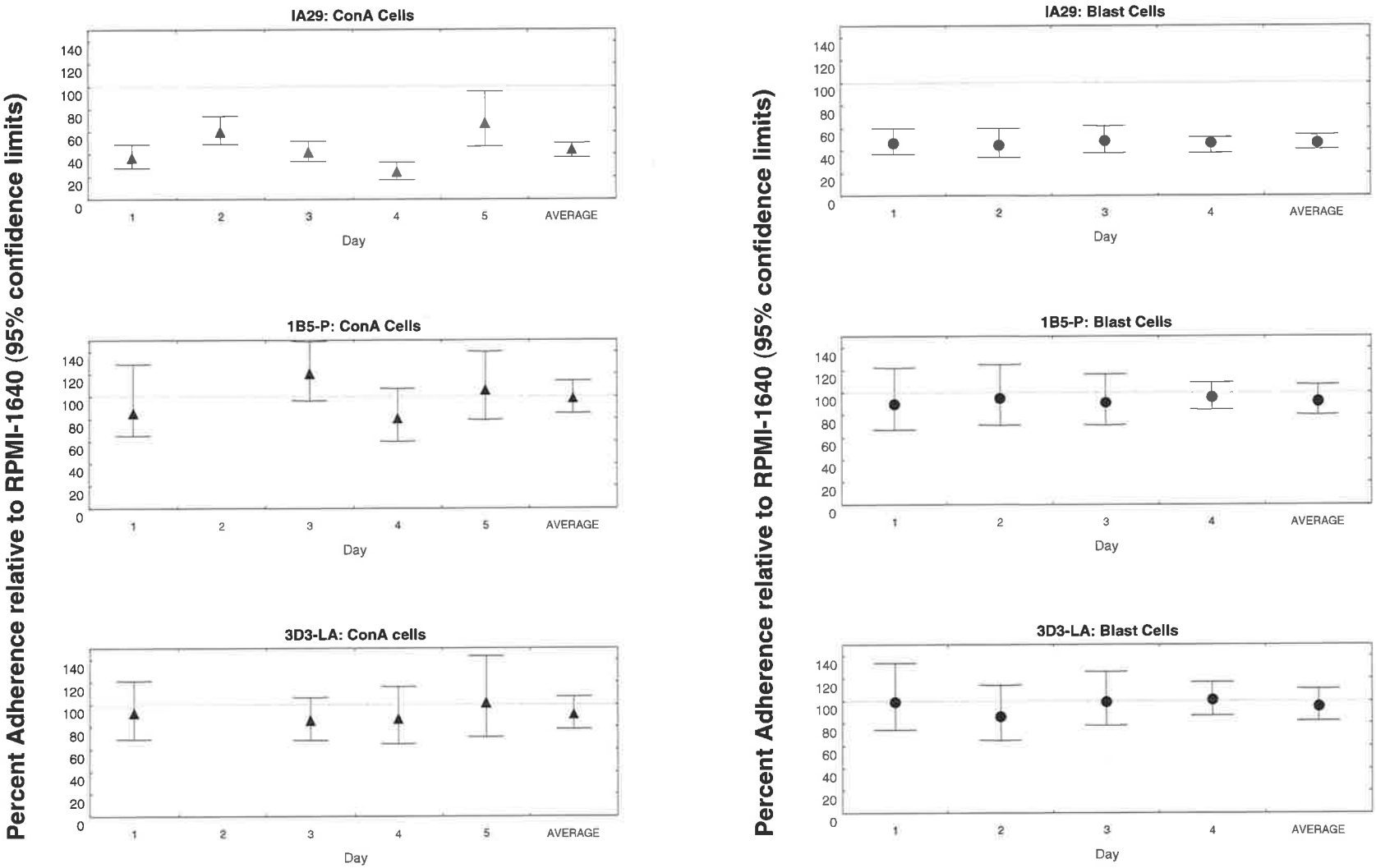
The results from 5 experiments using Con A blasts and 4 experiments using CD4+ lymphoblasts from TD lymph were analysed for inter-experiment variation in lymphocyte adherence (Figure 6.5). The data were analysed by Main Effects ANOVA (2-way) analysis performed in the General Linear Models module of Statistica Version 6. This method of analysis makes allowance for random effects in replicate experiments carried out on different days. The response variable was the $\log_e$ of the number of adherent cells and the two categorical predictor variables were "Antibody"(fixed effects) and "Day" (random effects). The regression coefficients from this analysis for each test antibody (with the coefficient for adherence in RPMI medium alone set to zero to avoid over-parameterisation) estimate a "common" effect (i.e. the same for each day) for each antibody. They actually represent the difference between the $\log_e$ (adherence) in the presence of the antibody and the $\log_e$ (adherence) in the presence of RPMI alone. Back transformation (i.e. exponential of the coefficient, upper and lower confidence intervals) is the ratio (with 95% confidence intervals (CI)) of the number of adherent cells in the presence of each antibody relative to adherence in RPMI alone. (95% CI for the regression coefficients were estimated by +/- 1.96*s.e).

It was noted from the raw data collected from replicate experiments carried out on separate days that there was some inter-experimental variability between experimental days. However, intra-experimental variation (replicate wells within an experiment) in data sets was not evident (see Figure 6.5). Data from experiments on different days, examining the effects of mAb 1A29 and two IgGl isotype negative control antibodies (mAbs 1B5 and 3D3) on lymphocyte adherence are shown in Figure 6.5. The inter-experimental variability appeared greater in the experiments using Con A stimulated lymphocytes, while there was little variability between experiments using CD4+ T lymphoblast cells. It is noteworthy that adherence of lymphocytes in the presence of either the control IgG1 mAbs was not significantly different to adherence in RPMI alone. The RPMI and the control IgG1 mAbs were included in each experiment, however, adherence in RPMI medium was used as the reference for the effect of antibodies on lymphocyte adherence.

Figure 6.5 Inter-experimental variability in lymphocyte adhesion assays.

Plots show the mean and 95% confidence limits, calculated from the raw data collected from 3 replicate wells per experiment. The individual experiments were carried out on separate days and the results are expressed as the percentage of lymphocytes bound in the presence of antibody relative to binding in the presence of buffer alone. Inter-experimental variability can be assessed by comparing the results from individual replicate experiments with the mean (average) of all of the experiments. Reproducibility of inhibition of adhesion by a mAb was assessed using mAb 1A29 (anti-ICAM-1) and compared with the effects of two IgGl isotype negative control antibodies (1B5 and 3D3). Data are presented utilising Con A stimulated LN lymphocytes (left) and CD4+ T lymphoblasts (right). The inter-experimental variability appeared greater in the experiments using Con A stimulated lymphocytes. There was no significant effect of control IgG1 mAbs on lymphocyte adherence but mAb 1A29 reproducibly inhibited binding of both Con A blasts and CD4+ T lymphoblasts from TD lymph.

Inter-Experimental Variability in Adhesion Assays



6.4 Discussion

Each of the experiments in this chapter investigated one of the variables in the lymphocyte adherence assay. The first variable investigated was the type of T lymphocyte that could be utilised in the assay. Analysis of the cell surface phenotypes expressed by lymphocytes that had been stimulated for different lengths of time with Con A revealed that a range of activation markers and adhesion molecules were upregulated. As anticipated, the cell surface expression of these molecules changed with the duration of Con A stimulation. Cells stimulated for 24 hours had the adhesion molecule expression profile that most closely matched that of T cells isolated from arthritic synovium.

Initially using lymphocytes that had been stimulated for 24 hours with Con A, conditions were established that distinguished clearly the adhesion of Con A activated lymphocytes from the adhesion of unstimulated LN cells. Although LN cells stimulated with Con A for 48 hours showed a generally high level of adhesion molecule expression than cells stimulated for 24 hours. Their adhesion to EC monlayers was similar.

A method was also established for the isolation of CD4+ T lymphoblasts from TD lymph obtained from rats during the prodromal period of AA. Analysis of the surface antigen phenotypes of these cells was carried out. The profile of adhesion molecules expressed by these blasts resembled closely T cells accumulating in arthritic synovium.

The expression of the LFA-1 was similar to that detected on cells from the inflamed joint. CD11a/CD18 (LFA-1) was expressed at high levels by TD T lymphoblasts, consistent with the involvment of the molecule in the early stage of the cascade of events leading to successful migration to inflammatory sites. LFA-1 is involved in the initial tethering of the leucocytes to ECs, thus initiating rolling prior to firm adhesion.

This finding illustrates the potential usefulness of the adherence assay to examine the effects of mAbs on the adherence of lymphocytes to endothelium. The other variables that

were examined were the density at which lymphocytes were seeded, a suitable EC growth matrix, pre- and post- adhesion wash protocols and the effects of protein concentration in the assay buffer.

In summary, the studies show that microvascular EC can be isolated from arthritic synovium and cultured in monolayers *in vitro*. The findings show that these cells display functional integrity, particularly in regard to preferential adhesive interactions with activated compared to unstimulated lymphocytes. Furthermore, adhesion was shown to be sensitive to blockade of a cognate interaction (between ICAM-1 and its ligand) known to be involved in lymphocyte recruitment to synovium. Adhesion of enriched TD lymphoblasts from arthritic rats indicated that T cells activated *in vivo* during the pathogenesis of AA display adhesive interactions with synovial microvascular endothelium, the cells with which they must interact *in vivo* in order to enter synovium (Cleland *et al.*, 2002).

The experimental system developed is thus suitable for dissection of adhesive events involved in recruitment of activated lymphocytes to inflamed synovium. While more convenient sources of EC and lymphoblasts may be available for screening new reagents, their relevance to lymphoblast recruitment to synovium is questionable. The system described has the advantage of utilising both synovial microvascular EC and lymphoblasts from animals with arthritis. Acceptable levels of reproducibility were achieved, with the caveat that absolute numbers of cells adhering between experimental preparations. However, the proportion of adherence attributable to the target adhesion molecule (ICAM-1) was reproducible between experiments.

Chapter 7

Effects Of Antibodies Against Known And Unknown Adhesion Molecules On Adherence of Lymphocytes To Endothelial cells from Synovial Microvasculature.

7.1 Introduction

The role of endothelium as the gatekeeper regulating entry of lymphocytes into extra-vascular tissues is complex. Activated lymphocytes adhere strongly to inflamed endothelium and play a critical part in the inflammatory process (Osborn, 1990). The interaction of lymphocytes with endothelium and their adherence is the first step in a cascade of events that commences with rolling, activation and firm adhesion and is followed by transendothelial migration at sites of inflammation. A number of different molecules are involved in this interaction of lymphocytes with EC (Butcher and Picker, 1996; Springer, 1994). Molecules from all five of the adhesion molecule families (Section 1.6) are involved at some stage of this complex and finely controlled sequence of events.

The interaction of adhesion molecules on lymphocytes and EC leads initially to rolling of leucocytes along the surface of the vascular endothelium. Members of the selectin family mediate this process. Later in the sequence of events, members of the integrin and immunoglobulin superfamilies are responsible for high affinity interactions that tether the lymphocyte to the EC, resisting the shear forces of the flow through the blood vessels.

The conformational change in lymphocyte-expressed integrins that is responsible for this high-affinity binding to complementary Ig-superfamily ligands is due to the effects of chemokines displayed on the EC apical surface and their signalling via complementary chemokine receptors on the lymphocyte surface membrane (Grabovsky *et al.*, 2000; Peled *et al.*, 1999) (see Section 1.6.2). The integrins, VLA-4 and LFA-1 and the immunoglobulin superfamily adhesion molecules, ICAM-1, ICAM-2, VCAM-1 and Mad-CAM play a role

here (Springer, 1995), depending on the vascular bed under consideration. The final stage of extravasation of lymphocytes through the intercellular junctions between EC appears to involve interactions with the Ig superfamily member PECAM-1.

Agents that are able to block interactions between adhesion molecules and their ligands on lymphocyte and ECs inhibit adhesion and migration of lymphocytes to inflamed tissues (Springer, 1994; Issekutz, 1992; Oppenheimer-Marks *et al.,* 1991; Haskard *et al., 1986*). Studies in rats have shown that β2 integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and the B1 integrin VLA-4 (CD49d) play a role in the development of rat AA (Issekutz *et al.,* 1996; Barbadillo *et al.,* 1995). Blocking P- and E-selectin was shown to inhibit the onset of AA. LFA-1 appears to be an important mediator of T lymphocyte migration in inflammatory reactions in the skin, but anti-LFA-1 treatment alone did not inhibit T cell migration in inflamed joints in AA (Issekutz & Issekutz, 1991).

Monoclonal Abs against known adhesion molecules have been investigated for their effects on lymphocyte adhesion and/or migration and the results have been interpreted as indicating that the particular adhesion molecules are involved to varying degrees at different sites and in different causes of inflammation. These studies have utilised various established *in vivo* or *in vitro* models. Previous *in vitro* studies have investigated adhesion of lymphocytes to EC derived from large vessels, such as the rat aorta or the human umbilical cord (Roth *et al.*, 1995). Relatively few studies have used EC from the microvasculature.

The present study was designed to investigate adhesion molecule interactions utilising EC prepared from microvasculature of joints affected by AA in the rat. Con A stimulated lymphocytes and preparations enriched for CD4+ lymphoblasts from TD of rats in the prodrome of arthritis were used as the source of activated T cells. A number of available mAbs known to react with defined adhesion molecules have been tested for their ability to block adhesion in the assay described in Chapter 6. In addition, the assay has been used to examine the activities of a number of mAbs raised against inflamed rat synovium and showing specificity for EC, for their ability to modify adhesion of these lymphocytes to

synovial microvascular endothelium. These mAbs were selected by their staining of vascular endothelium in immunohistochemical studies using tissue sections as the source of EC.

7.2 Hypotheses and aims

7.2.1 Hypotheses:

- Monoclonal Abs against certain defined adhesion molecules will block adhesion of Con A stimulated LN lymphocytes to monolayers of microvascular EC from inflamed synovium.

- Monoclonal Abs against these adhesion molecules will block adhesion of cells in preparations, enriched for CD4+ lymphoblasts from TD lymph of rats in the prodrome of AA, to monolayers of microvascular EC from inflamed synovium.

- Inoculation of mice with cells from inflamed synovium will raise antibodies against rat synovial ECs, some of which will detect synovium-specific vascular addressins.

- Some EC-specific antibodies raised against inflamed synovium will block adhesion of Con A stimulated LN lymphocytes to monolayers of microvascular EC from inflamed synovium.

- These EC specific antibodies raised against inflamed synovium will block adhesion of cells in preparations enriched for CD4+ lymphoblasts from TD lymph of rats in the prodrome of AA, to monolayers of microvascular EC from inflamed synovium.

7.2.2 Aims:

- To investigate, using a panel of mAbs to defined adhesion molecules, the role of adhesion molecules in interactions between EC from inflamed synovial microvasculature and LN lymphocytes stimulated *in vitro*.

- To investigate, using a panel of mAb to defined adhesion molecules, the role of adhesion molecules in the interactions between EC from the inflamed synovial microvasculature and CD4+ lymphoblasts in TD lymph from rats in the prodrome of AA, using a range of existing anti-adhesion molecules specific mAbs.

- To raise antibodies against cells from inflamed synovium, in order to generate novel mAb that detect synovium-specific vascular addressins.

- To investigate the ability of mAb that bind to rat synovial microvasculature EC to affect adhesion of Con A stimulated LN lymphocytes to EC from the microvasculature of inflamed synovium.

- To investigate the ability of mAb that bind to rat synovial microvasculature endothelium to affect adhesion of CD4+ lymphoblasts from TD lymph of rats with AA to EC from the microvasculature of inflamed synovium.

7.3 Results

The lymphocyte–EC adhesion assay was carried out as described in Section 2.15.2. The Abs tested were either against known adhesion molecules or they were (as yet uncharacterised) culture supernatants from uncloned hybridomas that displayed reactivity with rat microvascular endothelium. The assay utilised EC obtained from the microvasculature of inflamed synovium (Section 2.13). Lymphocytes were either Con A-activated LN cells (Section 2.8) or CD4+ lymphoblasts obtained from TD lymph during the prodrome of AA. The latter were enriched from TD lymphocytes using density gradient separation (Section 2.10.1), followed by negative selection of CD4+ cells with immunomagnetic beads (Section 2.15.1).

In the experiments, aliquots of lymphocyte suspensions were pre-incubated with antibodies that are known to bind to lymphocyte cell surface adhesion molecules (eg L-selectin) before

they were added to the wells containing EC monolayers. In the case of adhesion molecules that are expressed by EC, or Abs reactive with unknown antigens expressed by EC, the antibodies were incubated with the EC before the addition of lymphocyte. In all experiments the antibody being investigated was present throughout the assay. Each antibody was tested in triplicate and the experiments were repeated at least 3 times, except in the case of TD lymph CD4+ T lymphoblasts, where the experiments were carried out twice.

7.3.1 The effects of monoclonal antibodies against known adhesion molecules on the adhesion of T lymphocytes to EC from synovial microvasculature.

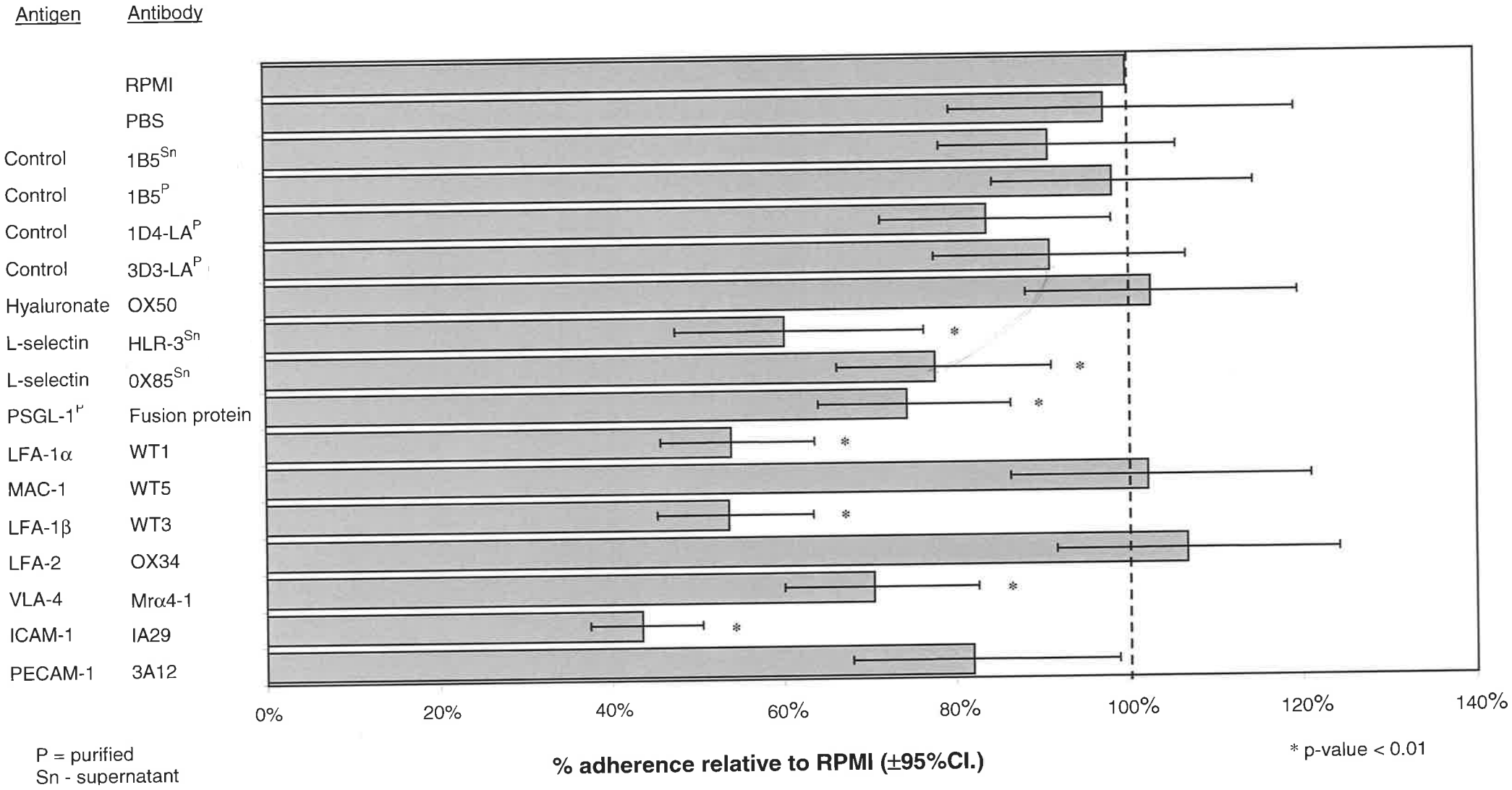
The antibodies investigated in this part of the study were directed against PECAM-1 (TLD-3A12), ICAM-1 (1A29), L-selectin (HLR-3 and OX85), LFA-1 (WT1), common chain of the β_2 integrins (WT3), Mac-1 (WT5), LFA-2 (OX34) and the hyaluronate receptor CD44 (OX50). P-selectin ligand (PSGL-1) fusion protein was also included. The mAbs were of either the IgG_1 or IgG_{2a} isotype. For comparison, two IgG1 (1B5 and 3D3) and one IgG_{2a} (1D4) negative control mAbs were incorporated, in addition to negative controls consisting of culture medium RPMI and PBS. Adherence in the presence of antibody was expressed as a percent of adhesion in the presence of RPMI.

7.3.1.1 The effects of specific antibodies on the adhesion of Con A stimulated lymphocytes to EC monolayers.

Statistical analysis showed a significant reduction of lymphocyte adherence in the presence of several of the mAbs (Figure 7.1). In the presence of mAbs against ICAM-1 (1A29), LFA-1 (WT1), common chain of the β_2 integrins (WT3), VLA-4 (Mrα4-1), L-selectin (OX85 and HLR-3) and also in the presence of P-selectin ligand (PSGL-1) soluble fusion protein, there was a significant reduction of adherence of between 25 and 56 % (Figure 7.1).

Figure 7.1 The effect of mAbs with known specificity against adhesion molecules on the adherence of Con A stimulated LN lymphocytes to endothelial cells from microvasculature of arthritic joints.

Monolayers of EC were prepared from the microvasculature of severely inflamed joints 13 days after inoculation with CFA. Lymph node cells from normal rats were stimulated *in vitro* with Con A for 24 hours prior to use in the assay (see Methods; Section 2.8.2). Test antibodies were present throughout the adhesion assay. The number of lymphocytes adhering to the monolayers, in the presence of the mAb against specified adhesion molecules was compared with adherence in control wells that contained assay medium alone. Main Effects ANOVA analysis was used to calculate the regression coefficients for each antibody, with the coefficient for RPMI (assay buffer) set at zero. Adhesion expressed as a percentage, was calculated from the lymphocytes adherent in the presence of antibody relative to the total number of adherent lymphocytes detected in the absence of antibody. Upper and lower 95% confidence intervals (calculated as +/- 1.96*standard error) are presented as error bars. Error bars for each antibody were calculated from the mean (replicate wells) percentage of lymphocyte adherence obtained from replicate experiments. Statistically significant inhibition of adherence is indicated by * on the histogram plot.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RPMI
PBS
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Control 1B5P
Control 1D4-LAP
Control 3D3-LAP
Hyaluronate OX50
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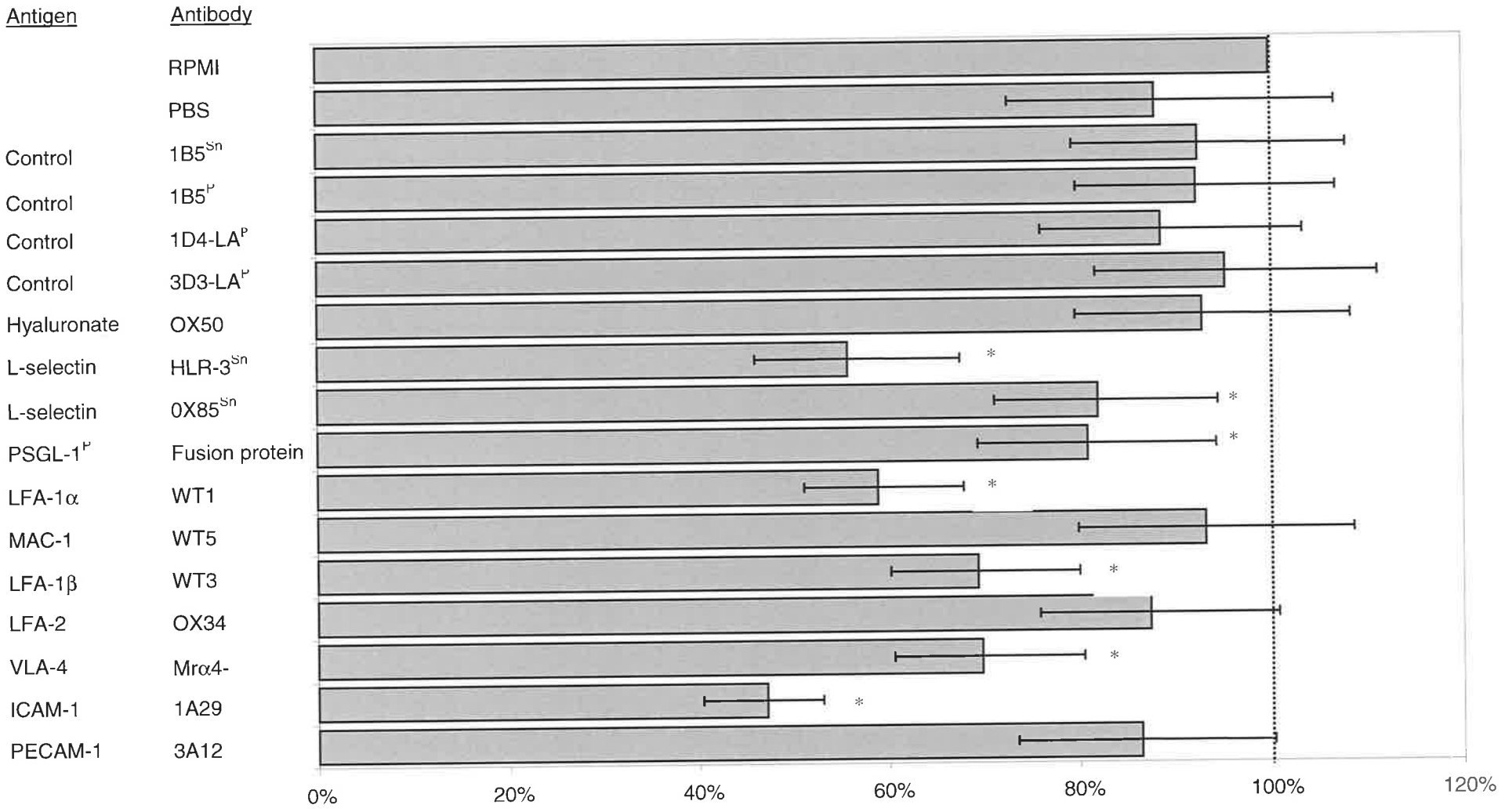
L-selectin 0X85Sn
PSGL-1P Fusion protein
LFA-1α WT1
MAC-1 WT5
LFA-1β WT3
LFA-2 OX34
VLA-4 Mrα4-1
ICAM-1 IA29
PECAM-1 3A12
0%
20%
40%
60%
80%
100%
120%
140%
P = purified
Sn - supernatant
% adherence relative to RPMI (±95%CI.)
* p-value < 0.01

Figure 7.2 The effect of mAbs with known specificity against defined adhesion molecules on the adhesion of CD4+ lymphoblasts from TD lymph of arthritic rats to EC from microvasculature of arthritic joints.

Monolayers of EC were prepared from the microvasculature of severely inflamed joints 13 days after inoculation with CFA. CD4+ lymphoblasts were isolated from TD lymph obtained from rats in the prodrome of AA, just prior to use in the assay (Section 2.15.2). Antibodies were present throughout the adhesion assay. The number of lymphocytes adhering to the monolayers in the presence of the specified mAb against adhesion molecules was compared with adhesion in control wells that contained assay medium alone. Main Effects ANOVA analysis was used to calculate the regression coefficients for each antibody, with the coefficient for RPMI (assay buffer) set at zero. Adhesion expressed as a percentage was calculated from the lymphocytes adherent in the presence of test antibody relative to the total number of adherent lymphocytes detected in the absence of antibody. Upper and lower 95% confidence intervals (calculated as +/- 1.96*standard error) are presented as error bars. Error bars for each antibody were calculated from the mean (replicate wells) percentage of lymphocyte adherence obtained from replicate experiments. Statistically significant inhibition of adherence is indicated by * on the histogram plot.


Antigen
Antibody
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PBS
Control
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Control
1B5P
Control
1D4-LAP
Control
3D3-LAP
Hyaluronate
OX50
L-selectin
HLR-3Sn
L-selectin
0X85Sn
PSGL-1P
Fusion protein
LFA-1α
WT1
MAC-1
WT5
LFA-1β
WT3
LFA-2
OX34
VLA-4
Mrα4-
ICAM-1
1A29
PECAM-1
3A12
*
0%
20%
40%
60%
80%
100%
120%
P = purified
Sn - supernatant
% adherence relative to RPMI (±95% CI.)
* p-value < 0.01

The mAbs against LFA-2 (OX34), CD44 (OX50) and Mac-1 (WT5), had no detectable inhibitory effect on lymphocyte adherence relative to control wells containing assay buffer alone.

7.3.1.2 The effects of antibodies on the adhesion of CD4+ lymphoblasts from arthritic donors.

The mAbs against ICAM-1 (1A29), LFA-1 (WT1) or common chain of the β_2 integrins (WT3), VLA-4 (Mrα4-1), L-selectin (OX85 and HLR-3) and the P-selectin ligand (PSGL-1) fusion protein, all produced a significant reduction in adherence of CD4+ T lymphoblasts of between 19% and 40% (Figure 7.2). No significant differences were observed in adherence in the presence of antibodies against LFA-2 (OX34), CD44 (OX50), Mac-1 (WT5) or PECAM-1 ligand.

7.3.2 Supernatants from uncloned hybridomas that stain endothelial cells.

The antibodies investigated in this part of the study were the products of uncloned hybridomas that were produced by fusion of spleen cells (Section 2.14) from mice immunised with a cell suspension obtained by enzymatic and physical disaggregation of inflamed rat synovium with mouse myeloma X63 cells (Section 2.13). The culture supernatants are referred to hereafter by the plate and well number (eg. antibody 3C3; plate 3, well C3).

7.3.2.1 Hybridoma selection

Wells that contained antibodies reactive with vascular endothelium were identified by screening on frozen sections of rat tissues using immunoperoxidase technique (Section 2.4.3). The tissues used for screening were normal rat lymph node, normal small intestine and inflamed synovium from arthritic rats. A summary of the results of the distribution of staining on vascular and non-vascular structures are found in Table 7.1.

After identifying culture supernatants that contained antibodies binding to EC, the next step was to examine whether the antibodies affected the adhesion of lymphocytes to monolayers of microvascular EC in the adherence assay described in Section 2.15.2. The culture

supernatants listed in Table 7.1 were tested for their effects on adherence of Con A activated LN cells and CD4+ T lymphoblasts from the TD lymph of rats in the prodromal phase of AA. The results described below are from an initial screening in the adhesion assay.

7.3.2.2 The effect of EC specific hybridoma supernatants on the adhesion of Con A stimulated lymphocytes from peripheral lymph nodes.

Each of the 34 antibodies was added to the wells containing EC monolayers. The antibody being investigated was present throughout the assay. Each antibody was tested in duplicate and the assay was repeated. Statistical analysis showed that 32 of the antibody supernatants produced no significant reduction of lymphocyte adherence. However, the culture supernatants designated 3C1 and 4C4 produced significant blocking of adherence of the stimulated lymphocytes, with mean reductions in adherence of 52% and 51% respectively (Figure 7.3).

7.3.2.3 The effect of EC specific hybridoma supernatants on the adhesion of CD4+ lymphoblasts from arthritic donors.

Aliquots of CD4+ lymphoblasts suspended in culture supernatants from the 34 uncloned hybridomas were added to the wells containing EC monolayers. The antibody was present throughout the assay and each culture supernatant was tested in triplicate. Results are shown in Figure 7.4.

Statistical analysis showed a significant reduction of lymphocyte adhesion only in the case of supernatant 3C1. In the presence of this supernatant, the adhesion of CD4+ lymphoblasts was reduced by 45%, when compared with lymphocyte adherence in the absence of antibody. It is important to note that this antibody supernatant also inhibited adhesion of Con A stimulated lymphocytes (Section 7.3.2.2). Interestingly, culture supernatant 4C4, which inhibited adhesion of Con A activated LN cells at a level similar to supernatant 3C1, had no significant effect on the adherence of CD4+ lymphoblasts from TD lymph of rats in the prodrome of AA.

Table 7.1 Staining of tissue with antibodies produced by hybridoma supernatant. Supernantants produced by immunisation of mice with cells from arthritic synovium (sub-Achilles tissue). Summary of results from immunoperoxidase screen on 3 different tissues. Fresh frozen sections of arthritic synovium, normal peripheral lymph node and small intestine (ileum) from rats. Shows the distribution of staining on vascular and non vascular structures.

Inflamed synovium			**Other tissues (normal)**	
Ab	**Vasculature**	**Non-vascular**	**Lymph Node**	**Small intestine**
1A5	small vessels		Subcapsular sinus	
1A6	small vessels, epimysium	CT PMN, Mast		
1B2	widespread		HEV	
1B3	artery	MNC,Mast	HEV	PP, ECs, Paneth
1B4	vein	CT, nerve	HEV-(weak)	Not vaculature
1B5	widespread -pale	PMN, Mast, MAC	CT capsule	Interfollicular vessels lymphocytes
1C2	widespread	mast, nerve, inflammatory cells	CT, EC(pale), HEV (pale)	HEV (pale), LP (diffuse)
1C3	small vessels (CD36-like)	PMN, MAC, adipocytes		
1C5	small vessels,	PMN, nerve, MNC	CT	BB, nerve, smooth musc-vessels
2A6	capillaries,		EC, (arterty), CT	EC, CT, smooth musc, enterocyte-(basal)
2B2	widespread	Mac	DC	BB, LP-(diffuse)
2B4	widespread	nerve, smooth muscle, Mast, FB.	CT, HEV	
2B5	widespread	Mast, nerve, PMN, MNC, CT(Bkgd)		
2C2	Few vessels (pale)	Inflammatory cells	DC	BB, enterocytes, few cells in LP
2D2	veins	–	Medullary cord, HEV-(weak)	BB, nerve, smooth muscle(capillaries)
2D3	widespread	nerve	Blood vessels, HEV,	
2D5	widespread	CT, nerve	HEV, CT	

Table 7.1 continued...

Abbreviations

FB- fibroblast (discrete cells)
CT- connective tissue (general stain)
Mast- mast cell
MAC-macrophage
PMN- polymorphonuclear cell
DC- DC
HEV- high endothelial venule
MNC- mononuclear cell
LP- lamina propri
PP- Peyer's patches
Musc- muscle
BB- brush border

Inflamed synovium			Other tissues (normal)	
Ab	**Vasculature**	**Non-vascular**	**Lymph Node**	**Small intestine**
3B4	small vessels	mast cells	HEV	PP-HEVs, myocytes
3C1*	Widespread	CT, PMN, Monocytes	CT	Enterocyte,BB, CT
3D2	widespread	Mac,	EC sm vessels	Sm vessels in LP, PP HEVs
4A5	Widespread		EC-(in CT),	
4B3	Widespread	FB, Mac, nerve, Syn-intima-typeB	CT	PP, sm musc, LP-diffuse
4C3	Widespread	mast Mac,	CT, HEV (weak),	not EC, not PP HEV
4C4*	Widespread	mast	EC, not HEV mast	BB only
4C5	Widespread		FB	lacteal
4D1	Widespread		HEV (strong)	EC (artery)
4D3	Widespread	CT, FB, nerve, synovial intima, mast PMN		PP-IF, BB
5A4	arterioles, venules (wk)			
5B1	widespread (weak),	PMN,Mast, MAC	WK HEV, DC, MAC	ENTEROCYTES,GP BLET , MAC, NOT PP HEV
5B4	Widespread	FB	CT,WK EC , NOT HEV	WK DIFFUSE BACKGR
5C1	Widespread	FB		
5C2	capillaries, veins, not arterioles,	Mast, MAC	CAPSULE, MAST	CT LAMINA PROPRIA, PP INTERFOLLICULAR, HEV
5C3	Widespread	MAC		MAC IN LP
5C4				
5D1	large vessels, venules, arterioles,	MAC, PMN(wk), tendon sheath	CT capsule, EC (weak) not HEV	BB
5D4	Widespread	nerve,CT, Mac, FB (tendon)	CT capsule, MAC, EC, HEV(weak)	LP (diffuse), HEV (weak)
5D6	Widespread	nerve	EC, Nerve,	Smooth muscle, CT, HEV-(basal)

7.3.2.4 Tissue distribution of EC specific hybridoma supernatants 3C1 and 4C4.

The hybridoma 3C1 showed a widespread distribution of binding to vascular structures, including EC of small and large calibre vessels in inflamed synovium. Antibody 3C1 also bound to components of loose connective tissue, polymorphonuclear cells and monocytes within the sections of inflamed synovial tissue. It also stained the connective tissue capsule and septa of normal LN and lamina propria of small intestine. Enterocytes and the associated brush border on the apical aspect of the small intestine are stained positive.

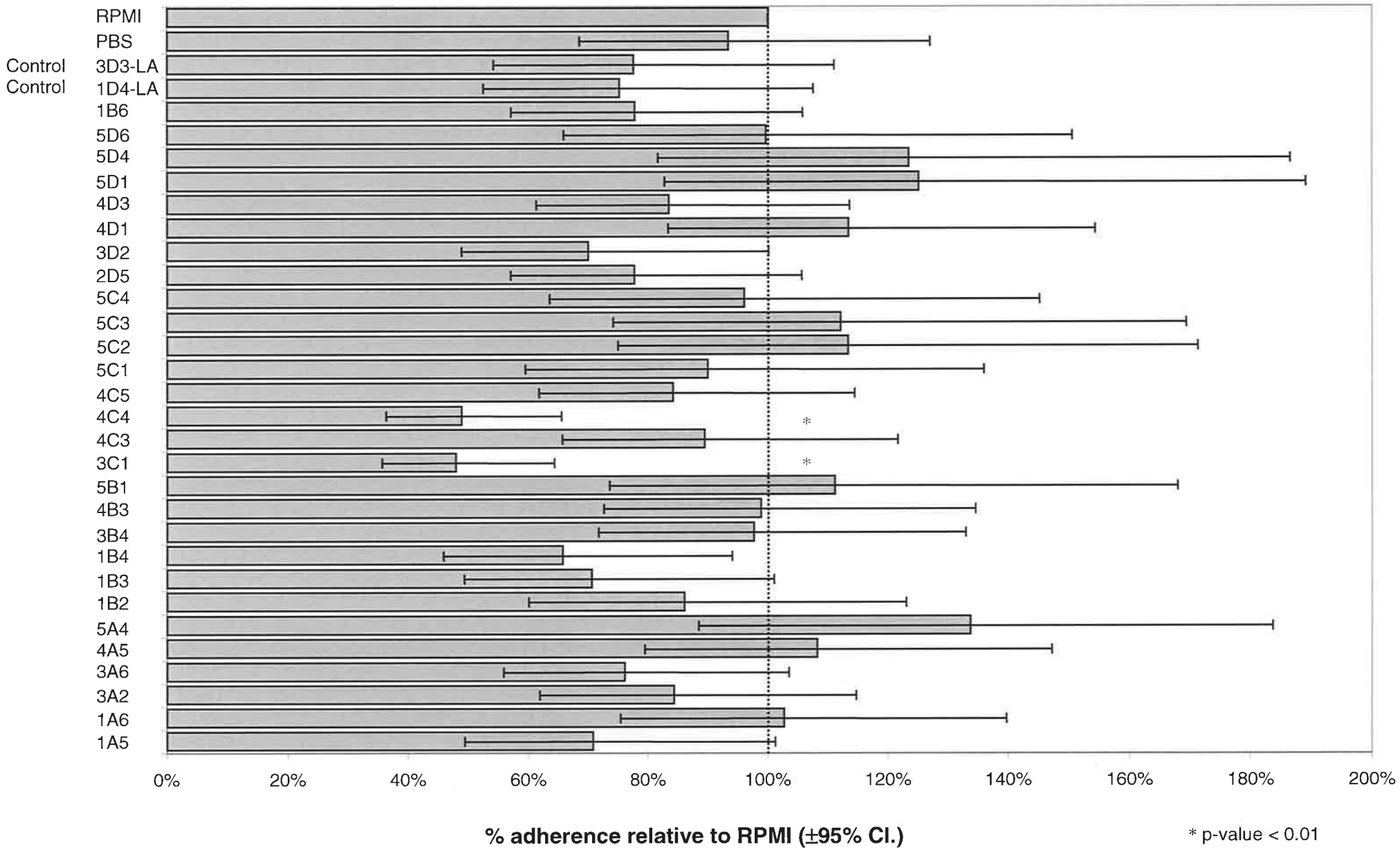
Similar to 3C1, 4C4 shows widespread distribution on vascular structures in synovium, including EC of small and large calibre vessels. It also stains mast cells in synovium and LN. In normal LN tissue, 4C4 stained vascular endothelium, however, the endothelium lining HEVs was not stained. Within small intestine, only the brush border of enterocytes stained.

7.4 Discussion

The present study utilised an experimental system that allowed direct investigation of the adhesion of activated lymphocytes to microvascular EC from the inflamed synovium of rats with AA. This is the first study to investigate lymphocyte adhesion to viable microvascular EC from arthritic rat joints. Such studies have not been carried out previously because of the difficulties of purifying the microvascular EC population from rat joints in adequate number and purity. Furthermore, despite the small numbers of cells available, it has been possible to examine freshly isolated cells (without growth *in vitro*) by miniaturisation of the assay system. Using this miniaturised system, we have shown partial blocking of lymphocyte adhesion with mAbs against several known adhesion molecules. In addition, partial inhibition of lymphocyte adhesion was detected with anti-endothelial antibodies contained in two of the uncharacterised hybridoma culture supernatants.

Figure 7.3 The effect of antibody supernatants on the adherence of Con A stimulated LN lymphocytes to EC from microvasculature of arthritic joints.

Monolayers of EC were prepared from the microvasculature of severely inflamed joints 13 days after inoculation with CFA. Lymph node cells from normal rats were stimulated *in vitro* with Con A for 24 hours prior to use in the assay (see Methods; Section 2.8.2). Antibodies were present throughout the assay. The number of lymphocytes adhering to the monolayers, in the presence of a range of antibody supernatants, was compared with control wells that contained assay medium alone. Main Effects ANOVA analysis was used to calculate the regression coefficients for each antibody, with the coefficient for RPMI (assay buffer) set at zero. Adhesion expressed as a percentage, was calculated from the lymphocytes adherent in the presence of antibody relative to the total number of adherent lymphocytes detected in the absence of antibody. Upper and lower 95% confidence intervals (calculated as +/- 1.96*standard error) are presented as error bars. Error bars for each antibody were calculated from the mean (replicate wells) percentage of lymphocyte adherence obtained from replicate experiments. Statistically significant inhibition of adherence is indicated by * on the histogram plot.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5D6
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5C4
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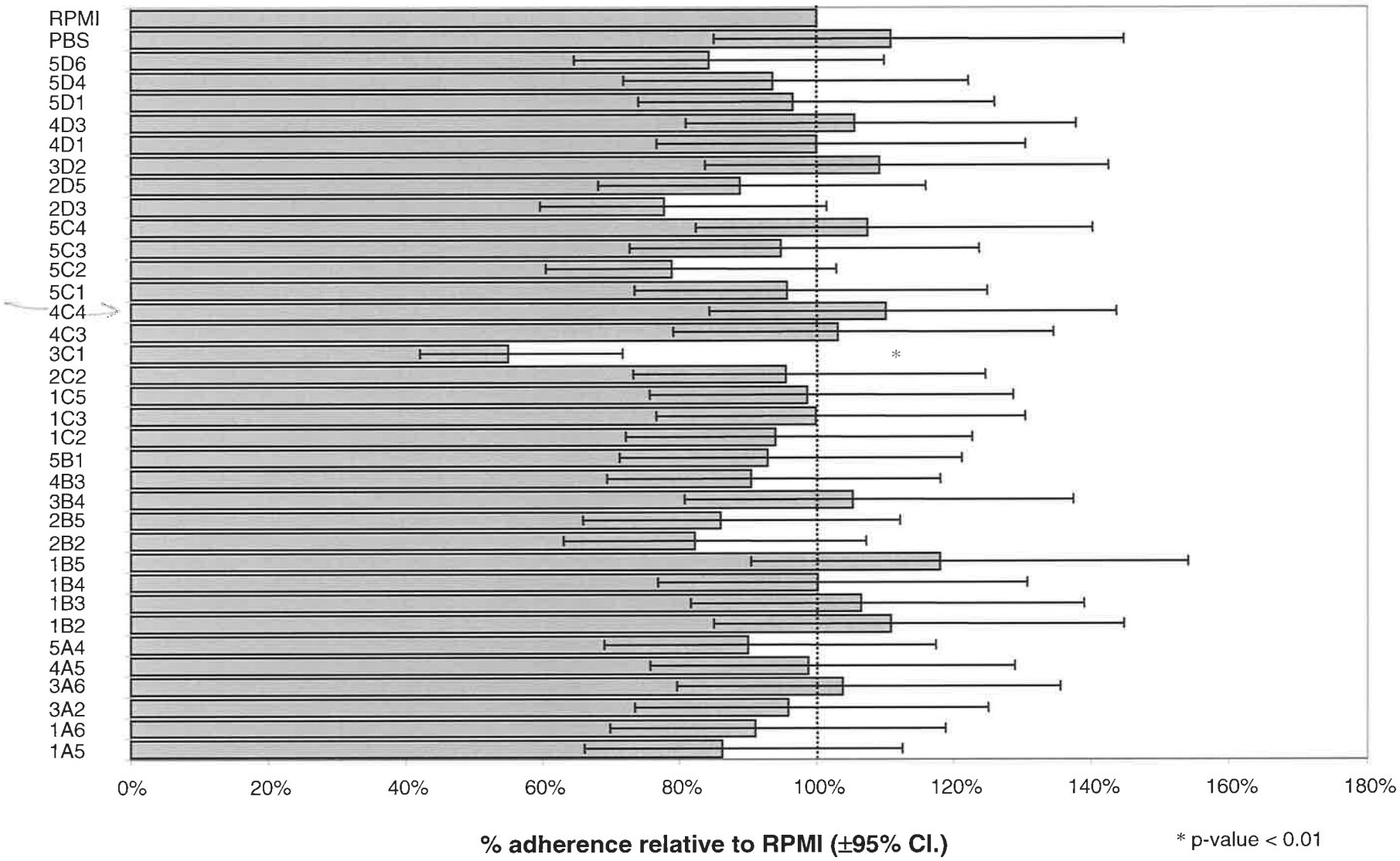
5C2
5C1
4C5
4C4
4C3
3C1
5B1
4B3
3B4
1B4
1B3
1B2
5A4
4A5
3A6
3A2
1A6
1A5
*
*
0%
20%
40%
60%
80%
100%
120%
140%
160%
180%
200%
% adherence relative to RPMI (±95% CI.)
* p-value < 0.01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2B2
1B5
1B4
1B3
1B2
5A4
4A5
3A6
3A2
1A6
1A5
*
0%
20%
40%
60%
80%
100%
120%
140%
160%
180%
% adherence relative to RPMI (±95% CI.)
* p-value < 0.01

Figure 7.4 The effect of antibody supernatants on the adhesion of CD4+ lymphoblasts from TD lymph of arthritic rats to EC from microvasculature of arthritic joints.

Monolayers of EC were prepared from the microvasculature of severely inflamed joints 13 days after inoculation with CFA. CD4+ lymphoblasts were isolated from TDL cannulated from rats in the prodrome of AA, just prior to use in the assay (Section 2.15.2). Antibodies were present throughout the assay. The number of lymphocytes adhering to the monolayers, in the presence of a range of antibody supernatants was compared with control wells that contained assay medium alone. Main Effects ANOVA analysis, calculated the regression coefficients for each antibody, with the coefficient for RPMI (assay buffer) set at zero. Adhesion expressed as a percentage was calculated from the lymphocytes adherent in the presence of antibody relative to the total number of adherent lymphocytes detected in the absence of antibody. Upper and lower 95% confidence intervals (calculated as +/- 1.96*standard error) are presented as error bars. Error bars for each antibody were calculated from the mean (replicate wells) percentage of lymphocyte adherence obtained from replicate experiments. Statistically significant inhibition of adherence is indicated by * on the histogram plot.

This study utilised two different sources of activated lymphocytes and EC purified from small vessels of inflamed joints. The microvascular EC from the hind limb of the AA rat had been stimulated *in vivo* during the inflammatory response to CFA injection and they were harvested when the joint was severely inflamed. The first population of lymphocytes investigated was harvested from LN cells that were stimulated *in vitro* with Con A. The period of Con A stimulation was chosen based on expression of adhesion molecules and activation markers (Section 6.3.1). These markers of activation were similar to those found on lymphocytes released from the synovium of inflamed joints by collagenase digestion. The second population consisted of CD4+ lymphoblasts purified from TD lymph (Section6.3.4) of rats that were in the late prodrome of AA. This population is believed to play an integral role in the progression and initiation of AA (Spargo *et al.,* 1996; 2001).

Several studies, using *in vitro* and *in vivo* experimental models, have examined lymphocyte-endothelial adhesion and/or recruitment by using mAbs against adhesion molecules. *In vitro* studies have utilised EC obtained from large vessels, such as human umbilical cord (HUVEC) or rat aorta. The results obtained from these models have been interpreted by assuming they are representative of the properties of microvascular endothelium. The validity of this comparison is problematic, for the reason that heterogeneity between EC from different sized vessels and different vascular beds is evident (Gumbowski *et al.,* 1994; Kumar *et al.,* 1987; Page *et al.*, 1992; Petzelbauer *et al.,* 1993). Furthermore, there is clear evidence that the surface adhesion molecules expressed by EC changes during extended periods of culture (Cines *et al.*, 1998; Grant *et al.,* 1989), so that EC cultures that have been expanded in culture may have altered phenotype. Wykretowicz and Issekutz, (1993) compared lymphocyte adhesion to normal cardiac microvascular EC and macrovascular EC (aorta). The number of adherent lymphocytes adherent to these EC varied significantly, pre- and post- stimulation.

Monoclonal Abs have been used widely as tools for studying the functions of adhesion molecules. Inhibition by mAbs of lymphocyte-EC adhesion *in vitro* and effects on

transmigration of lymphocytes to inflamed tissues has been utilised by a number of researchers (Springer, 1994; Oppenheimer-Marks, 1991; Haskard, 1986; Issekutz, 1992; Issekutz, 1991). Migration studies were used by some groups, but because T cells represent only a small proportion of the inflammatory cells present in a joint during AA, quantification is a difficult process. Furthermore, antibodies against cell surface antigens can affect lymphocyte survival and recirculation, in addition to effects locally in sites of inflammation.

In vivo analysis of adhesion molecule actions have involved migration studies of radiolabelled lymphocytes, to different tissue sites. In this type of study, it is difficult to isolate the effects of individual components of the cascade of events that leads to adhesion and finally to migration to an inflammatory site. In *in vitro* studies, however, individual components of the cascade can be studied. Furthermore, sub-populations of lymphocytes have shown preferential migration patterns to different tissues. This has been reported in tissues such as skin, lymph node or gastrointestinal tract (Griscelli, 1969; Gowans and Knight, 1964; Issekutz, 1991; Issekutz and Issekutz, 1991a; Issekutz and Issekutz, 1991b; Salmi *et al*, 2001). Lymphocytes from the gastrointestinal tract migrated poorly to LN, whilst lymphocytes from LN migrated well to LN and inflamed joints but did not migrate well to skin (Issekutz *et al., 1991*b). For this reason, EC from the microvasculature of the arthritic joint should be the preferred model to study aspects of the migratory cascade in arthritis.

In this chapter, the blocking effects of antibodies against known adhesion molecule (L-selectin, P-selectin (using PSGL-1), LFA-1α, LFA-1β, MAC-1, LFA-2, VLA-4, ICAM-1, PECAM-1 and CD44) have been examined. This was done to assess the sensitivity of the assay developed in Chapter 6 to the effects of blocking a range of adhesion molecules that have been implicated in lymphocyte-EC adhesion. The assay was then used to assess the effects on lymphocyte- EC adherence of a large number of hybridoma culture supernatants that contain antibodies that had been shown to bind to endothelium in tissue sections of inflamed synovium from rats with severe AA.

7.4.1 L-selectin

L-selectin is one of 3 members of the selectin family of adhesion molecules. It is expressed on naïve lymphocytes and some memory T cells (Lewinsohn *et al.,* 1987), although the level, if it is expressed, is reduced on most memory T cells and on activated T cells (Tedder, 1995). The molecule was identified originally in mice, where a mAb (MEL-14) was found to block almost completely the migration of lymphocytes to peripheral LNs (Gallatin *et al.,* 1983).

L-selectin plays a vital role in T lymphocyte recirculation through LN and migration of lymphocytes to inflammatory sites (Tedder *et al.,* 1995). *In vivo* studies have shown that anti-L-selectin reduces lymphocyte accumulation in the peritoneum by 90% (Pizcueta *et al.,* 1994), while L-selectin deficient mice are reported to exhibit impaired recruitment of lymphocytes into inflammatory sites (Tedder *et al.,* 1995).

Two anti-L selectin mAbs (HLR-3 and OX85) were studied. The mouse anti-rat antibody (OX85) reduced the number of adherent lymphocytes to 77.7 % of the control in the case of Con A stimulated lymphocytes and to 82% of control in the case of TD lymph CD4+ lymphoblasts. The hamster anti-rat mAb (HLR-3) reduced adherence of the respective lymphocyte population to 62% and 55% of control. The anti- adhesive effects of these antibodies are consistent with the expression of CD62L by the Con A stimulated (23.4%) (Section 6.3.1) and TD CD4+ lymphoblasts. CD62L appears to have mediated selective adherence of this subpopulation, although other adhesion molecules are presumably important for the anti-L selectin resistant adherent cells most of which are likely to be $CD62L^{-}$.

7.4.2 P-selectin-glycoprotein fusion protein

PSGL-1 is a recognised ligand of P-selectin (Goetz *et al.*, 1997). The other members of the selectin family, E-selectin (Goetz *et al.*, 1997; Hirata *et al.,* 2000) and L-selectin (Walcheck *et al.,* 1996) are also known to bind the P-selectin ligand. The PSGL-1 molecule is a

glycoprotein that contains the sialyl-Lewis^x -like tetrasaccharide. A sub-population of T lymphocytes express PSGL-1 strongly (Austrup *et al.*, 1997) and it appears to have a critical role in lymphocyte recruitment. Adhesive interactions with EC have only been observed with activated endothelium (Piccio *et al.*, 2002).

Purified P-selectin-IgG fusion protein produced a modest but significant inhibition of the adhesion of Con A stimulated lymphocytes (25%) and CD4+ blasts (19%). P-selectin and its ligand play a role of inflammation in some tissues. Lymphocyte migration to inflamed skin is reduced to between 20-48% by P-selectin blockade (Issekutz and Issekutz, 2002). PSGL-1 is a key molecule in the migration of lymphocytes to the central nervous system (Piccio *et al.*, 2002). However, in the development of AA, it is E-selectin and not P-selectin that has been implicated in playing a role (Issekutz *et al.*, 2001). In the present study (Section 5.3), PSGL- 1 was detected on a small population of CD4+ lymphocytes isolated from inflamed synovium by collagenase digestion.

Inhibition of adhesion by P-selectin-Ig fusion protein was unexpected, because surface expression of PSGL-1 was barely detected on lymphocytes after 24 hours Con A stimulation, although expression of the molecule had increased significantly by 48hrs (Section 6.3.1.2). The level of inhibition by P-selectin-Ig fusion protein that was observed may reflect blocking of other selectin ligands. It could also be due to a relatively higher sensitivity of the assay compared with flow cytometric detection of the molecule.

7.4.3 LFA-1 and ICAM-1

The β_2 integrin LFA-1 (CD11a/CD18) is expressed on the lymphocyte surface (Arnaout, 1990). The immunoglobulin super family members ICAM-1 and ICAM-2 are expressed by ECs and both are ligands for LFA-1. ICAM-1 expression is also upregulated by activated T cells. LFA-1 is involved in the adhesion and migration of lymphocytes into tissues (Haskard *et al.*, 1986). However, the relative importance of the molecule depends on the nature of the lymphocytes and the target tissue (Kulidjian *et al.*, 1999).

In the present study, anti-LFA-1 inhibited adhesion of Con A stimulated lymphocytes by more than 50% and adhesion of CD4+ TD lymphoblasts by nearly 60%. Furthermore, the inhibition of adhesion of both sorts of lymphocytes by the mAb directed against the β2 integrin chain (WT3) was similar to that caused by antibody specific for LFA-1 (WT-1). The CD4+ TD cells had a higher proportion of cells expressing LFA-1 on their surface than the Con A activated LN cells and this may explain the greater inhibitory effect observed with the former.

In vitro adhesion of lymphocytes to stimulated HUVECs is strongly (but not completely) inhibited by anti-LFA-1 (Haskard *et al.*, 1986). *In vivo* migration and *in vitro* studies have shown that although LFA-1 is important in lymphocyte recruitment to inflammatory sites in some tissues, such as skin (Issekutz, 1993; Issekutz, 1992), on its own it appears less important in recruitment to inflamed joints in the rat adjuvant arthritis model (Issekutz and Issekutz, 1991; Issekutz *et al.,* 1996) or human inflammatory bowel disease (Salmi and Jalkanen, 2001). The incomplete blocking by mAb against LFA-1 indicates that adhesive interactions that do not involve LFA-1 are important in adhesion of activated T cells to synovial microvascular EC. LFA-2 had no such inhibitory effect on lymphocyte adhesion.

In normal tissues, expression of ICAM-1 is relatively low. However, it is upregulated in response to inflammatory mediators (Dustin *et al.*, 1986). In RA (Hale *et al.*, 1989; Szekancz *et al.*, 1994; Tak *et al.*, 1995) and AA, ICAM-1 is expressed at high levels on EC in the inflamed synovium. In the current study, ICAM-1 blockade (mAb 1A29) produced significant inhibition of adhesion of activated T cells to synovial EC. Monoclonal Ab 1A29 inhibited binding of Con A stimulated T cells and CD4+ TD lymphoblasts by 50-60%. An earlier *in vitro* study by Iigo *et al.*, (1991) has reported inhibition in the development of AA by mAb 1A29 in an adoptive transfer model. These findings suggest a potential role for ICAM-1 in both actively induced disease, in the induction phase of the disease and on migration of activated T cells to joints in the effector phase of the disease. The present findings support an important role for the interaction of LFA-1 and ICAM-1 in the adhesion of activated T cells to endothelium from inflamed synovium, and a role in the

transmigration of CD4+ lymphoblasts through the microvascular EC in the pathogenesis of AA. In contrast, the related integrin Mac-1 does not appear to have a significant role in the process.

7.4.4 VLA-4

The β_1 integrin VLA-4 (CD49d/CD29) is expressed on the surface of several types of leucocytes, including lymphocytes (Hemler *et al.,* 1990). VLA antigens are known to appear on lymphocytes several days after mitogen activation (Albeda and Buck, 1990). The major ligand of VLA-4 is the immunoglobulin super family member VCAM-1 and lymphocytes expressing VLA-4 will bind to ECs expressing VCAM-1 (Issekutz and Wykretowicz, 1991; Elices *et al.,* 1990). Normal (resting) EC either express low levels of VCAM-1 or they do not express the molecule at all. Recently, it was discovered that VLA-4 could bind also to the connecting segment-1 (CS-1) portion of fibronectin (Elices *et al.,* 1995; Johnston *et al.,* 2000). Integrins are expressed constitutively at low levels and furthermore, these molecules do not bind with high affinity to their counter receptors unless the integrin is activated (Mackay and Imhof, 1993). The VLA-4 ligand, VCAM-1 has been detected on EC following stimulation by cytokine or LPS (Issekutz and Wykretowicz, 1991; Thornhill *et al.,* 1991, Pober *et al.,* 1986, Rice *et al.,* 1990; Schwartz *et al.,* 1990; Carlos *et al.,* 1990). It is expressed also by EC in inflamed synovium from rats immunised with CFA (Johnston *et al., 2*000) and is present on EC from arthritic human synovium (Akin *et al.,* 2001).

In vivo studies in the rat have indicated that administration of anti-VLA-4 (mAb Mrα4-1) causes partial blocking of T cell migration to inflammatory sites. Blockade with anti-VLA-4 (mAb Mrα4-1) inhibited migration of resting and activated lymphocyte to inflamed skin by approximately 60% (Issekutz, 1991). The migration of a sub-population of isolated lymphocytes from peritoneal exudates, to inflamed joints (AA), after intravenous injection, was inhibited by approximately 50% but migration of unstimulated lymphocytes from peripheral LN to the joints was not affected (Issekutz and Issekutz, 1991).

In the present study, mAb Mrα4-1 (anti-VLA-4), inhibited adhesion of lymphocytes (both Con A activated LN cells and CD4+ TD cells) to EC monolayers by approximately 30%. This level of inhibition of adhesion is lower than the reported inhibition by this mAb of migration into sites of inflammation. The difference could be attributable to the very different methods of detection and the complexity of the *in vivo* system, which in fact examines the outcome of the multi-step process. However, it is also important to note the heterogeneity between vascular beds and also the levels of VLA-4 expression by different lymphocyte sub-populations. Activation of T cells with mitogen Con A for only 24 hours would not be expected to induce high levels of expression of VLA-4, as this molecule is expressed after longer periods of stimulation (Albeda and Buck, 1990). In the case of inhibition of adhesion of Con A stimulated lymphocytes, surface expression levels of VLA-4 (Table 6.2) approximated inhibition by anti-VLA-4 mAb. The level of surface expression of VLA-4 by lymphocytes from peritoneal exudates was not mentioned in the Issekutz study (Issekutz and Issekutz, 1991).

An interesting observation that inhibition of adherence by CD4+ TD lymphoblasts by anti-VLA-4 antibody is greater than the proportion of VLA-4 positive lymphocytes in the T cells applied to monolayers. Approximately 12% of the CD4+ TD lymphoblast population expressed VLA-4 (Table 6.5), while anti-VLA-4 inhibited adhesion by between 19% and 40%. This result suggests that the adherence is biased towards cells that express VLA-4. If this is the case, interpretation of the effects of antibodies on adhesion of lymphocytes from a heterogeneous population will be sensitive to the proportion of lymphocytes in that population that express the respective adhesion molecule.

In some tissues, such as skin, blockade of both VLA-4 and LFA-1 by antibodies inhibits the migration of lymphocytes to inflamed sites by 98% (Issekutz, 1993). The combined blockade observed in skin was not, however, mirrored in inflamed joints. Although joint inflammation in AA was reduced significantly by injection of anti-VLA-4 and anti-LFA-1 mAbs, arthritis was not suppressed completely. *In vivo*, this combination of mAbs inhibited migration of spleen lymphocytes to arthritic joints by only 66-79% (Issekutz *et al.*,

1996). When combined with anti-VLA-4 antibody, anti-P-selectin did not augment the levels of inhibition of migration. (Issekutz and Issekutz, 2002). It appears therefore, that adhesion molecules in addition to LFA-1, VLA-4 and PSGL-1 may be involved in recruitment of lymphocytes into inflamed synovium.

7.4.5 Effects of uncharacterised hybridoma culture supernatants containing anti-EC antibodies.

Of the hybridoma supernatants tested, two out of 34 caused significant inhibition of adherence of Con A stimulated lymphoblasts. The mean inhibitory effects of the two culture supernatants (3C1 and 4C4) were similar. Supernatants 3C1 and 4C4, inhibited adherence by approximately 52% and 51% respectively. The level of inhibition was of a magnitude comparable to that detected in the presence of mAb 1A29, (anti-ICAM-1) (see Figure 7.1 and 7.2). Interestingly, supernatant 3C1 also inhibited adherence of CD4+ TD lymphoblasts but adherence of these cells was not affected by supernatant 4C4. This observation suggests that the culture supernatants contain different antibodies.

In the initial immunohistochemical survey investigating supernatant, 3C1 was found to contain antibodies that reacted with antigen expressed by EC, inflammatory cells and connective tissue in inflamed synovium. Supernatant 4C4 reacted with EC, mast cells and macrophages in inflamed synovium. These observations support the conclusions that the culture supernatants contain different antibodies, although both react with EC and both inhibit lymphocyte adhesion. Further investigation of the antibodies requires that the hybridoma cells in these wells should be cloned and that the resulting mAbs are used for further investigation of blocking activity, tissue distribution of the antigens and characterisation of the respective antigens.

In summary, the use of mAbs directed at known adhesion molecules verifies the application of the miniaturised adhesion assay to study lymphocyte adhesion to synovial microvascular EC. Several of these antibodies exhibited partial blockade of lymphocyte adhesion. The incomplete inhibition of lymphocyte adherence by blockade of individual adhesion

molecules leads to the conclusion that adhesion involves multiple pathways, mediated by several adhesion molecules. Other groups, utilising lymphocytes from different sources, have reported similar results. Further studies, using cocktails of existing antibodies against known adhesion molecules, could be carried out using this novel assay system.

In vivo migration studies have not looked specifically at the adhesive interactions between EC microvasculature and lymphocytes. In the work described herein, both *in vitro* activated T cells and T cell from TD lymph of rats in the prodrome of AA (and known to contain arthritogenic cells) have been examined for adherence to microvascular EC from inflamed synovium. Of great importance, these EC were freshly isolated, rather than expanded for lengthy periods *in vitro*. The results demonstrate that several adhesion molecules (LFA-1, ICAM-1, VLA-4 and L-selectin) are active in promoting lymphocyte adherence to microvascular EC and that these molecules may have a role in recruitment of activated CD4+ T cells to inflamed synovium.

Antibodies in the culture supernatants that contain anti-EC antibodies inhibited lymphocyte adhesion. These results are preliminary and in particular, the anti-adhesive effects cannot be attributed to a single antibody because the respective hybridomas were not cloned at the cessation of laboratory work. If mAbs produced from cloned hybridomas also inhibit lymphocyte adhesion, these antibodies can be used in further studies, both *in vitro* and *in vivo*. Furthermore, they can be used in immunohistochemistry and flow cytometric analysis to define the distribution of the respective antigen. Once these objectives are achieved, the nature of the antigen can be investigated and compared with recognised adhesion molecules.

8 General Discussion and Conclusion

8.1 Discussion

This work has investigated adhesive interactions between freshly isolated populations of synovial microvascular ECs and activated T cells from (Con A activated) LN and TD lymph (from arthritic donors). Previous studies, utilising various models, have highlighted a role for a number of adhesion molecules in the recruitment of inflammatory cells during the pathogenesis of immunologically mediated polyarthritis. Earlier *in vitro* studies have used either EC derived from large vessels, such as the rat aorta or the human umbilical cord with few having used EC from the microvasculature, in the latter case, for example, endothelium from the hearts of normal rats. These EC populations required *in vitro* stimulation with pro-inflammatory mediators prior to use in an adhesion assay.

In contrast, the current study has examined adhesive interactions using EC activated *in vivo*. A novel aspect of this work is that it uses monolayers of EC of microvascular origin prepared as primary cultures from inflamed synovium obtained from rats with AA. This rat model of polyarthritis is well established and a number of mAbs are available for use in this species that have been shown previously to modify lymphocyte recruitment. This model has, therefore, advantages for investigation of novel lymphocyte-EC adhesive interactions and novel assay systems, using these available mAbs. The study has identified two hybridoma supernatants that contain antibodies that inhibit lymphocyte adhesion and the microassay of lymphocyte–EC adhesion that has been developed could have wider application in examining new mAbs and interactions between immune cells and EC from other sites of inflammation.

In addition to the work carried out utilising EC from inflamed synovial tissue, the method developed in this study, in the production of a single cell suspension by enzymatic synovial digestion has proved to be a very useful investigative tool for other research projects within our research group. Work that has been carried out utilising cells obtained from synovium

(from rats with polyarthritis) includes Dr Melissa Brasted and work on cytokines in effector CD4+ T lymphocytes (Thesis 2001), Dr Mahin Moghaddami's work on isolation of DCs (Mogadambi *et al*, submitted for publication), and Mr Llewelyn Spargo's investigation of isolated CD4+ T lymphocytes in vivo migration, and in the characterisation of T cell phenotype over a time course of adoptively transferred AA (Spargo *et al.*, in preparation).

8.1.1 Endothelial Cells

EC from different sources have been utilised to investigate adhesion *in vitro*. HUVECs in particular have been a useful tool for *in vitro* studies. They have provided a means to elucidate an immense amount of information pertaining to the biology of EC. They are not, however, a completely reliable model in which to investigate mechanisms of inflammation. The reasons for this stem from the fact that these EC originate from large vessels and it would be unlikely for them to reflect the properties of microvascular EC, which are the cells that are usually involved at sites of inflammation. It is the microvasculature that is the main site of adhesion and migration of lymphocytes during inflammatory processes (Cines, 2001).

The heterogeneity between EC of different sized vessels and between different microvascular beds is well documented. For these reasons, the validity of *in vitro* studies that have utilised HUVECS and EC from other vessels, have been questioned (Gumbowski *et al.*, 1994; Kumar *et al.*, 1987; Page *et al.*, 1992; Petzelbauer *et al.*, 1993; Wykretowicz and Issekutz, 1993; Bassenge *et al.*, 1996). The responses of HUVECs or cells from large blood vessels to proinflammatory stimuli might be different from the responses by microvascular EC. The work herein, using microvascular EC from inflamed synovium, has provided an experimental model that avoids the problems posed by EC heterogeneity and the known effects of prolonged growth *in vitro* on the phenotype of EC and possible differences of responsiveness to proinflammatory stimuli between HUVECs and microvascular EC.

8.1.2 Assessing adhesion and migration

A range of methods has been used in the past to assess lymphocyte adhesion or migration. These include histology, direct or indirect anatomical measurements, intravital microscopy, the Stamper-Woodruff adhesion assay on frozen sections, detection of accumulation of radioactively labelled cells *in vivo* and more recently, the use of 'Knock-out' mice with specific deficiencies in adhesion molecules. Each is an established investigative tool, yet all have their limitations.

The use of histological methods to assess extravasation of cells has the difficulty that immigrant lymphocytes are difficult to identify and it is usually impossible to state the direction of migration with confidence. Indirect measurements using indirect effects of migrated leucocytes, such as footpad or ear swelling in DTH reactions, do not necessarily indicate that the observed inflammation is due to cellular infiltrate or to oedema and infact may generally indicate the latter. Intravital microscopy, which allows direct observation of leucocytes and their interactions with vascular endothelium *in vivo*, is difficult to quantify and it is usually not possible to identify leucocyte sub-populations of interest. Furthermore, while suitable for some tissues, such as mesenteric spreads and Peyer's patches, it cannot be carried out easily in the synovium.

The Stamper-Woodruff binding assay for leucocyte adhesion to frozen tissue sections has been very useful. In studies described herein, the assay resulted in large variation on the adhesion of lymphocytes to sections of synovium. There were technical difficulties in removing unbound lymphocytes from the section, without accidental removal of adherent lymphocytes. On the other hand, unbound lymphocytes remaining associated with the section may become permanently adhered to the section by the chemical fixation step. The Stamper-Woodruff assay has achieved greatest success in identifying adhesion between lymphocytes and specialised HEV in secondary lymphoid tissues (Stamper and Woodruff, 1976; Rosen *et al.*, 1989; Sawada *et al.*, 1993; Hamann *et al.*, 1994; Toppila *et al.*, 1997) and in lymphoid nodules in chronic synovial inflammation (Grober *et al.*, 1993). These are the sites of intensive lymphocyte traffic and they appear to have high expression of

adhesion molecules. The method has been less effective in identifying adhesive events in the microvasculature of non-lymphoid tissues.

Several groups have utilised *in vivo* migration methods to investigate migration of lymphocytes from the blood to sites of inflammatory stimuli. This type of investigation, although useful to study extravasated cells after their migration to the site of inflammatory stimulus, does not allow the examination of individual components of the cascade of events that leads to extravasation and migration. Such studies of leucocyte migration endeavour to block migration with mAbs, and although presence or absence of labelled cells at the inflammatory site does indicate whether some aspect of the process migration has been blocked and the processes that have taken place in the cascade of events leading to migration can not be identified. Because *in vitro* investigations of adhesion or migration are carried out in isolation, a single molecule may be examined in the absence of other factors that may be exert an influence in a whole animal model. For example, adhesion molecule deficient mice can possess "compensatory alterations in the expression of adhesion molecules, compared with wild type mice" (Cines *et al.,* 1998) which may effect the progression of the inflammatory response.

The present investigation targeted the early stages of the adhesive process leading up to extravasation; as the model developed here facilitates the study of these early interactions in the adhesion cascade. Using this model the specific effects of individual anti-adhesion molecules were able to be tested in a reductionist approach, ie in the absence of unknown systemic factors, which are beyond the control of an investigator in a complex *in vivo* model system (Pitzalis *et al.,* 1996).

It is important to examine the effects of anti-adhesion molecule antibodies upon aspects of the migration process at each step of the cascade and migration process. This understanding is essential, so that, with regard to the interactions of any potential *in vivo* therapies, all facets of the process can be understood in detail, e.g. adhesion, entry into the tissues, extra-vascular transit to the site of inflammation and exit from inflammatory foci.

This is important, since adhesion-molecule-therapy may influence more than entry into the tissues (Westermann *et al.*, 2001). If each part of the process is understood, it will give a full understanding of the mechanism of preferential accumulation of lymphocytes.

8.1.3 *In vitro* models using endothelial cell monolayers

Endothelial cells are obtained usually from large vessels, and the differences between these cells and microvascular EC have been discussed in Section 1.3.5. Normally culture of microvascular EC requires expansion of the EC and passage performed several times to obtain adequate numbers of cells. This is because usually the assays are carried out in culture vessels that require relatively large numbers of EC per well. The major advantages of the micro-assay developed herein, are that expansion of the EC *in vitro* was not necessary and could be used in the assay just hours after plating and without the need for *in vitro* stimulation. The use of Terasaki plates made it possible to test a considerable number of antibodies using a single preparation of freshly isolated EC. For example, 6 rats provided enough freshly isolated EC to test approximately 20 antibodies in triplicate.

8.1.4 Development of the assay

Development of the micro-assay required solution to a number of challenging problems. Solving these problems constituted a large proportion of this project.

8.1.4.1 The source of synovial tissue

The starting point of the project was to identify a suitable area of synovium. The histological assessment showed that the sub-Achilles region of the ankle undergoes a transformation from a loose connective tissue with abundant adipocytes to a dense, highly cellular tissue during the pathogenesis of AA. These changes were a result of proliferation of connective tissue cells and infiltration of inflammatory cells.

The synovium is referred to often as the superficial layers of specialised cells that line the synovial joint spaces. More correctly, however, this superficial lining is the synovial intima and it is just one of the two structural areas that together form the synovium. The second layer is the subintima, which is connective tissue that underlies the intimal layer

(Wilkinson, 1994). The tissue identified in the sub-Achilles region of the inflamed ankle captured both of these components of synovium.

In the ankles of rats with AA, the joint spaces are expanded, with the synovial intima and the synovial space occupied by a fibrinous and cellular exudate. The surrounding synovial intima and subintima from the sub-Achilles region was highly vascular and contained a cell mass that was reckoned sufficient to yield enough EC for the development of a micro-assay for lymphocyte-EC adhesion. Having identified a tissue source, the next task was to isolate EC from this tissue in sufficient numbers and purity that they could be used in the assay, without the need for expansion or stimulation. This is a major problem faced in cell work using EC, where the main difficulty is that of avoiding contamination with non-EC such as FBs and macrophages. Both of these cells are, like EC, adherent, and furthermore, FBs replicate rapidly in culture and tend to overgrow EC.

Having developed a method to recover viable cells from the sub-Achilles tissue, using collagenase and hyaluronidase enzyme digestion, a number of methods were explored with which to purify EC. These included differential adhesion of cells from the mixed suspension, attempts to obtain EC by outgrowth from manually selected vessel segments, the use of density gradient centrifugation and employment of immunomagnetic beads in conjunction with EC-specific lectins or EC-specific mAbs (using positive and negative selection strategies).

Attempts to find an antibody with which to remove FBs selectively were not successful. The antigen Thy-1, detected by OX7, is expressed in the rat by FBs, nerve tissue, vascular pericytes and a small population of peripheral T cells. The initial promise of mAb OX7 as a reagent for use in depleting FBs was negated when it was found that Thy-1 was also strongly expressed by EC in synovium from inflamed rat joints. An attempt to develop mAb specific for FBs, using cell suspensions from the sub-Achilles region as immunogen, was not successful

8.1.4.2 Isolation of EC

The shear numbers of non-EC in collagenase digests of sub-Achilles tissue presented a barrier to EC isolation. This situation was partially overcome by using mAb OX1 to remove all cells of haematopoietic origin. In addition to removing many other inflammatory cells, macrophages were also removed. This was important because microvascular EC and macrophages both express CD36 on their surface and both are adherent (Section 3.3.2.1). The depletion of macrophages meant that the mAb UA009 (against CD36) could be utilised as a tool to positively select microvascular EC.

Of all the endothelial specific mAbs tried, UA009 proved to be the most suitable for positive selection of EC. This mAb stained only EC from small vessels. The yield of EC obtained by positive selection was high compared with other mAbs trialed and the numbers of cells obtained were adequate for setting up the micro-assay. Furthermore, CD36 was shown to be a surface molecule not involved in lymphocyte adhesion. Initial attempts at purification of EC by positive selection using immunomagnetic (Dynal) beads were successful but it was found that the beads remained attached to the surface the of EC for long periods of time in culture. However with the introduction of Dynal CELLection beads, it became possible to remove the beads after selection of the EC. The successful release of beads from EC was a critical breakthrough and allowed the development of the micro-assay to finally move forward.

A further problem that had to be addressed was loss of EC from the Terasaki wells following initial adhesion. This was solved by a change of the surface binding substrate from gelatin to fibronectin and the development of a standardised gentle washing method that allowed the efficient removal of non-adherent cells, without loss of adherent EC. The seeding density of lymphocytes in the assay was also standardised, to avoid clumping of lymphocytes and non-specific binding that would make accurate quantification difficult.

8.1.5 Lymphocytes

8.1.5.1 Synovial lymphocytes from rats with AA

A relatively large proportion of lymphocytes from within the joint were CD4+ T cells and presented an activated phenotype. High levels of MHC Class II, CD25 and CD71 are characteristic of activated lymphocytes and these were detected on lymphocytes from the inflamed joint.

In RA and other forms of synovitis in humans, high levels of $CD45RA^-$ cells and higher levels of CD54RO+ cells are reported in the synovium (Kingsley *et al.*, 1988). This preferential accumulation of activated CD45RO+ cells in the inflamed joint may occur in response to *in situ* proliferation due to local antigens. However, since only approximately one third of these cells express CD71, a marker usually expressed by proliferating cells, this is unlikely to be the only mechanism. Increased migration of activated T cells is likely to be important also, aided by the increased adhesion of cells to EC at sites of inflammation.

The precise contribution of migration of activated cells, entry as naïve cells with *in situ* changes in their phenotype, proliferation of activated cells *in situ* or activation elsewhere and preferential retention after migration; have not been elucidated at this stage. The characterisation of the phenotype of T cells in synovium provided a guide for selection of lymphocyte for use in the adhesion assay.

8.1.5.2 Selection of lymphocytes for use in the adhesion assay.

Initially the work was carried out using lymph node lymphocytes that were stimulated *in vitro* for 24hr with Con A. These cells were selected because of the ease with which they can be obtained. The cells were activated with Con A and a period of activation was selected, at which time the expression of activation markers was similar to the pattern of surface activation markers expressed by lymphocytes from arthritic joints. Preliminary experiments revealed that the number of adherent Con A stimulated cells per well was enhanced by about 7 fold compared to control wells containing unstimulated LN

lymphocytes.

With this information, attention was turned to adhesion of CD4+ T lymphoblasts in TD lymph from rats during the late prodromal phase of AA (Spargo *et al.*, 2002). This blast cell sub-population, isolated from the TD lymph of arthritic rats, expressed relatively high levels of the surface activation markers MHC class II, CD25, CD71 and KI67, and increased expression of the adhesion molecules CD44, CD11a, CD18, CD45 and CD49d. These cells can transfer AA adoptively, and it has been shown that TD lymphoblasts from arthritic donors are recruited to synovium (Spargo *et al.*, 1996).

Other studies have shown differences in the recruitment of activated and resting cells to sites of inflammation (Young and Hay, 1999). It has also been reported, that lymphocyte accumulation in the joints of rats with AA is 6 fold greater than in the joints of normal rats (Issekutz and Issekutz, 1991) and that blockade of adhesion molecules or their ligands with mAbs against ICAM-1, VCAM-1 (Oppenheimer-Marks *et al.*, 1991), LFA-1 (Haskard *et al.*, 1986; Issekutz, 1992), and VLA-4 (Issekutz, 1991) inhibits lymphocyte-endothelial adhesion and migration.

8.1.6 Adhesion assays

The adhesion assay developed in this work is a static assay that measures lymphocyte adhesion in the absence of shear forces. The adhesion molecules LFA-1, (Issekutz, 1992; Issekutz and Issekutz, 1993: Issekutz *et al.*, 1996; Jasin *et al.*, 1992; Salmi and Jalkenen 2001); ICAM-1 (Iigo *et al.*, 1991; Salmi and Jalkenen 2001), VLA-4 (Issekutz, 1991; Issekutz and Issekutz, 1991; Issekutz and Wykretowicz; Issekutz and Wykretowicz; Issekutz *et al.*, 1996; Salmi and Jalkenen 2001), E-selectin (Issekutz *et al.* 2002; 2001), L-selectin (Salmi and Jalkanen, 2001; Tedder *et al.*, 1995; Pizcueta *et al.*, 1994) and the selectin ligand PSGL-1 (Salmi and Jalkanen, 2001) have all been implicated in lymphocyte adhesion and in the pathogenesis of AA (Barbadillo *et al.*, 1995; Issekutz *et al.*, 1996; Jasin *et al.*, 1992; Mikecz *et al.*, 1995; Iigo *et al.*, 1991).

For each adhesion molecule, the extent of inhibition of lymphocyte adhesion by mAbs reported by other research groups has been quite variable. Part of this variability is probably related to the origin of the lymphocytes used in the assays. Various workers have used cells from spleen, peritoneal exudate, lamina propria of inflamed bowel, lymph node or peripheral blood. When reviewing these studies, a relatively large amount of variation was found both within and between experiments. For example, in the case of adhesion molecule LFA-1, the inhibition ranged between 10% and 60%, while *in vivo* inhibition was in the range from 30 to 88% (depending upon the cells used). Similarly, blocking of adhesion by anti-VLA-4 mAbs ranged from 0 to 70% (*in vitro* or *in vivo*). For this reason, care was given to the choice of lymphocytes utilised in this assay (Section 8.5.1.2).

The choice of EC source used for *in vitro* studies is critical. Most studies have used EC from human umbilical cord vein, or from animal tissues such as rat cardiac microvasculature, aorta or dermis. However, since these EC were not harvested from tissues affected by any pathology, *in vitro* stimulation was used in attempts to emulate inflammatory situations where the expression of adhesion molecules is upregulated. Culturing of EC *in vitro* may lead to changes in their phenotype. In work described herein, the period of culture *in vitro* was kept to a minimum and the assay was completed within 24 hours of commencing preparation of the EC. Furthermore, the microvascular EC were obtained from inflamed target tissues of the arthritogenic T cells *in vivo*.

This study has demonstrated the ability of mAbs against the CAMs ICAM-1, L selectin, LFA-1, β_2 integrin, VLA-4, and PSGL-1 (fusion protein) to significantly block adhesion of Con A stimulated lymphocytes from normal lymph node and CD4+ lymphoblasts from TDL of rats in the prodrome of AA, to microvascular EC from the same arthritic model. The results obtained support in general the findings of previous investigations that have used a variety of assays including *in vitro* adhesion assays using cytokine stimulated microvascular EC from normal rat heart or aorta, Stamper Woodroofe adhesion assay using human synovium from arthritic joints, and *in vivo* models of migration (rat or rabbit) to articular and non-articular sites.

The role for L-selectin in the T lymphocyte migration to inflammatory sites is supported in this study, by the inhibition of around 20 % of lymphocyte adhesion in the presence of anti-L selectin mAb. This was at a similar level of inhibition to that reported by Salmi and Jalkanen, (2001) to sections of human synovium.

LFA-1, is one of the first adhesion molecules to be reported to play a role in lymphocyte adhesion to EC and its partial role (50%) in lymphocyte adhesion is supported in this study. Similar levels of lymphocyte binding inhibition with anti-LFA-1 antibodies were detected *in vitro* to cytokine-stimulated microvascular EC from normal rat heart (Wykretowicz and Issekutz, 1993) and HUVECs (Haskard *et al.*, 1986). Inhibition of lymphocyte adhesion to EC in sections of human synovium was less marked (10-15%) (Salmi and Jalkanen, 2001).

Partial inhibition of lymphocyte migration to the inflamed joint of rats with AA was previously reported using the mAb WT3 against LFA-1 β chain (Issekutz *et al.*, 1996). No inhibition of lymphocyte migration to inflamed rat joint was observed when anti LFA-1 mAb TA3 was used as a blocking agent (Issekutz and Issekutz, 1993), however, significant inhibition of migration was observed to dermis (Issekutz, 1992). Development of AA was prevented by treatment with anti-LFA-1 mAbs in the rat (Issekutz *et al.*, 1996) and rabbit (Jasin *et al.*, 1992).

In this study, over 50% of lymphocyte adhesion was mediated by ICAM-1. Inhibition of lymphocyte adhesion to sections of human synovium was approximately half that observed in this study (Salmi and Jalkanen 2001). However, Iigo and co-workers (1991), reported suppression of the development of AA following treatment with anti-ICAM-1 mAb.

The role of VLA-4 in T lymphocyte migration has also been investigated *in vitro*. In the current study, around 30% of lymphocyte adhesion was inhibited by mAb against VLA-4, similar level of inhibition in human synovial tissue reported by Salmi and Jalkanen (2001). Approximately twice this amount of total inhibition however, was reported using

microvascular EC from normal rat heart (Issekutz and Wykretowicz, 1991) or EC from aorta (Wykretowicz and Issekutz, 1993). Treatment with VLA-4 blocking antibody also inhibited T cell migration to inflamed joints in AA (Issekutz and Issekutz, 1991). Treatment with anti-LFA-1 and anti-VLA-4 mAbs combined, completely inhibited lymphocyte migration to stimuli in skin. However, this combination of antibodies only inhibited lymphocyte migration to AA joints by up to 75% (Issekutz *et al.*, 1996).

8.1.7 Antibody supernatants raised against cells from AA synovium.

An important objective of developing the adhesion assay was to use it with the aim of detecting new adhesion molecules involved in recruitment of activated lymphocytes to inflamed synovium. This involved the generation of new mAbs against synovial EC. Using the assay to screen culture supernatants from hybridomas generated against rat synovial EC, at least two antibody supernatants of interest were identified. The antibodies produced by these uncloned hybridomas have been shown to partially block adhesion of Con A-activated LN cells. One of the antibodies also reduced adhesion of CD4+ T lymphoblasts from TD lymph to synovial EC. Constraints of time meant that the results obtained with these supernatants were preliminary and further experimental work is required to validate them. Furthermore the hybridomas must be cloned and the resultant mAbs tested for activity. This is important, because the results obtained could be due to the combined activities of two or more mAbs in the uncloned culture supernatants. Furthermore, of the nearly 20 other EC specific antibodies assayed, some exhibited inhibition of adhesion that did not reach statistical significance. It is possible that the wells that produced these supernatants contain antibody-producing cells whose product is at a concentration below that needed to saturate adhesion molecules. Cloning of cells from the wells may yield mAbs of higher titre and reveal further adhesion inhibitory activity. It is possible therefore, that other antibodies of interest may be identified by further work.

Importantly, these preliminary screening studies have established the utility of the adherence assay system for screening large numbers of antibody containing preparations efficiently. Furthermore, in principle, the assay should be readily adaptable to examine

adherence of other cell types, e.g. monocytes and putative DC precursors, to synovial microvascular EC.

When cloned mAb producing hybridomas that inhibit adhesion have been identified, the next step will be to identify the endothelial antigen against which they are detected. Should these mAbs identify novel EC surface molecules, they will be studied to determine whether they have an effect on the course of the disease in the AA model, or on the recruitment of effector cells in the adoptive transfer model. The rat model of AA is ideal for studying the benefits of adhesion molecule blockade in polyarthritis.

The identification of a synovium-specific adhesion molecule that facilitates the adhesion and subsequent migration of lymphocytes to the joint might make it possible to block entry of antigen-specific cells into synovium, while having minimal effects on systemic immuno-competence.

8.1.8 Ambiguities and Limitations

8.1.8.1 The functional significance of adhesion.

It has been postulated that only about one third of adhered cells actually migrate into the tissues (Bjerknes *et al.,* 1986). With this in mind, the current assay cannot, of itself, illuminate whether an adherent cell would migrate into the tissue or detach and return to the circulation. In particular, it is not known whether the washing steps in the assay approximate the shear forces in the microvasculature. The stability of adherence could be studied by adapting the flow chamber studies of Lawrence and Springer (1991), where dynamic forces can be calculated, to a chamber containing a microvascular EC monolayer. Further aspects of lymphocyte-EC interactions could be investigated by modification of this model. The assay could also be modified to study extravasation through the monolayer, using EC grown on an extracellular matrix.

8.1.8.2 EC growth matrices

Fibronectin is a multifunctional protein that is capable of homeotypic interactions as well as

heterotypic adhesion with molecules such as collagen, heparin and proteoglycans. It has binding sites for cell surface adhesion molecules and can, therefore, function as an anchor molecule between cellular and extracellular matrix components (Ruoslahti, 1988). T cells can interact with fibronectin via members of the β1 integrin superfamily, VLA-4 and VLA-5 (Takada *et al.,* 1987;Ruoslahti, 1988).

Observation of adherent lymphocytes in the assay showed that they attached to the EC, rather than to the exposed fibronectin matrix between the cells. However, if some lymphocytes were attached to fibronectin via the adhesion molecule VLA-4, blocking with anti-VLA-4 mAb may inhibit binding to extracellular matrix as well as binding to EC via the counter receptor VCAM-1. In retrospect, binding of lymphocytes to fibronectin-coated plates should have been investigated. Physiologically, binding of extravasated lymphocytes to extracellular matrix may be an important factor in retaining the cells in the tissues and determining the transit times of cells that leave the tissues via the lymphatics or by re-entry into blood vessels. Blocking this adhesion is actually exploring a later step in the adhesion/ trans-endothelial migration process concerning receptors involved with adhesion to extracellular matrix components that have a role in migration within the extravascular, interstitial compartment to facilitate trafficking and retention in the tissues.

8.1.8.3 *In vitro* changes to adhesion molecule expression

An important strength of the adhesion model developed in this work is that it uses freshly isolated EC that have been maintained *in vitro* for less than 24 hours. Nevertheless, it cannot be excluded that expression of adhesion molecules may have been changed by culture conditions or by the processes used to prepare and purify the cells. The EC have been exposed to collagenase, have lost contact with the natural extracellular matrix and been exposed to to surface labelling with anti-CD36 antibody. However, the micro-assay system has avoided extensive growth *in vitro* and numerous rounds of trypsinisation. It is likely that the EC retain expression of more surface molecules associated with the differentiated state of synovial microvascular EC than cells that have been expanded by growth *in vitro* for use in assays that require larger numbers of cells.

8.2 Future Work

This work has shown that it is possible, through the development of micro-assays, to study lymphocyte adhesion to freshly isolated microvascular endothelium. This model can be used to investigate the cascade of events that lead to adhesion and migration of lymphocytes into normal and inflamed tissues. In addition to use in exploring new tissue specific adhesion molecules and their roles in attachment, the assay could be adapted to study migration into a sub-endothelial matrix on which the EC are seeded. Migrated cells could be collected and their phenotype identified, to compare them against the phenotype of cells found in synovium. Adhesion and migration assays could also be carried out under shear-flow stress to emulate the flow conditions normally experienced by marginated leucocytes *in vivo*. This could investigate molecules that are important in rolling and adhesion under sheer flow stress or flow conditions in specific vascular beds. This could also be extended to investigate the combined effects of anti-adhesion molecule antibodies. Additive interaction between some adhesion molecules has been reported previously (Issekutz *et al.*, 1996). It is likely that the process of adhesion and migration is mediated by a combination of adhesion molecules, rather than by single molecules acting at each stage of the cascade.

The method of producing a mixed cell suspension from the synovial tissue of rats with polyarthritis has already shown great utility in a number of projects within our research group. Future use of this method will continue in further investigations, studying the role of different cell types in this model of inflammation.

The micro-assay of adhesion has advantages for studying lymphocytes also. It is known that the arthritogenic cells in the thoracic duct lymph from arthritic donors belongs to a subset of activated CD4+ T cells. It would be possible to purify subpopulations of activated CD4+ T cells, using fluorescence activated cell sorting, and to examine their adherence to synovial microvascular endothelium. The adhesion molecules responsible for adhesion could be identified by the use of specific mAbs and subsets of adherent lymphocytes identified in this way could be tested for arthritogenicity *in vivo*.

The hybridomas that were studied in this work have not been cloned. Cloning those that produce antibodies with anti-adhesive properties is an obvious next step to characterising the molecules to which they bind. The resulting mAbs can be used to immunoprecipitate the cognate antigens and to identify them in Western analysis. Furthermore, they can be used to monitor expression cloning of the relevant genes. The main objective of this work is the identification of normal adhesion molecules that function as addressins in the synovial microvascular endothelium.

Although the causes of RA are still not well defined, an improved understanding of the pathogenesis of this disease will allow the development of new and innovative therapies. An understanding of each facet of the adhesion cascade leading to T cell migration into synovium should help to target molecules that are directly involved with the process of synovial inflammation. By targeting these molecules, treatments might be developed that are more efficacious, specific and systemically less toxic than current therapies. Adhesion molecules are established in their role in lymphocyte recruitment and the progression of the disease. Novel therapeutic approaches aimed at blocking the function of adhesion molecules may prove beneficial in the management and prevention of arthritis. If adhesion molecules can be identified that have expression limited to synovium, they could offer an immunotherapy for arthritis that is limited in its affects on the systemic immune system.

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