STUDIES ON MONONUCLEAR CELL ADHESION TO MUCOSAL ENDOTHELIAL CELLS IN RHEUMATOID ARTHRITIS.

Thesis submitted for the degree
of Doctor of Philosophy at the University of Leicester

by

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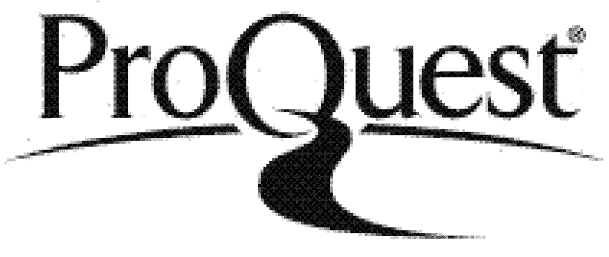

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Studies on lymphocyte adhesion to mucosal endothelial cells in rheumatoid arthritis.

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The aetiology of rheumatoid arthritis (RA) remains largely unknown. This thesis investigated two questions (1) the likely origin of synovial membrane and synovial fluid lymphocytes in patients with RA, and (2) the effects of corticosteroid therapy on the adhesion of rheumatoid lymphocytes to endothelium.

The migratory patterns of lymphocytes known to be of mucosal and peripheral lymph node origin were compared to those of rheumatoid lymphocytes from peripheral blood (PB), synovial fluid (SF) and synovial membrane (SM). The adhesion of such lymphocytes to mucosal and lymph node endothelium was studied and the possible adhesion molecules involved were analysed by a series of experiments that included monoclonal antibody inhibition studies. Synovial fluid lymphocytes from RA patients exhibited adhesion properties more in keeping with lymphocytes of mucosal than of lymph node origin. Synovial membrane lymphocytes, by contrast, showed poor adherence to mucosal endothelium, suggesting the involvement of different adhesion molecules, than those used by SF lymphocytes.

The adhesion of RA PB lymphocytes to mucosal endothelium was inhibited by over 50%, 24 hours following intravenous pulse steroid (methylprednisolone) therapy. To identify whether this effect was due to fluctuations in circulating lymphocyte subsets or changes in adhesion molecule expression, circulating as well as adherent lymphocyte subsets were analysed by flow cytometry and immuno-histochemistry respectively. Percentage changes in the CD3 population of circulating lymphocytes and the HLA-DR population of adherent lymphocytes were statistically significant at $P = 0.03$ and 0.01 respectively. Levels of circulating VCAM-1 were also analysed before and after pulse steroid therapy, with no significant changes observed.

For my family,

To whom this work is dedicated to..

Acknowledgements

I would like to thank Dr. Peter Sheldon for his supervision and advice throughout my PhD, his good humour and his attempts at trying to make a "good scientist" out of me. I hope he has succeeded. Thanks for all the conferences too!

I would also like to thank all of the following for their friendship and company over the years, its been great knowing you; Fred "Full House" Esumeh, Dave "One pair" Simpson, Steve "Amnesty International" Gordon, Hasan "Can't stay, can't come, got to work" Yesilkaya, Andrew "one more pint" Wallace, Ala'eddin "Yet another paper in Thorax" Ahmed, Nebil "Ratcliffe wrist (forward)" Behar and "Little Stinky" (who knows who she is).

Also a big thanks for Pat, who has always helped me with computer problems, no matter how silly they may have sounded to her.

Finally, a special dedication to Abby, who has stuck by me in good times and bad, and has managed to calm me down in times of despair, as well as be a source of happiness. Thank you.

Statement of originality

The accompanying thesis submitted for the degree of Ph.D. entitled "Studies on mononuclear cell adhesion to mucosal endothelial cells in rheumatoid arthritis" is based on work conducted by the author in the Department of Microbiology & Immunology of the University of Leicester mainly during the period between September 1992 and October 1995.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: Date:

Abbreviations

APAAP	=	Alkaline phosphatase anti-alkaline phosphatase
ARA	=	American rheumatism association
Ca^{2+}	=	Calcium
CLA	=	Cutaneous leukocyte antigen
Co^{2+}	=	Cobalt chloride
CRP	=	C-reactive protein
CSM	=	Cell suspension medium
DAB	=	Diaminobenzidine tetrahydrochloride
DMARDs	=	Disease modifying anti-rheumatic drugs
DNA	=	Deoxyribonucleic acid
DSM	=	Dextran sedimentation mixture
DTT	=	Dithiothreitol
EC	=	Endothelial cell
EDTA	=	Ethylene diamine tetra-acetic acid
EGF	=	Epidermal growth factor
EGTA	=	Ethylene glycol tetra-acetic acid
ELISA	=	Enzyme linked immuno-sorbent assay
ESL-1	=	E-selectin ligand-1
ESR	=	Erythrocyte sedimentation rate
FGFR	=	Fibroblast growth factor receptor
GALT	=	Gut associated lymphoid tissue
GLP	=	Gut lamina propria
GlyCAM-1	=	Glycosylation dependent cell adhesion molecule-1
HA	=	Hyaluronic acid
HBSS	=	Hank's balanced salt solution
HEC	=	High endothelial cell

HEV	=	High endothelial venule
HUVEC	=	Human umbilical vein endothelial cell
IC	=	Immune-complex
ICAM	=	Intercellular adhesion molecule
ICM	=	Iscove's culture medium
Ig	=	Immunoglobulin
IL	=	Interleukin
IFN-γ	=	Interferon-gamma
IV	=	Intravenous
LAD	=	Leukocyte adhesion deficiency
LFA	=	Leukocyte function associated molecule
LFM	=	Lymphocyte freezing mixture
LN	=	Lymph node
LNL	=	Lymph node lymphocytes
LPAM-1	=	Lymphocyte Peyer's patch adhesion molecule-1
LPS	=	Lipopolysaccharide (bacterial)
MAdCAM-1	=	Mucosal addressin cell adhesion molecule-1
MALT	=	Mucosa associated lymphoid tissue
MC	=	Mononuclear cell
mg	=	Milligram
Mg^{2+}	=	Magnesium
MHC	=	Major histocompatibility complex
µl	=	Microlitre
ml	=	Millilitre
mm	=	Millimeter
mM	=	Millimolar
Mn^{2+}	=	Manganese
MNL	=	Mesenteric lymph node lymphocytes
MP	=	Methylprednisolone

ng	=	Nanogram
Ni^{2+}	=	Nickel chloride
NSAIDs	=	Non-steroidal anti-inflammatory drugs
PAF	=	Platelet activating factor
PB	=	Peripheral blood
PBS	=	Phosphate buffered saline
PECAM-1	=	Platelet endothelial cell adhesion molecule
PP	=	Peyer's patch
PPL	=	Peyer's patch lymphocytes
PS	=	Pulse steroid
PSGL-1	=	P-selectin glycoprotein-1
RA	=	Rheumatoid arthritis
ReA	=	Reactive arthritis
RF	=	Rheumatoid factor
RNA	=	Ribonucleic acid
SCR	=	Short consensus repeats
SF	=	Synovial fluid
SM	=	Synovial membrane
TCR	=	T cell receptor
TGF-β	=	Transforming growth factor-beta
TNF-α	=	Tumour necrosis factor-alpha
VAP-1	=	Vascular adhesion protein-1
VCAM-1	=	Vascular cell adhesion molecule-1
VLA	=	Very late antigen

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The aim of the first part of the thesis was to develop and validate an in vitro adherence assay involving porcine mononuclear cells (MCs) and porcine endothelium, present within gut and lymph node. Factors involved in MC / endothelium interactions were determined. In summary we found that cell adhesion in our assay system was temperature, Ca^{2+} and Mn^{2+} sensitive, required metabolic activity, was inhibited by the phosphorylated monosaccharide galactose 6-phosphate, and unaffected by the presence of mucus. These findings reflected certain aspects of in vivo cell adhesion, present within the in vitro assay used. The adhesion characteristics of porcine Peyer's patch (PP), peripheral blood (PB), and lymph node (LN) MCs to porcine gut and lymph node endothelium was examined and used as an guiding model for the future study of human MCs adherence. It was found that PP MCs adhered significantly better to gut endothelium than to LN endothelium and similarly LN MCs adhered significantly better to LN endothelium than to gut endothelium. PP MCs exhibited significantly greater levels of adherence to gut endothelium as compared to PB and LN MCs.

The second part of the thesis was to apply the assay in order to study the adherence of MCs from patients with rheumatoid arthritis (RA) and to compare results with those obtained from porcine MCs. In summary we found that RA synovial fluid (SF) MCs exhibited adherence characteristics in keeping with those of PP MCs, with the adherence of RA SF MCs to gut endothelium significantly greater than to lymph node, and also significantly greater than RA synovial membrane (SM), PB and control PB MCs adherence to gut endothelium. This pattern of adhesion was maintained when adherence to human gut endothelium was also examined. The possible adhesion molecules involved were also studied by inhibition experiments using various monoclonal antibodies.

The third part of the thesis, studied the in vitro effects of corticosteroid (methylprednisolone) treatment on cell adhesion, using porcine MCs. We then proceeded to study the effects of in vivo methylprednisolone (MP), given intravenously to RA patients. The effects on ex-vivo adherence to porcine gut endothelium showed on average a 58% inhibition of adherence of PB MCs, 24 hours following MP therapy. The question of whether this was a quantitative or qualitative effect was addressed by the immunophenotyping of circulating and adherent PB MC subsets. We observed no significant effect on the majority of circulating PB MC subset populations, except for the CD3+, CD4+ and CD45RA+ subsets, no significant effect on adherent subsets except for the HLA-DR+ subset was observed and additionally no significant effect upon the circulating levels of soluble VCAM-1 was observed.

In conclusion, this thesis has shown a reliable and reproducible in vitro cell adhesion assay, RA SF MCs which share adhesion characteristics with porcine PP MCs and differ from porcine LN and RA SM MCs. In vivo corticosteroid treatment in RA patients which significantly reduces adhesion and strengthens the likely relevance of the assay in understanding adhesion properties and likely origin of MCs involved in the rheumatoid process.

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Appendix 1 : General reagents

Appendix 2 : American rheumatism association revised criteria for rheumatoid arthritis classification.

Appendix 3 : Publications which cover work presented in thesis

CHAPTER 1

Introduction

1.1 Lymphocyte recirculation

The most important feature of the immune system, is its ability to protect the host against pathogenic micro-organisms. Lymphocytes play a major role in such a defence system. It is these cells of the immune response that specifically recognise and respond to foreign antigen (Abbas et al., 1994). To achieve this role, lymphocytes need to continuously travel into and out of the organs of the body, by means of the bloodstream and lymphatic system (Ager 1994). The cornerstone of this process are the organised secondary lymphoid organs; lymph nodes, Peyer's patches and spleen (Imhof & Dunon 1995). They are of vital importance, because it is these organs that drain the sites of antigen entry, and subsequently produce "samples" of such collected antigens to lymphocytes. Therefore it is at these sites, that lymphocytes for the first time since their production, development and differentiation in the primary lymphoid organs (bone marrow and thymus), encounter the molecules which their antigen receptors can recognise (Gray 1992). It is crucial therefore, for lymphocytes to migrate continuously in and out of these secondary lymphoid organs, and maximise the probability of an immune interaction (Yednock & Rosen 1989). This highly complex, yet elegantly developed scenario is called lymphocyte recirculation.

1.2 Historical background

The continuous recirculation of lymphocytes from blood to lymphoid organs and back to blood again, was first shown by the pioneering work of Gowans and his colleagues (Gowans 1957, 1959, Gowans & Knight 1964). It was shown that when tritium labelled small

lymphocytes obtained from the thoracic duct of the rat were transfused intravenously into other rats of the same inbred strain, the labelled small lymphocytes rapidly migrated to the spleen, Peyer's patches and peripheral lymph nodes, and that a large proportion of these labelled cells could be recovered from the thoracic duct within a 5-day period (Gowans & Knight 1964). These findings taken together with earlier work (Gowans 1957, 1959) showed that transfused small lymphocytes recirculate in large numbers from blood to lymphoid tissues to blood again. In contrast to this, tritium labelled large lymphocytes from thoracic duct lymph localised primarily in the lymphoid tissue of the gut following transfusion into the blood, with few being found in the spleen and virtually none in peripheral lymph nodes, which led to the suggestion that this selective migration may be due to the fact that most large lymphocytes in the thoracic duct where derived from intestinal lymphoid tissue, where they may have been "sensitised" by antigenic material originating in the gut (Gowans & Knight 1964). These studies not only provided the experimental basis for lymphocyte recirculation, but more importantly established that certain lymphocytes migrated in an organ specific fashion. This paved the way for numerous other investigators to reveal a migratory system of remarkable specificity.

Initially two organ specific lymphocyte migration pathways were demonstrated, one to mucosal lymphoid organs and the other to peripheral (subcutaneous) lymph nodes (Griscelli et al., 1969). It was found that tritiated thymidine labelled large lymphocytes obtained from lymph nodes of rats exhibited migration to lymphoid tissue after intravenous injection into the thoracic duct of sysgeneic recipients (Griscelli et al., 1969). In addition to finding that large lymphocytes possessed the same capability of recirculation as small lymphocytes did, it

was also discovered that they showed different patterns of migration, depending upon the source of the donor cells, such that cells from the peripheral nodes localised preferentially in the peripheral lymph nodes and mesenteric lymph node cells accumulated preferentially in lymphoid tissue within the gut, whereas small lymphocytes did not display a similiar tendency to accumulate selectively in lymphoid tissues of certain regions (Griscelli et al., 1969).

Subsequent work substantiated and further developed these earlier findings. Amongst these was the observation that tritiated thymidine labelled large lymphocytes (by this time being called lymphoblasts) from the mesenteric lymph node and thoracic duct of mice and rats, when transferred into same species animals bearing a graft of fetal intestine (placed under the kidney capsule and devoid of exposure to antigenic stimulus provided by intestinal bacteria or food) migrated into the graft's mucosa in the same manner as observed within the hosts own gut. This led to the suggestion that such selective migration was not antigen driven but could instead be due to the existence of membrane receptors which could mediate gut selective migration (Guy-Grand et al., 1974). Similiar patterns of organ selectivity to those demonstrated in mice and rats, were also demonstrated in sheep using unfractionated cells (Scollay et al., 1976) and purified T cells (Cahill et al., 1977), where lymphocytes from intestinal and peripheral lymph recirculate through the gut and the peripheral nodes, respectively. The study of the migration patterns of tritiated thymidine labelled donor mesenteric lymph node B cell immunoblasts and their subsequent accumulation within recipient gut, cervix and vagina, uterus, mammary glands and mesenteric lymph nodes over a 24 hour period, in contrast to the small numbers of labelled peripheral lymph node donor B cell immunoblasts found in these tissues

led to the suggestion of a common mucosal immunological system in mice (McDermott & Bienenstock 1979). The overall consensus to emerge from these studies, was that small lymphocyte recirculation took place in a general, random pattern to secondary lymphoid organs and that the migration of large lymphocytes took place in an organ specific manner dependent upon the lymphoid tissue origin of the cells involved. It was further suggested that this preferential migration was in some manner mediated by specific surface receptors on lymphocytes and their counter-receptors on tissue. This process of migration was termed "lymphocyte homing". The details of which was to emerge in the following years.

As more was understood of lymphocyte migratory pathways, the more important became the role of high endothelial venules (HEV). Since their discovery by Thome in 1898 (Schoefl 1972), these specialised vessels have been described in all secondary lymphoid organs including lymph nodes, Peyer's patches, tonsils, adenoids, appendix and within lymphoid aggregates associated with the mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts (Yednock & Rosen 1989, Girard & Springer 1995). It was initially demonstrated by electron microscopy in the rat that lymphocytes selectively migrated from blood circulation into lymph nodes through these high endothelial venules (Marchesi & Gowans 1964, Howard et al., 1972). Initial studies focused on morphology and histology, and the interaction of lymphocytes with HEV were further examined by scanning and transmission electron microscopy (Schoefl 1972, Van Ewijk et al., 1975, Anderson & Anderson 1976). Such studies revealed how lymphocytes interacted with endothelial cells through numerous microvilli, forming initial contacts and subsequent close contact and adherence. The prevailing notion was that somehow, certain structures on the surface of endothelial cells and on

lymphocytes assisted and augmented this contact, though receptors or ligands had not, as yet, been demonstrated.

An advancement in the understanding of such interactive adhesive contacts, and their role in lymphocyte homing, was achieved due to a newly developed in vitro adherence assay (Stamper & Woodruff 1976). Basically, the selective adherence of thoracic duct lymphocytes to the endothelium of HEV when overlaid onto glutaraldehyde fixed frozen sections of lymph nodes was demonstrated, with the numbers of adherent lymphocytes being proportional to the endothelial area. Adherence was selective in the fact that lymphocytes did not adhere to any other vascular structure in the node and that there was little adherence to other cells in the sections. All of which led to the suggestion that thoracic duct lymphocytes had surface membrane structures specific for lymph node HEV recognition (Stamper & Woodruff 1976). It was also indicated that HEV-adherent lymphocytes were capable of dissociation under physiological conditions, which supported prevalent suggestions that lymphocytes migrated through lymphoid tissues by binding to HEV with subsequent transmigration into the parenchyma (Anderson & Anderson 1976). Data which indicated that lymphocyte adherence is an energy dependent, calcium requiring event that involves membrane receptors which are sensitive to trypsin treatment also emerged from experiments using this new adherence assay (Woodruff et al., 1977). By means of this assay it was now possible to answer some of the questions asked with regards to lymphocyte-HEV interactions, to quantify lymphocytes layered over cryostat cut sections of lymphoid tissue, and also to quantify the "selective" adherence of these lymphocytes to HEV. It was the development of this in vitro adherence assay that allowed researchers to

substantiate their theories on organ specific lymphocyte homing, and the roles that complementary ligands were to play.

1.3 Development of lymphocyte homing

Studies carried out by means of using the in vitro adherence assay, demonstrated the existence of organ specific migratory pathways, based on the role that adhesive receptors played, on endothelial cells and lymphocyte cell surfaces. Based on such experiments, a model was proposed, in which two different receptor sets existed, one specific for Peyer's patch (mucosal lymphoid tissue) and the other for peripheral lymph nodes (Butcher et al., 1979, 1980). It was suggested that HEV in mouse Peyer's patches and peripheral lymph nodes present different, distinctive surface receptors for lymphocytes, and that lymphocytes could express surface receptors specific for these organ-restricted endothelial cell determinants, hence selectively directing their traffic through these organs (Butcher et al., 1980). This was done primarily by studing the adherence of peripheral lymph node lymphocytes (PLN), mesenteric lymph node lymphocytes (MNL) and Peyer's patch lymphocytes (PPL) adherence to Peyer's patch and peripheral lymph node HEV and also by the relative localisation of ^{51}Chromium labelled PLN, MNL, PPL in several HEV bearing organs 30 minutes after intravenous injection (Butcher et al., 1980). A close resemblance was observed between in vitro adherence characteristics and in vivo localisation of aforementioned lymphocyte populations, with HEV in Peyer's patches demonstrating a selective affinity for PPL, HEV in peripheral lymph nodes selectively binding PLN and mesenteric lymph node HEV appearing to demonstrate an almost equal affinity for both PPL and PLN (Butcher et al., 1980).

This work was central to further advance the understanding of lymphocyte homing insofar as the proposal that lymphocyte surface receptors specific for organ restricted endothelial cell determinants mediated the antigen independent organ specificity of lymphocyte migration (Butcher et al., 1980).

This model was confirmed by further studies and the development of specific monoclonal antibodies, which inhibited the adherence of lymphocytes to HEV in selected organs. Of these the development of the monoclonal antibody Mel-14 which reacted with the cell surface of mouse lymphoma cell lines that adhered selectively to peripheral lymph node HEV (Gallatin et al., 1983) and the development of the monoclonal antibody Meca-367 which recognised HEV within gut associated mouse lymphoid tissues (Streeter et al., 1988) was of great value. Mel-14 was demonstrated to inhibit peripheral lymph node lymphocyte adherence to peripheral lymph nodes, but not to Peyer's patch HEV in vitro and furthermore treatment of lymphocytes with MEL-14 prior to intravenous infusion inhibited their localisation within peripheral lymph nodes but not within Peyer's patches (Gallatin et al., 1983). In contrast, MECA-367 stained all HEV in Peyer's patches, but fail to stain HEV within peripheral lymph nodes and likewise in vitro and in vivo work demonstrated inhibition of localisation and inhibition of adherence of lymphocytes to Peyer's patch HEV, but not to peripheral lymph node HEV (Streeter et al., 1988).

By this time, the receptors present on lymphocyte cell surfaces were being called "homing receptors" and their complementary ligands (previously called determinants) on endothelial cell surface, "vascular addressins". At present today, an overwhelming abundance of these

adhesion molecules have been studied, finally leading to a coherent model of lymphocyte homing, in an multistaged adhesion cascade. The important features of this process will be examined in section 1.7.5.

1.4 Lymphocyte activation and the recirculation of naive and memory cells.

In common with secondary lymphoid organs, Peyer's patches are sites of lymphocyte extravasation, and subsequent activation (more detail of Peyer's patches in section 1.5). Initially, B and T lymphocytes which have differentiated in the bone marrow and thymus respectively, migrate to the Peyer's patches and other secondary lymphoid organs, as mature, but non the less naive lymphocytes, since they have not yet encountered antigen to which their antigen receptors can respond to. During their extravasation from blood into the Peyer's patches (through HEV) if they fail to be presented with antigen which can be recognised, they remain as naive lymphocytes and return back to the blood stream through the efferent lymphatics and subsequently the thoracic duct. Hence naive lymphocytes travel continuously from the blood lymphocyte pool, into different secondary lymphoid organs, out through the lymphatics and back again to the blood lymphocyte pool, until they respond to their specific antigen, or until they die. The migration of naive lymphocytes to lymphoid organs is not organ specific, rather, it is an non-specific recirculation of lymphocytes, with equal migration patterns to peripheral lymph nodes, spleen and Peyer's patches (Hamann & Rebstock 1993). To participate in an immune response these naive cells must be induced to proliferate and differentiate into cells capable of contributing to the

removal of antigen. Such a capability is achieved by the presentation of specific antigen and subsequent activation.

Lymphocytes which are presented with antigen within the secondary lymphoid organs, and respond to it, transform into lymphoblasts. These cells are the progeny of lymphocytes that have been activated by antigen, and are dividing cells larger than naive lymphocytes in size and which can be distinguished from such cells by labelling with thymidine analogues. These large cells eventually divide and differentiate into "effector" cells which eliminate antigen from the body. B lymphoblasts generate immunoglobulin secreting plasma cells and T lymphoblasts generate cytotoxic lymphocytes (CD8) and cytokine secreting lymphocytes (CD4). Effector T cells can be placed into three functional classes that detect processed antigens presented as peptide fragments on the cell membrane surface of antigen presenting cells (APCs) such as macrophages, dendritic cells and B cells. This antigen presentation is done in association with specialised cell surface glycoproteins, which are encoded in a cluster of genes called the Major Histocompatibility Complex (MHC). Two different classes of these molecules are required for antigen presentation and subsequent lymphocyte activation; the MHC class-I and MHC class-II. Antigens derived from pathogens that multiply within the cell (viruses for example) are carried to the cell surface by MHC class-I molecules. Briefly, antigenic material processed into peptides in the cytoplasm by large protease complexes called proteasomes, are transported to the lumen of the endoplasmic reticulum where pre-assembled MHC class-I molecules bind to it and form a peptide : MHC complex which is then exported out of the endoplasmic reticulum through the Golgi complex to the cell surface and presented to CD8 cytotoxic T lymphocytes that kill the

infected cell (Janeway & Travers 1996). Antigens derived from pathogens multiplying in intracellular vesicles, and those derived from ingested extracellular bacteria and toxins, are not accessible to proteasomes, instead antigenic proteins are degraded by vesicular proteases into peptides and these peptide containing vesicles fuse with other vesicles containing newly synthesized MHC class-II molecules, which bind the peptide fragments of the pathogens and deliver them to the cell surface where they are recognised by CD4 T cells. These cells in turn, can further differentiate into two types of effector T cell; the inflammatory T lymphocytes (T_H1) which activate infected macrophages to destroy intracellular pathogens, and the helper T lymphocytes (T_H2) which activate specific B cells to produce antibody (Janeway & Travers 1996).

It is these T_H cells which orchestrate the immune response, by promoting intracellular killing by macrophages, antibody production by B lymphocytes and clonal expansion of cytotoxic T lymphocytes (Fearon & Locksley 1996). T_H1 lymphocytes secrete cytokines such as Tumour Necrosis Factor-α (TNF-α) Interferon-γ (IFN-γ) and lymphotoxin, which induces nitric oxide synthase in macrophages (to promote the killing of intracellular pathogens), and IFN-γ causes B lymphocytes to switch their immunoglobulin isotype to IgG1 (in humans) which promotes phagocytosis by activating the classical pathway of complement and binding to receptors on macrophages (Fearon & Locksley 1996). T_H2 lymphocytes on the other hand secrete cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 which in combination mediate the growth and activation of mast cells, eosinophils, cause B lymphocyte immunoglobulin switching to IgE and IgG4 (in humans) and inhibit macrophage activation (Fearon & Locksley 1996). Clearly this last function described has important immune regulatory implications in countering the tissue damaging effects

of T_H1 lymphocytes, since by virtue of their ability to phagocytose and present antigen, macrophages may also bias the development of T_H1 lymphocytes. Additionally, cytokines such as IL-12 and TNF-α, secreted by activated macrophages leads to the differentiation of naive T cells to the T_H1 phenotype, which in turn secretes IFN-γ which augments IL-12 secretion, hence IL-12 and IFN-γ make up a positive feedback system that amplifies the levels of IFN-γ for macrophage activation and IL-12 for the proliferation of T_H1 lymphocytes (Fearon & Locksley 1996).

Other than the effector cells described above, lymphoblasts can also differentiate into "memory" lymphocytes which upon re-exposure to antigen can rapidly proliferate and differentiate back into effector cells. This rapid response is vital in controlling the extent of infection and preventing further disease. Memory lymphocytes are morphologically indistinguishable from naive lymphocytes (and hence are smaller in size than lymphoblasts) except for a few cell surface markers, one such distinguishing molecule being the CD45 cell surface protein. Naive T cells that have not been exposed to their specific antigens express a 200kD isoform of this protein that contains a segment encoded by an exon designated "A". This CD45 isoform can be recognised by antibodies specific for the A encoded segment, and is therefore called CD45RA (RA standing for "restricted A"). CD45RA does not associate with either the T cell receptor or co-receptors. In contrast most activated and memory T cells express a 180kD isoform of CD45 in which the A exon has been spliced out, this isoform is thus called CD45RO (Abbas et al., 1994). CD45RO is known to associate with both the T cell receptor and the co-receptor, enabling this isoform to transduce signals more effectively than the receptor on naive cells (Janeway & Travers 1996). Cell surface markers which distinguish between memory T lymphocytes from naive T

lymphocytes are not useful in differentiating between memory and effector cells (since both express the CD45RO marker), hence size and functional differences have been generally used instead for identification.

Not much is known about the precise conditions and signals that lead to the development of memory versus effector cells. It has been suggested that large amounts of antigen plus the presence of inflammatory signals and cytokines may favour the development and differentiation of effector T cells, whereas the presence of antigen in the absence of such cytokines or signals may result in the generation of memory T lymphocytes (Ahmed & Gray 1996). The convention at present is to suggest that memory lymphocytes are derived directly from effector cells. Another view is that the balance between memory and effector cells is governed by the level of antigenic stimulation (Ahmed & Gray 1996). Indeed there has been substantial debate regarding the role of specific antigen in maintaining T cell memory. The two current theories would put forward the view that memory is due to long lived memory lymphocytes that do not require contact with specific antigen for their survival, while the other view would suggest that long term memory is dependent upon the continous stimulation of T cells by persisting antigens (such as a low grade infection) (Ahmed & Gray 1996). It is possible that memory lymphocytes are long lived but need antigen stimulation to maintain a state of readiness, or that they cycle because of a unspecific stimulus rather than specific antigenic stimulation. The patterns of memory/effector cell recirculation is better known however, and they are substantially different from naive lymphocyte recirculation.

In general, naive lymphocytes recirculate through secondary lymphoid tissues (lymph nodes, Peyer's patches, spleen) in a random

manner, that is, they show no preference in their migration to these lymphoid organs (Butcher & Picker 1996). Their migratory properties appear to be determined ontogenically as a function of their class, and they probably traffic continously between the different secondary lymphoid organs using lymph and blood circulation systems untill they die, or they respond to their specific antigen which is accumulated and presented in these lymphoid organs (Imhof & Dunon 1995). Upon activation however, (from the naive to the effector cell and memory lymphocyte stage) these recirculation patterns change substantially (see diagram-1 for both naive and organ specific lymphocyte circulation). The main difference is that memory/effector cells preferentially return to the site of their activation, rather than randomly recirculate throughout the secondary lymphoid organs.

Most memory and effector cells may indeed traffic through lymphoid organs as well, but unlike naive lymphocytes, they can also recirculate through non-lymphoid immune sites such as the gut lamina propria, pulmonary interstitium, inflamed skin and joints (Butcher & Picker 1996). For example, naive lymphocytes activated in the Peyer's patches, will drain into the mesenteric lymph node, where they will undergo further differentiation and development, drain via the efferent lymphatics and thoracic duct back to blood and finally, home selectively to non-lymphoid peripheral tissues (such as the gut lamina propria) before returning back to the Peyer's patch, by means of the afferent lymphatics. This pattern of homing targets memory lymphocytes and effector cells to sites where they are most likely to encounter (or re-encounter) their specific antigen. Therefore this organ specific homing pattern, enables lymphocytes to seek out and eliminate the specific antigen to which they are activated against, throughout the lymphoid tissue and

DIAGRAM I a : RANDOM RECIRCULATION OF NAIVE LYMPHOCYTES

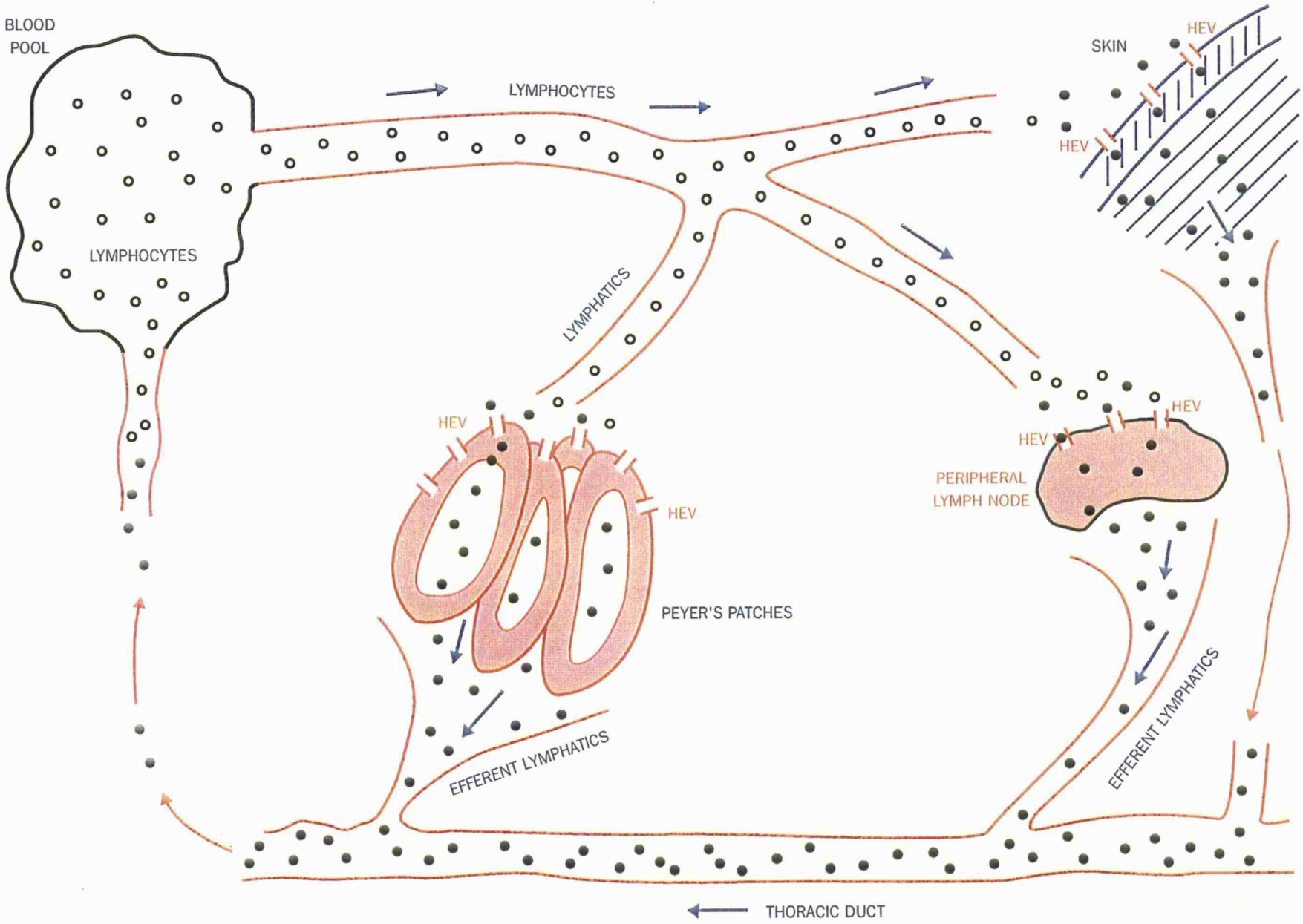
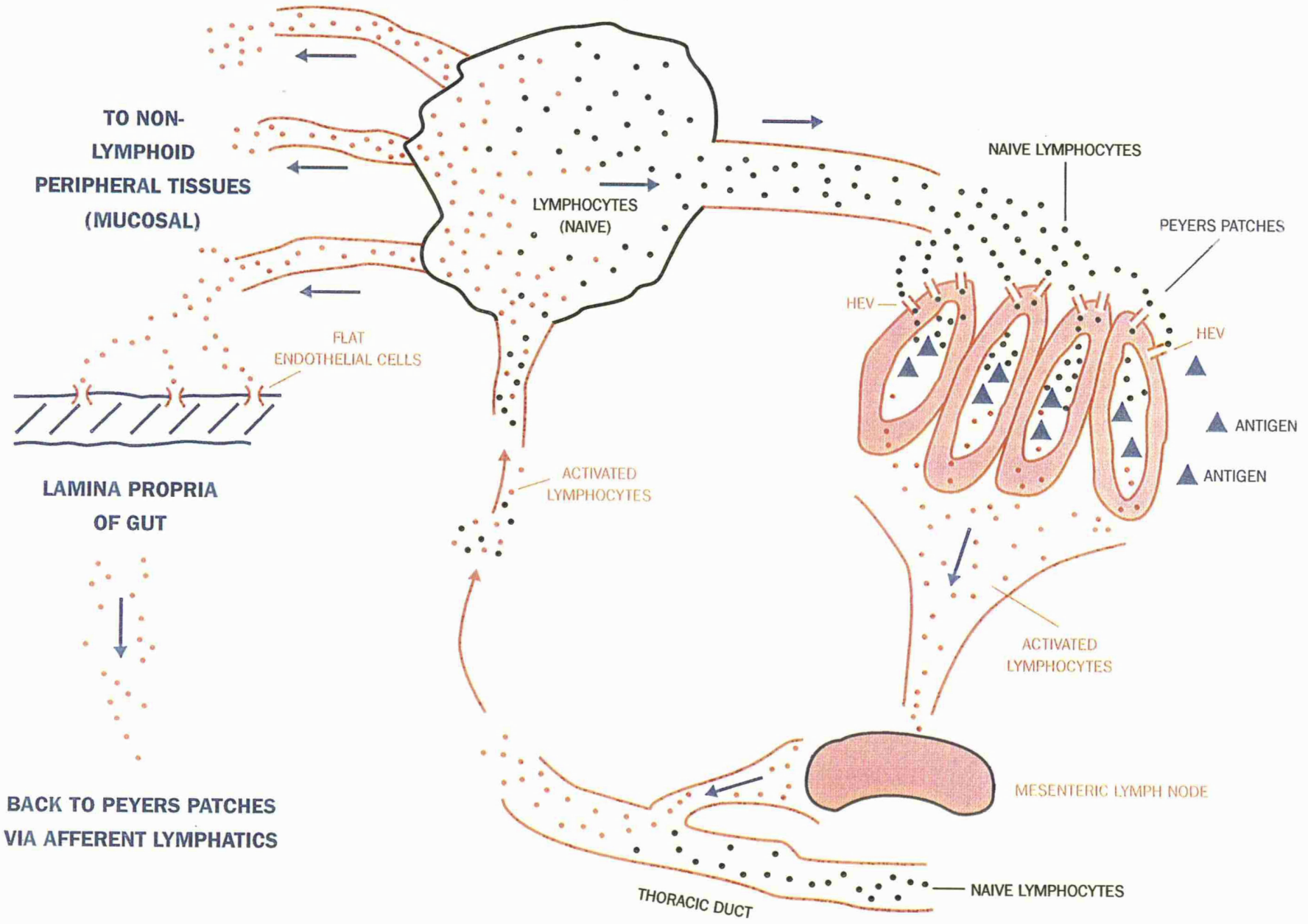


DIAGRAM 1b : ORGAN SPECIFIC CIRCULATION (MUCOSAL)

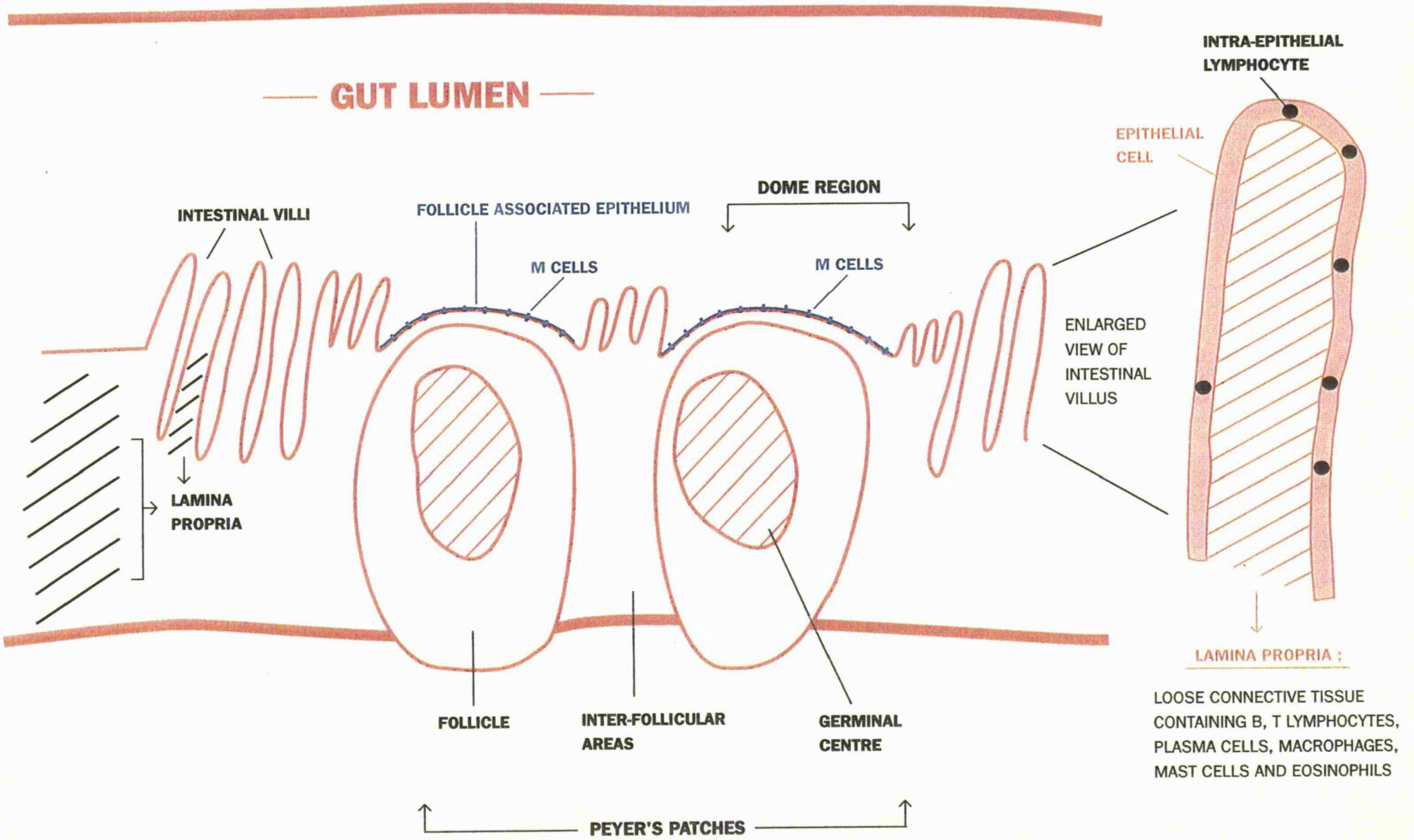


the associated peripheral non-lymphoid tissues concerned. Such changes in recirculation require changes in the expression of homing receptors, which are brought about by antigen induced activation. Homing receptor regulation during memory/effector T cell differentiation is analogous to that of T helper type T_H1 versus T_H2 lymphocyte differentiation, with immunoregulatory cytokines, nature of antigen and costimulatory signals all participating in the process of regulation (Butcher & Picker 1996). Adhesion molecules in general, and the ones involved in naive and memory lymphocyte homing, will be discussed in detail between sections 1.6 - 1.8.

1.5 The Gut Associated Lymphoid Tissue (GALT).

It is now generally acknowledged, that three separate lymphocyte homing pathways exist; peripheral lymph node homing, skin homing and homing to mucosa associated tissues (Imhof & Dunon 1995). The gut associated lymphoid tissue (GALT) is part of the larger mucosa associated lymphoid tissue (MALT), which consists of respiratory, intestinal and genital tissues (McDermott & Bienenstock 1979). The GALT itself, which includes the tonsils, adenoids and appendix also consists of aggregated lymphoid follicles called Peyer's patches, the surrounding lamina propria tissue and the overlying intra-epithelial areas within the small intestine (see diagram-2) (Targan 1992). The GALT contains several lymphoid populations that can be distinguished on the basis of anatomic and morphological considerations. Lymphocytes and plasma cells populate the loose connective tissue of the lamina propria, and lymphocytes are present between intestinal epithelial cells lining the intestinal villus (i.e., intraepithelial lymphocytes). Collections of organised lymphoid nodules

DIAGRAM 2 : REPRESENTATION OF PEYER'S PATCHES AND ASSOCIATED TISSUE



(i.e., Peyer's patches) as well as scattered solitary lymphoid follicles also line the intestinal tract (Kagnoff 1981).

The intestinal lamina propria contains B lymphocytes, plasma cells (with the majority of antibody production being of the IgA class, with approximately 20% of the IgM class), T lymphocytes (mainly of the CD4 population), macrophages, eosinophils, and mast cells interspersed in the vascular and lymphatic rich connective tissue of the villi and surrounding areas (Kagnoff 1981). Intraepithelial lymphocytes (IELs) are most often situated between the intestinal columnar epithelial cells lining the villous surface of the small intestine. They are present near the basement membrane in the basal part of the epithelium. Intraepithelial lymphocytes consist of nearly 90% CD8 phenotype T lymphocytes, in contrast to the lamina propria with its majority of the CD4 phenotype (Brandtzaeg 1996). The focal point of the GALT however, is the organised lymphoid structures, found in the wall of the gut tissue; the Peyer's patches. They are specialised secondary lymphoid organs in which draining and sampling of antigens from the gut lumen occurs with subsequent presentation to lymphocytes. They were first discovered in 1677 by the Swiss physician Johann Conrad Peyer (Griebel & Hein 1996).

Peyer's patches contain several distinct regions, such as the follicles, domes and thymus dependent areas between the follicles, with a compartmentalisation of T and B lymphocytes. The follicles contain germinal centres consisting largely of B lymphocytes. Cells in the deeper and lateral aspects of the follicle are actively dividing. T lymphocytes are found in the inter-follicular areas between follicles, as well as the dome region overlying the follicles (Kagnoff 1981). The epithelium overlying Peyer's patches (also refered to as follicle associated epithelium) differs

from surrounding villous surface in that it contains specialised membranous cells (M cells). These cells are different from adjacent cells, in that they lack fully developed microvilli, have luminal surfaces consisting of many small folds and have the ability to take up and transport material from the gut lumen to the sub-epithelial tissue by means of their endocytic domains (Kagnoff 1981, Neutra et al., 1996). M cells are believed to play a key role in the induction of immune responses in Peyer's patches, since they appear to be capable of transporting macromolecules, particles and microorganisms, which are unable to penetrate mature intestinal absorptive cells from the intestinal lumen to underlying lymphoid follicles. The M cell surface molecules that serve as receptors for this process have not been identified as yet however. M cells are not thought to express MHC molecules on their surface, hence even though they are responsible for the uptake and the transport of antigens, they are not thought to be involved in processing and presentation even though MHC class-II antigens have been documented in some subpopulations of M cells in the rat (Allan et al., 1993, Veys et al., 1996).

Instead M cells are in close contact with dendritic and B cells, both of which will communicate via MHC expression with the germinal centres of Peyer's patches containing activated B cells and antigen presenting cells. This contact is thought to be achieved by means of basal processes that extend 10μm or more into the underlying tissue, where they are thought to make direct contact with dendritic cells (Neutra et al., 1996). Adjacent to the germinal centres are the intrafollicular areas which contain predominantly CD4+ T lymphocytes. Therefore, antigens are presented to these T cells by macrophages and other antigen presenting cells (including B cells), leading to activation, resulting in the generation of memory lymphocytes by retaining and presenting antigen on follicular

dendritic cells under the influence of CD4+ T cells (Brandtzaeg 1996). Intestinal B cells differentiate in the lamina propria under the influence of macrophages, antigen presenting cells and CD4+ T cells into plasma cells, which mainly produce joining chain containing polymeric IgA that is transported to the gut lumen by epithelial transmembrane secretory component to become secretory IgA (Brandtzaeg 1996). Antigen uptake also occurs through the enterocytes at the secretory sites of the gut (villi). These enterocytes are in close contact with the intraepithelial lymphocytes which they interact with, possibly initiating cytotoxic and suppressor activity (Veys et al., 1996).

It is possible to define two broad groups in terms of the amount of Peyer's patch (PP) tissue found in different segments of the small intestine. The first group consists of pigs, dogs, horses, ruminants and humans, where PP tissue is unequally distributed along the small intestine, with more occuring in the ileum than in the jejunum. In these animals ileal PPs occur as a single continuous aggregation of lymphoid follicles and jejunal PPs as multiple, discrete accumulations of lymphoid follicles (Griebel & Hein 1996). In the second group of animals which include rabbits, rodents and chickens, PPs are found more or less at random intervals throughout the ileum and jejunum (Griebel & Hein 1996). The development of PPs are distinct as well, in these two different groups of animals. In the first group PPs develop in the ileum and jejunum prenatally, with ileal PPs reaching maturity early in life and then commencing involution, with the jejunal PPs persisting throughout the life span of the animal. In the second group of animals PPs develop 2-4 weeks postnatally, with jejunal PPs again remaining throughout the animals life span (Griebel & Hein 1996). These differing developmental features also present differing immunological features. It has been

demonstrated in sheep (less than five months of age) that ileal PP follicles contain a majority of B cells (of which 95% express surface IgM), with less than 1% T cells, and 2-3% macrophages in contrast to jejunal PP follicles which consist of fewer surface IgM+ B cells, more plasma cells (IgM, IgA and IgG1) and up to 15% T cells of which the majority are CD4+ (Griebel & Ferrari 1995). Additionally it has been demonstrated that labelled sheep thoracic duct lymphocytes that are transfused back into blood circulation exhibit low levels of localisation to ileal PP tissue in contrast to jejunal PP tissue, leading to suggestions that ileal PPs do not have a prominent role in lymphocyte recirculation (Reynolds 1988).

1.6 Endothelium and its role in cell migration

The endothelium consists of a single layer of tightly connected cells forming a boundary between the vascular lumen and the underlying tissues. Endothelial cells are polarised, consisting of a luminal membrane in contact with the blood and an abluminal membrane adherent to the subendothelial matrix (Defilippi et al., 1993). These two membranes are functionally distinct, with the non-adhesive luminal membrane allowing the free flow of blood cells and the abluminal membrane being firmly adherent to the basal lamina, providing anchorage to the subendothelium (Defilippi et al., 1993). Endothelium is an active tissue which mediates the traffic of cells and metabolites across the vessel wall, and is involved in inflammatory and immune responses. In contrast to endothelial cells from other vessels, the high endothelial cells of HEVs (which are found in all secondary lymphoid organs, with the exception of spleen) exhibit a number of distinguishing characteristics, such as different morphology, the expression of specialised ligands for the adherence of lymphocytes and

the ability to support high levels of lymphocyte extravasation (Girard & Springer 1995). The endothelial cells of HEVs have a plump, cuboidal shape to them, with the luminal side presenting a "cobblestone-like" appearence composed of dome shaped endothelial cells separated by deep intercellular clefts (Freemont & Jones 1983, Yednock & Rosen 1989). Ultrastructurally, high endothelial cells of HEVs have the characteristics of metabolically active, secretory type cells, exhibiting a prominent Golgi complex, abundant mitochondria, many ribosomes (frequently in polyribosome clusters), rough endoplasmic reticulum and a large rounded nucleus with one to two nucleoli (Yednock & Rosen 1989, Girard & Springer 1995). Endothelial cells from other vessels which do not exhibit this "high endothelial cell" morphology (and are therefore sometimes refered to as "flat endothelial cells") also do not exhibit the biosynthetic activity observed in the high endothelial cells of HEVs. Another feature of HEVs, is the existence of discontinuous junctions between the high endothelial cells. These junctions differ from the tight junctions found in capillary and arterial endothelium, and are thought to facilitate the passage of lymphocytes between adjacent high endothelial cells, allowing lymphocyte transmigration in HEVs (Girard & Springer 1995).

Unfortunately, only a small number of HEV-specific monoclonal antibodies have been described to date, although a much wider range of monoclonal antibodies exist, directed against specific adhesion molecules present on lymphocyte cell surfaces. Two of the HEV-specific monoclonals have been described in the mouse; Meca-325 which is known to define an antigen that can be induced in non-lymphoid endothelial cells by IFN-γ (Girard & Springer 1995) and has also been reported to react with high endothelial cells in mouse peripheral lymph node and Peyer's patches (Duijvestijn et al., 1987) and Meca-367 which recognises the

mucosal addressin MAdCAM-1 (which is a counter-receptor for the gut homing receptor $\alpha4\beta7$) and is expressed in mucosal HEV and in venules of intestinal lamina propria (Andrew et al., 1994). Other monoclonals include Meca-79 which stains all HEVs within lymphoid tissues and HECA-452, which is known to recognise a carbohydrate epitope related to the sialyl-Lewis X and sialyl-Lewis A oligisaccharides, in addition to reacting with high endothelial cells. While the Meca monoclonal antibodies were initially produced against mouse HEVs, these antibodies have also been demonstrated to show reactivity against rat, sheep, pig and human HEV (the question of cross species reactivity shall be discussed in detail within the relative section of the discussion chapter).

Differences in endothelial structure have an important role to play in the recirculation of naive and memory/effector lymphocytes. Naive lymphocytes enter secondary lymphoid organs through HEV, whereas activated lymphocytes showing organ specific migration, home to and enter non-lymphoid tissues via non-cuboidal (flat) vascular endothelium, as shown in the mouse by short term in vivo migration experiments (in combination with immunocytochemistry) of labelled lymphoblasts migrating and adhering to flat walled endothelium within the lamina propria of the small intestine (Jeurissen et al., 1987). Furthermore these cells recirculate back to the lymphoid organ where they were initially activated, through afferent lymphatics, unlike naive cells which recirculate through blood and enter the lymphoid organs via HEV. Hence, activated lymphocytes tend not to use high endothelial venules throughout their migratory patterns, either when homing to the non-lymphoid peripheral tissues (flat endothelial cells are used), or when homing back to the secondary lymphoid organ (afferent lymphatics). Therefore it would seem that HEV are not required for the migration of antigen

activated lymphocytes to non-lymphoid peripheral tissues and to lymphoid tissue, though they play a vital role in the recirculation and migration of unactivated naive lymphocytes into secondary lymphoid organs (Hamann & Rebstock 1993).

The use of flat endothelium by activated lymphocytes in homing to non-lymphoid peripheral tissues does not however suggest an alternative adhesion pattern to that of its naive state. On the contrary, an overall adhesion specificity similar to that of the naive lymphocyte-HEV interaction is maintained. Flat walled endothelial venules appear to be phenotypically and functionally similar to HEV from lymphoid tissues (Girard & Springer 1995). Indeed vessels with HEV characteristics (plump, cuboidal endothelial cells and incorporation of high amounts of $^{34}SO_4$) appear in human tissue in association with long standing chronic inflammation, such as the synovial membrane tissue in rheumatoid arthritis (Freemont 1987). However it is known that 40-45% of lymphocytes migrate in an unactivated naive state through HEV (in peripheral lymph nodes and Peyer's patches) while only 10-15%, following activation, will home to non-lymphoid tissue through flat endothelium (Ager 1994, Young & Hay 1995). This is in keeping with the slow, low rate migration of lymphocytes through flat endothelium, and the large scale migration of lymphocytes through HEV (Ford 1975, Ager 1994).

It has been suggested that structural characteristics common to HEV and those to non-lymphoid flat endothelial venules may facilitate these differences observed in lymphocyte migration rates (Ager 1994). The properties that enable HEVs to support the large scale migration of unactivated lymphocytes is not known, though it has been suggested that

inter-endothelial cell junctions that regulate the passage of lymphocytes are differentially regulated (or even absent) on HEVs allowing greater levels of lymphocyte migration (Ager 1994). Interestingly, it has been shown that while both antigen activated and naive lymphocytes can bind to non-lymphoid endothelium, only the transendothelial migration of antigen activated lymphocytes is promoted, whilst the migration of naive lymphocytes is restricted (Greenwood & Calder 1993, Masuyama et al., 1992). It would seem appropriate therefore, to assume that part of this variation in migration can be attributed to the differences in the expression of adhesion molecules on the surface on HEV and on non-lymphoid endothelium (along with possible endothelial structural differences) as well as the changes in adhesion molecule expression on the surface of activated lymphocytes as compared to naive lymphocytes.

These structural changes may be under the control of the local microenviroment insofar as HEV / flat walled endothelium morphology is concerned. It has been shown that following the cessation of afferent lymph flow into peripheral lymph nodes, HEV convert from a high to flat walled endothelial morphology, losing their characteristic ability to support large scale lymphocyte traffic (Hendricks & Eestermans 1983, Drayson & Ford 1984). Also consistant with this, is the observation that direct injection of antigen into the afferent lymph deprived node, does restore the typical HEV morphology (Hendricks & Eestermans 1983). Although the exact nature of how HEVs maintain large scale lymphocyte migration is not clear, it makes sense as an evolutionary strategy. Large numbers of naive lymphocytes do not randomly migrate throughout all tissues, instead they are routed into specific migratory pathways and eventually gathered into secondary lymphoid organs, and brought together with specialised antigen presenting cells and antigen drained

from local tissue, within the microenviroment. If lymphocytes fail to be antigen activated, then they are re-routed again to another lymphoid organ, where they have another chance to meet their special antigen, and this process carries on, until they are antigen activated. At this stage they develop into memory/effector lymphocytes as described earlier, which are sent to non-lymphoid tissues via specific homing pathways to re-encounter antigenic threat in a similar anatomic location, to where they were first stimulated.

Hence the travel of lymphocytes within the immune system is governed by distinct migratory pathways, specially suited for the requirements of both naive and activated lymphocytes, which ultimately provide an antigen "seek and destroy" traffic system.

1.7 The main adhesion molecule families

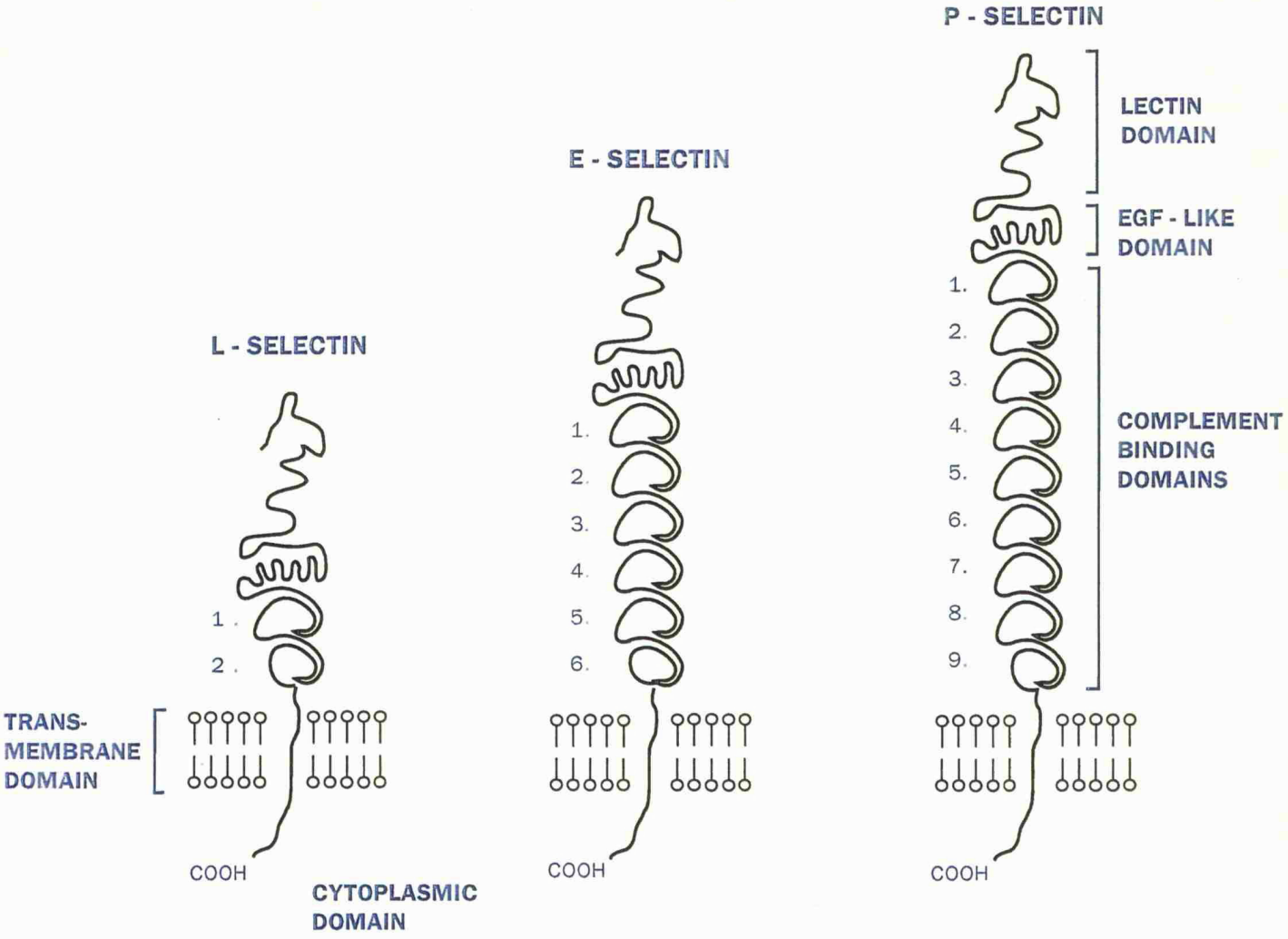
A large number of adhesion molecules have been identified over the past 10 - 15 years, and subsequently arranged together into three main groups, according to their biochemical properties and to a lesser extent, their physiological functions. In the following section, these families will be discussed in general, with particular emphasis on adhesion molecules which play important roles in leukocyte - endothelium interactions.

1.7.1 The Selectin family

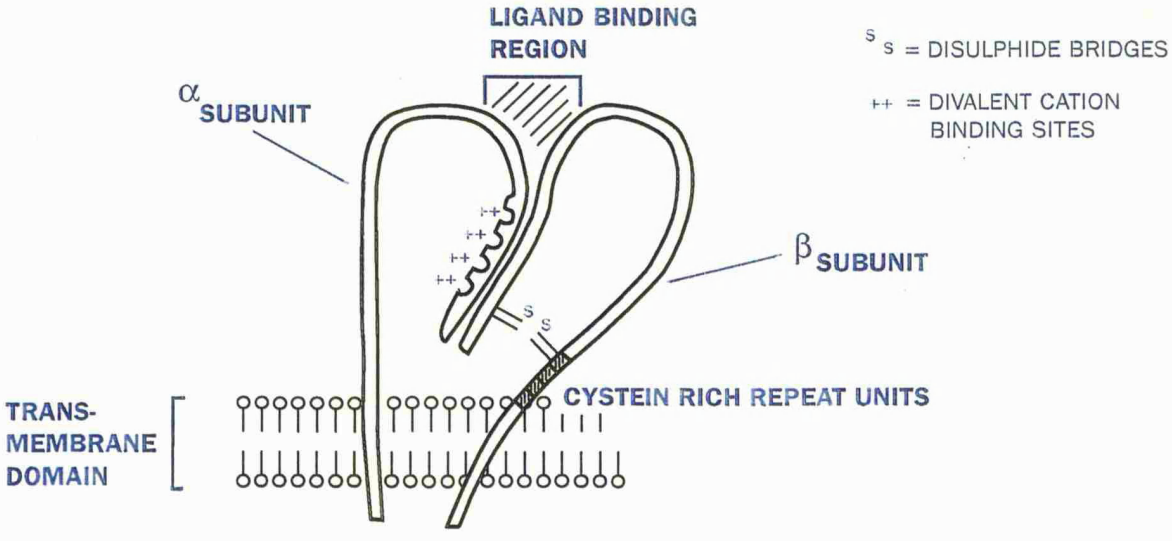
Three adhesion molecules are found in this family. They are E-, P- and L-selectin (Bevilacqua et al., 1991). E-selectin is expressed only on endothelial cells (Fairburn et al., 1993, Imhof & Dunon 1995, Tsang et al., 1995), P-selectin on endothelial cells and platelets (Johnston et al., 1989, Cronstein & Weissmann 1993) and L-selectin on all leukocytes, except for activated, memory lymphocytes (Gallatin et al., 1983, Geoffroy & Rosen 1989, Lasky et al., 1989, Imhof & Dunon 1995). These molecules belong to the same family because they share a common structure consisting of an N terminal calcium dependent lectin (sugar binding) domain (which is found in a variety of proteins including serum glycoproteins and proteoglycans of the extracellular matrix) followed by a single epidermal growth factor (EGF) like domain, several short consensus repeats (SCR) similar to those found in regulatory proteins that bind complement, and finally a transmembrane domain and C-terminal cytoplasmic domain (Drickamer 1989, Imhof & Dunon 1995). A high degree of homology is found between the selectin molecules, which some suggest was produced by duplication of an ancestral gene prior to the evolutionary divergence of mouse and man, due to the fact that the genes for all three adhesion molecules are clustered over a short region on human and mouse chromosome 1 (Pigott & Power 1993). Selectins are known to bind to carbohydrate ligands via their lectin domains. Tetrasaccharides, such as sialyl Lewis X (sLex) and sialyl Lewis A (sLea), or their sulfated forms, have been shown to exhibit ligand activity for all three selectins (Springer & Lasky 1991, Lasky 1992).

PART 1:

THE SELECTIN FAMILY



GENERAL OUTLINE OF THE STRUCTURE OF INTEGRINS



E-selectin

E-selectin, previously known as ELAM-1 (endothelial leukocyte adhesion molecule-1) and LECAM-2 (leukocyte cell adhesion molecule-1) is believed to play an important role in mediating the initial adhesive interactions between leukocytes and endothelium, during inflammation (Pigott & Power 1993, Tsang et al., 1995). It is found as a cytokine inducible endothelial adhesion molecule for neutrophils, monocytes and some memory T lymphocytes (Bevilacqua et al., 1987, 1989, Carlos et al., 1991, Picker et al., 1991, Shimizu et al., 1991). It is expressed on inflamed endothelium only, being absent on normal endothelium. However expression on normal, resting endothelium can be induced by a number of inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) and by bacterial endotoxin such as lipopolysaccharide (LPS) (Bevilacqua et al., 1987, Wellicome et al., 1990, Weller et al., 1992). Upon activation, expression is achieved by approximately 1 hour, maximal by 4-8 hours and is over by 48 hours (Zimmerman et al., 1992, Fries et al., 1993). Endothelial E-selectin expression upregulation can also be achieved by two cations; nickel chloride (Ni^{2+}) and cobalt chloride (Co^{2+}) both of which are known to be common contact sensitisers, leading to contact hypersensitivity (Goebeler et al., 1993). E-selectin is known to be rapidly downregulated by internalisation and degradation in lysosomes, though the exact nature of this process, or the signalling mechanisms that lead to internalisation, is not yet clear (Imhof & Dunon 1995)

E-selectin has a number of ligands. Besides sialyl Lewis X and sialyl Lewis A, which show ligand activity for all three selectins, E-selectin can also bind some ligands which do not bear sLex. One such

ligand is the cutaneous leukocyte antigen (CLA). These ligands consist of a family which is highly sialylated and found in a population of memory T lymphocytes present in inflammatory lesions of the skin. The connection between the recruitment of CLA expressing T lymphocytes and the expression of E-selectin on endothelium in inflamed skin, has suggested that this adhesive interaction between E-selectin and CLA may be regulating lymphocyte homing to skin (Picker et al., 1991, Ager 1994). CLA has been shown to be recognised by the anti-carbohydrate antibody HECA-452 (Berg et al., 1991). Another high affinity ligand for E-selectin is the recently described E-selectin ligand-1 (ESL-1), which is not a mucin, but instead fucosylated and 94% identical to a chicken variant of fibroblast growth factor receptor (FGFR). This homology has led some to suggest that this receptor may also be used by E-selectin for signalling (Steegmaier et al., 1995). Another novel E-selectin ligand has been identified recently on ruminant γδ T cell receptor (TCR) type lymphocytes (Walchek et al., 1993). It would seem to play a role in the homing of γδ T cells, since the TNF-α induced expression of E-selectin results in γδ T cell recruitment to skin. The binding of this ligand to E-selectin has also been shown to be uninhibited by antibodies to CLA or to sLex, though it is cation dependent and inhibited by EDTA (Imhof & Dunon 1995). Finally the sialyl stage specific embryonic antigen (SSEA-1) expressed on natural killer cells, is also a recent addition to the growing number of E-selectin ligands (Ohmori et al., 1993).

P-selectin

P-selectin, also known as GMP-140 (granule membrane protein), LECAM-3 (leukocyte cell adhesion molecule-3) and PADGEM (platelet

activation dependent granule external membrane), is expressed on activated platelets and inflamed endothelium and is known to mediate the adhesion of neutrophils, monocytes and macrophages (Larsen et al., 1990, Lawrence & Springer 1991, McEver 1991). P-selectin initiates the earliest stage of leukocyte-endothelium interactions in inflammatory conditions, and recently its role as the predominant adhesion molecule controlling leukocyte rolling over the surface of endothelial cells has been confirmed by experiments with P-selectin deficient mice (Mayadas et al., 1993). These strains of mice were obtained by isolating the normal P-selectin gene and replacing it in vivo with a defective copy, using the technique of homologous recombination followed by transgenesis, or "gene knockout" as it is more commonly known. This technique allows the replacement of a functional target gene with a copy which is inserted with erroneous sequences resulting in a non-functional gene, with subsequent abnormalities in gene function. In the case of P-selectin deficient mice obtained by this method, a number of defects were exhibited in leukocyte behaviour in contrast to normal P-selectin expressing mice strains. First, circulating neutrophil levels were elevated by 2.4 fold. Second, despite this elevation, the rolling of leukocytes on endothelial surfaces was virtually abolished and third, there was a 1-2 hour delay in the recruitment of neutrophils to an inflammatory site (Mayadas et al., 1993). The elevation in circulating neutrophil numbers was attributed to the failure of neutrophils to adhere to endothelium due to the lack of P-selectin expression (although other possibilities such as increased production or extended lifespan of neutrophils were not ruled out). The lack of leukocyte rolling on endothelial cell surfaces suggested that rolling required adhesion mediated by P-selectin and that the extravasation of neutrophils in inflamed endothelium required P-selectin involvement too (Mayadas et al., 1993). Such studies using gene knockout

mice are of considerable value due to the complete removal of a target adhesion molecule without affecting the expression of other adhesion molecules, thereby allowing the analysis of the relative contributions of individual molecules.

Similar to E-selectin, the expression of P-selectin is also inducible by TNF-α with maximal expression by 3-4 hours and down-regulation by 12 hours (Weller et al., 1992). Unlike E-selectin however, P-selectin also has another very interesting and extremely quick mode of cellular expression. P-selectin is stored intracellularly, in the Weibel-Palade bodies of endothelial cells and the α granules of platelets. It can be translocated to the plasma membrane within minutes following cell stimulation by thrombin, histamine, the complement factor C5q, oxygen radicals or various neuropeptides (McEver 1991, Sugayama et al., 1992, Smith et al., 1993). Hence P-selectin is unique amongst adhesion molecules, because it has two completely different mechanisms of cell surface expression; within minutes by the transport of stored molecules and within a few hours via the appearance of newly synthesised molecules. It would seem therefore that both regulatory systems can work in the same cells and yet function independently of each other, since it is known that TNF-α induced newly synthesised P-selectin molecules reach the cell surface directly, circumventing the route of the storage granules and that TNF-α itself cannot induce the transport of stored intracellular P-selectin (Vestweber 1993). The exact nature of how these two alternative systems work and coexist together is still not clear however.

As well as interacting with the sialyl Lewis x moiety found on neutrophils (as E-selectin can do), P-selectin also has two other ligands. The more established of these is the P-selectin glycoprotein ligand-1

(PSGL-1), which has recently been isolated and characterised (Sako et al., 1993). It is a transmembrane, highly glycosylated, dimeric mucin of 220 kDa, expressed on various leukocytes (Sako et al., 1993). It has been suggested that PSGL-1 can also act as an ligand for E-selectin (Hogg & Berlin 1995). The other ligand, expressed only on myeloid cells, is the so called 120-kDa P-selectin ligand (Moore et al., 1992), which is Ca^{2+} dependent and thought to be similar in structure to PSGL-1, though more work needs to be carried out on this ligand to make conclusive comparisons.

L-selectin

Also known as leukocyte adhesion molecule-1 (LAM-1), leukocyte cell adhesion molecule-1 (LECAM-1) and $gp90^{MEL-14}$, L-selectin is by far the most widely distributed and multifaceted selectin. It is expressed on peripheral blood B and T lymphocytes, neutrophils, basophils, eosinophils, monocytes, natural killer cells, bone marrow myeloid progenitor cells, erythroid precursor cells, some mature thymocytes and by some malignant leukocytes (Gallatin et al., 1983, Griffin et al., 1990, Tedder et al., 1990, Spertini et al., 1991). Though it is now known to perform many functions, it was initially described as a homing receptor, mediating lymphocyte adhesion to peripheral lymph node HEV (Gallatin et al., 1983). It was identified on rodent lymphocyte cell surface membranes as a ligand for a receptor molecule on the cuboidal endothelium of peripheral lymph node HEV (Gallatin et al., 1983). It was demonstrated that the monoclonal antibody Mel-14 blocked the adherence of lymphocytes to peripheral lymph node HEV, in vivo and in vitro (Gallatin et al., 1983). Besides its role as a homing receptor, L-selectin

contributes to neutrophil, monocyte and lymphocyte adhesion to endothelial cells at sites of inflammation. It is also involved in the initial, transient adhesion of leukocytes to vascular endothelium in organ specific adhesion under flow conditions known as "attachment and rolling" or "tethering" which slows down the leukocytes, enabling them to adhere more firmly in a second step (Palecanda et al., 1992, Shimizu et al., 1992, Mackay & Imhof 1993, Vestweber 1993, Girard & Springer 1995). L-selectin is well suited for this function due to its unique localisation on cell surface microvilli tips, which are thought to represent the principle sites of initial contact with the vascular endothelium under flow conditions (Picker et al 1991., Erlandsen et al., 1991). Hence where E and P-selectin initiate adhesion via the endothelial surface in inflammatory conditions, L-selectin initiates it via the leukocyte cell surface, either within the context of lymphocyte homing or in an inflammatory scenario together with E and P-selectin, all within the initial step of leukocyte attachment and rolling.

Another important aspect of L-selectin function is its apparent dual role in lymphocyte homing. Though primarily involved in lymphocyte homing to peripheral lymph node HEV, it does not perform this role exclusively. Indeed it is now known that L-selectin also plays a role in the initial attachment and rolling of lymphocytes in vivo to Peyer's patch HEV (Hamann et al., 1991, 1994), thus defining a more widespread role in homing. L-selectin was originally thought to mediate lymphocyte homing to peripheral lymph node HEV based upon short term in vivo homing experiments with the anti-L-selectin monoclonal antibody Mel-14, which demonstrated the inhibition of lymphocyte adherence to peripheral lymph node HEV and not to Peyer's patch HEV (Gallatin et al. 1983). However in these experiments lymphocytes were pretreated with Mel-14

and washed before injection, whereas more recent experiments where ^{51}Chromium labelled lymphocytes were pretreated with Mel-14 and then injected together into the tail vein of mice demonstrated substantial inhibition of adherence to Peyer's patches (Hamann et al., 1994). This Mel-14 mediated inhibition of adherence to Peyer's patches and peripheral lymph node HEV was only achieved in resting small lymphocytes however, and not in activated blast cells to either lymphoid organ, hence it was concluded that L-selectin also participates in the homing of small lymphocytes to Peyer's patches, but not significantly to the homing of blasts to non-lymphoid gut tissue (Hamann et al., 1994).

The regulation of expression of L-selectin is quite unique. Within 5-15 minutes of activation with chemotactic factors or phorbol esters, L-selectin is shed by proteolytic cleavage from the cell surface of neutrophils and monocytes and in 15-30 minutes from lymphocyte cell surfaces (Palecanda et al., 1992, Zimmerman et al., 1992). In neutrophils, downregulation of L-selectin is also related to exposure of IL-1 activated endothelial cells (Pigott & Power 1993). So far the only cytokine known to upregulate the cell surface density of L-selectin, is interferon-α (IFN-α) (Imhof & Dunon 1995).

All three of L-selectin's ligands are highly glycosylated mucin type proteins, which bear the sialylated or sulfated Lewis x or Lewis a tetrasaccharides. All are expressed on endothelial cells. One of these ligands, the glycosylation dependent cell adhesion molecule-1 (GlyCAM-1, also previously known as MECA-79) is expressed mainly in HEV of peripheral lymph nodes, acting as a vascular addressin for lymphocytes homing to peripheral lymph nodes (Lasky et al., 1992). This endothelial addressin role is however, questioned by some, who suggest that since

GlyCAM-1 is secreted by peripheral lymph node HEV, and has no transmembrane domain, at most it can be associated with HEV in some form, possibly through another protein, rather than being an endothelial adhesion molecule in its own right (Vestweber 1993, Ager 1995). Nevertheless there is no doubt that GlyCAM-1 does play an important role in lymphocyte trafficking to peripheral lymph nodes. Messenger RNA, encoding GlyCAM-1 has in addition been found in lung endothelium (Mebius et al., 1993). The regulation of GlyCAM-1 is also interesting, since it appears to be regulated directly or indirectly by exogenous factors in afferent lymph (Mebius et al., 1993). Loss of expression of GlyCAM-1 and flattening of HEV (accompanied by addressin downregulation) in de-afferentiated peripheral lymph nodes has been observed, in keeping with the observed loss of lymphocyte traffic to such lymph nodes. This suggests that not only has a system evolved where lymphocytes traffic to peripheral lymph nodes that have an influx of lymphatic material derived from peripheral sites, but also that the levels of GlyCAM-1 and HEV morphology are regulated by such lymphatic material (Mebius et al., 1993). The exact nature of the lymphatic components involved in this process is as yet undetermined. However it would seem logical to assume that antigen challenge would play an important role in the regulation of GlyCAM-1 expression and subsequent lymphocyte adherence, as well as the restoration of HEV morphology. It has been shown for example that direct injection of antigen into the peripheral lymph node, restores typical HEV morphology in de-afferentiated nodes (Girard & Springer 1995). However, it is also known that HEV can support lymphocyte migration in the absence of antigenic stimuli, therefore other factors must be involved, one possibility being the delivery of soluble factors such as chemoattractants (Ager 1994).

Another important ligand for L-selectin is CD34. This highly glycosylated ligand is expressed on the surface of endothelial cells in most organs, and has been demonstrated on the luminal side of small vascular endothelium, with expression also abundant in villi and sprouting processes (Ager 1995, Imhof & Dunon 1995). Though CD34 expression is widespread, it can only function as an L-selectin ligand when associated with specific sulfated oligosaccharides, with inhibition of sulfation leading to the loss of recognition by L-selectin (Girard & Springer 1995). It is now assumed that sulfation of L-selectin ligands is the key process for the recognition, and subsequent adherence by L-selectin expressing leukocytes. It is known that HEV endothelium can incorporate large amounts of $^{35}SO_4$ (Freemont 1988), unlike other endothelium, and the requirement for sulphate by L-selectin ligands on HEV endothelium such as CD34 and GlyCAM-1 provides a functional basis for such a metabolic requirement. The expression of CD34 can also be regulated by cytokines, such as IL-1, TNF-α and INF-γ, however this regulatory effect is opposite to the effect on E or P-selectin for example. It has been observed that cytokines, while upregulating E or P-selectin expression, can downregulate CD34 expression, leading to the assumption that CD34 is more likely to be involved in selectin interactions under normal conditions, rather than during inflammation (Imhof & Dunon 1995).

The recent demonstration that MAdCAM-1 (mucosal addressin cell adhesion molecule) can also act as a ligand for L-selectin, would explain the role that L-selectin has in the attachment and rolling stage of lymphocytes on Peyer's patch HEV. As in the case of peripheral lymph node homing lymphocytes, Peyer's patch homing lymphocytes also make use of L-selectin as an initial contact adhesion molecule. However, antigen activated lymphocytes migrate to Peyer's patches and to sites of

inflammation independently of L-selectin, hence the initial attachment and rolling stage of adhesion is mediated by another adhesion molecule in this instance. It has recently been demonstrated that the integrin $\alpha4\beta7$ (which is expressed on microvilli tips, like L-selectin) mediates adhesion to MAdCAM-1 in the initial attachment and rolling stage of activated lymphocytes (section 1.7.2.3 which contains the $\alpha4\beta7$ heading will address this issue in more detail), as well as in the latter firm adhesion stage, taking over the role from L-selectin (Ager 1994, Berlin et al 1995).

MAdCAM-1 has been demonstrated to act as a ligand for L-selectin under conditions of flow (in which lymphocytes are passed over purified MAdCAM-1 coated glass slides under shear), but not under non-flow conditions, such as a frozen section assay or with cultured endothelial cells (Berg et al., 1993, Berlin et al., 1993). MAdCAM-1 is a highly glycosylated mucin type transmembrane protein, which is different from the other selectin ligands, in that it exhibits a complex structure combining three Ig domains and a mucin like region between Ig domains 2 and 3 (Imhof & Dunon 1995). It is expressed on Peyer's patch, mesenteric lymph node HEV, gut lamina propria endothelial venules, and is the main mucosal vascular addressin for gut seeking lymphocytes (Andrew et al., 1994, Erle et al., 1994, Hamann et al., 1994). MAdCAM-1 will be discussed again in conjunction with the integrin $\alpha4\beta7$, which plays a more prominent role in MAdCAM-1 interactions (see section 1.7.2.4).

1.7.2 The Integrin family

The integrins are a family of heterodimeric membrane glycoproteins expressed on a wide range of cell types, which function as adhesion receptors for extracellular matrix and cell to cell interactions. They are involved in various biological processes, including inflammation, wound healing, tumour metastasis, embryonic development and lymphocyte homing (Hynes 1992, Pigott & Power 1993, Sonnenberg 1993). All integrins consist of two non-covalently associated subunits; α and β of 150 and 100-kDa respectively. So far, 15 α and 8 β chains have been identified, making up at least 21 different heterodimers (see table-1). Leukocytes can express 13 of these different integrins, 6 of which belong to the β_1, β_2 and β_7 integrin subfamilies, and which are important in leukocyte-endothelium interactions. The α subunits all have large extracellular domains containing up to four divalent cation binding domains (Ca^{2+} or Mg^{2+}) and a single transmembrane region, ending with a short cytoplasmic domain. The β subunits also have large extracellular domains, with 48-56 conserved cysteine residues arranged in four repeating units, followed by a transmembrane region ending with a short cytoplasmic domain. The exception is the $\beta4$ subunit, which contains a long cytoplasmic domain (Pigott & Power 1993, Sonnenberg 1993). The integrins bind to a variety of extracellular matrix proteins, such as fibronectin, laminin, collagen, vitronectin and von Willebrand factor, and also to members of the immunoglobulin superfamily, such as ICAM-1, -2, -3 and VCAM-1 (Sonnenberg 1993). Most members of the integrin family can bind to more than one ligand, depending on the cell type in which they are expressed.

1.7.2.1 The β1 integrins

The β1 integrins consists of ten heterodimeric glycoproteins (combining the β1 subunit with variable α subunits, from α1 to α9), seven of which belong to the VLA (very late antigen) group. This subfamily includes receptors that bind to extracellular matrix components such as fibronectin, laminin and collagen, as well as the Ig superfamily adhesion molecule VCAM-1 (vascular cell adhesion molecule). They are expressed on many non-haematopoietic and leukocyte cell types, and are called very late antigens because two of them (VLA-1 and VLA -2) appear on T lymphocytes 2-4 weeks after antigen stimulation in vitro (Springer 1990, Sonnenberg 1993).

VLA-1, VLA-2 and VLA-3

VLA-1 (also known as α1β1) is expressed on activated T lymphocytes, monocytes, neuronal cells in culture, melanoma cells and smooth muscle cells (Pigott & Power 1993). Its ligands are type I and IV collagen and laminin (Imhof & Dunon 1995). It is expressed on T lymphocytes 2-4 weeks after activation, and expression has been reported to be upregulated in a number of inflammatory diseases of the human intestine, though the exact role that VLA-1 plays is still unclear (MacDonald et al., 1990).

VLA-2 (α2β1) is expressed on B and T lymphocytes, fibroblasts, and platelets and its ligands are type I, II, III, IV collagen and laminin (Pigott & Power 1993). It is known to mediate Mg^{2+} dependent adhesion of platelets to collagen, and is assumed to play an important role in wound

healing. More recently it has been shown to act as a receptor for echovirus (Hogg & Berlin 1995). Expression can be upregulated on lymphocytes upon antigen stimulation, by TGF-β (transforming growth factor) and on fibroblasts in response to serum (Pigott & Power 1993).

VLA-3 (α3β1) is expressed on B lymphocytes, kidney glomerulus, thyroid and nearly all cell culture lines, except lymphoid (Pigott & Power 1993). Its ligands are fibronectin, type I collagen, laminin, epiligrin, and nidogen/entactin (Imhof & Dunon 1995). Unlike VLA-2, VLA-3 is downregulated by TGF-β.

VLA-4

VLA-4 (α4β1, also previously known as LPAM–2) is expressed on peripheral blood lymphocytes, monocytes, B and T cell lines, natural killer cells, eosinophils, many melanomas and functions as both a matrix and cell receptor (Springer 1990, Pigott & Power 1993). As a matrix receptor it binds to an alternatively spliced domain (containing the CS-1 peptide) of fibronectin (distinct from that to which VLA-5 can bind). As a cell receptor it binds to VCAM-1 (vascular cell adhesion molecule) of the immunoglobulin superfamily, a cytokine inducible (IL-1, TNF, also in some cases IFN-γ and gram negative bacterial lipopolysaccharide) endothelial vascular addressin, mediating cell adherence to inflamed tissue in particular (Rice et al., 1990, Shimizu et al., 1992, Issekutz 1993, Hamann et al., 1994). VCAM-1 will be discussed in detail within the context of Ig superfamily molecules in section 1.7.3.

The VLA-4 / VCAM-1 adhesion molecule pairing is an important interaction involved in strong lymphocyte adherence to endothelium of inflamed tissue. This pairing is not however, involved in lymphocyte adherence to HEV, since VCAM-1 is not present on unactivated endothelium (Girard & Springer 1995). Therefore this adhesion pair would appear not to play a role in organ specific lymphocyte homing to non-inflamed tissues.

VLA-4 has also been suggested to play an important role in the trans-endothelial migration of lymphocytes, due to its binding capacity for the extracellular matrix component fibronectin (Hogg 1993). It has recently emerged however that the state of activation of VLA-4 largely determines its ability to interact with either VCAM-1 or fibronectin. It would seem that there are three activation states for VLA-4; the fully active state, where VLA-4 can adhere to VCAM-1 and fibronectin, the partially active state, were it can adhere to VCAM-1 only, and the inactive state, were adherence is not possible to either ligand (Mackay & Imhof 1993). VLA-4 is activated by cytokines, and cellular activation. The up-regulation of this adhesion molecule can also be induced by increasing the concentrations of either Mn^{2+} or Ca^{2+} and Mg^{2+}, with maximal levels of adhesion induced by Mn^{2+} (Chiu et al., 1995). It has also been recently stated that VLA-4 is regulated by CD24 (Kilger et al., 1995). It would appear that CD24 promotes the efficient adhesion of VLA-4 to VCAM-1, how this is achieved is yet to be clarified however.

VLA-5 and VLA-6

VLA-5 ($\alpha 5\beta 1$) is widely distributed. It is expressed on monocytes, memory T lymphocytes, B lymphocytes (very early on during development and again later after activation), fibroblasts, platelets and muscle cells (Pigott & Power 1993). VLA-5 functions as a fibronectin receptor, mediating the adherence of B and T lymphocytes to fibronectin (Szekanecz et al., 1992, Imhof & Dunon 1995). It has been demonstrated recently that antibodies directed against VLA-5, or fibronectin can significantly inhibit the in vitro adherence of lymphocytes to peripheral lymph node HEV in frozen tissue section assays (Girard & Springer 1995), hence implicating a role for VLA-5 and its ligand in the strong, arrest stage of lymphocyte adherence (which in vitro frozen section assays are thought to simulate), rather than the initial rolling stage of adherence. Others have suggested that transmigration through endothelium following strong adherence of lymphocytes, may depend on VLA-5 mediated recognition of the extracellular matrix molecule fibronectin (Szekanecz et al., 1992). The precise role of VLA-5 in this proposed trans-endothelial migration is not yet clear however, and warrants further research.

VLA-6 ($\alpha 6\beta 1$) is expressed on platelets, monocytes, T lymphocytes, thymocytes and endothelial cells (Pigott & Power 1993, Imhof & Dunon 1995). Its ligands include laminin (to which it mediates the adhesion of platelets) and an as yet unidentified ligand on the cell surface of lymphocytes, which is believed to play a role in signal transduction (Pigott & Power 1993, Imhof & Dunon 1995). Most interesting of all however, VLA-6 is believed to promote progenitor T lymphocyte homing to the thymus (Dunon et al., 1993). Though a fairly new development in terms of lymphocyte adhesion research, the prospect

of understanding more about lymphocyte homing at such an early stage of T cell development is fascinating and clearly demands attention.

1.7.2.2 The β2 integrins

The β2 subfamily includes three integrins, which came to prominence following the analysis of immunocompromised patients suffering from a rare inherited disorder called leukocyte adhesion deficiency (LAD). This disease was characterised by the absence of adhesion between leukocytes and endothelium, especially the failure of migration of neutrophils to sites of extravascular inflammation, eventually leading to recurrent and often fatal bacterial infections (Pigott & Power 1993). It was found that LAD results from a mutational defect in the gene encoding for the β2 subunit and the three integrins sharing the β2 chain, LFA-1 (αLβ2), Mac-1 (αMβ2) and p150, 95 (αXβ2) were found to be missing in patients suffering from LAD (Imhof & Dunon 1995). More recently a new β2 integrin has been identified and provisionally termed αDβ2, which so far has only been found to be expressed on tissue macrophages (Hogg & Berlin 1995).

LFA-1

LFA-1 (leukocyte function associated molecule, also known as αLβ2) is expressed on the cell surface of all T lymphocytes (though at one to twofold higher levels on memory T lymphocytes than naive T lymphocytes), neutrophils, monocytes and macrophages, and interacts with its ligands ICAM (intercellular adhesion molecule) -1, 2 and 3 of the

immmunoglobulin superfamily (Shimizu et al., 1992, Pigott & Power 1993). LFA-1 has an important role in mediating leukocyte adhesion to inflamed endothelium via ICAM-1 (the ICAMs will be discussed in detail in section 1.7.3) and in lymphocyte trafficking, though this role would appear to be of a more general non-organ specific strengthening of adhesion (Hamann et al., 1988). Indeed it has been shown that LFA-1 mediates strong adhesion, not only at sites of inflamed endothelium but also in secondary lymphoid organs (though not in an organ specific manner), such as peripheral lymph node and Peyer's patches, where ^{51}Chromium labelled resting lymphocytes pretreated with antibodies directed against LFA-1 can inhibit lymphocyte migration by 80% to peripheral lymph node, by 50% to Peyer's patches and by 15% to non-lymphoid gut endothelium (Hamann et al., 1994).

LFA-1 / ICAM interactions have also been invoked in trans-endothelial migration, and although this has been shown to be the case for migration across human umbilical vein endothelial cells (Oppenheimer-Marks et al., 1991), more work needs to be carried out, to justify a more general role for LFA-1 in trans-endothelial passage. Other than lymphocyte / endothelium interactions, LFA-1 is also known to participate in cell to cell interactions, such as the killing of virus infected cells by cytotoxic T lymphocytes (in fact, LFA-1 was originally identified by monoclonal antibodies which inhibit T cell mediated killing) and the activation of T lymphocytes by antigen presenting cells (Springer 1990, Imhof & Dunon 1995).

Divalent cations have an important role to play in the regulation of LFA-1 activation within the context of resting peripheral blood CD4+ T cells. Mn^{2+} and Mg^{2+} has been shown to upregulate LFA-1 adherence to

ICAM-1 in such cells, while Ca^{2+} has been shown to inhibit Mn^{2+} dependent adhesion to ICAM-1 (Shimizu & Mobley 1993). However results also suggest that divalent cation upregulation is not complete, since phorbol ester treatment can further enhance LFA-1 adhesion in the presence of Mn^{2+}, or even in the presence of Mn^{2+} and Ca^{2+}. This suggests that a partial increase in LFA-1 integrin functional activity is achieved by divalent cations such as Mg^{2+} and Mn^{2+}, and that LFA-1 is receptive to other stimuli that may increase its activity yet further. Overall it can be assumed that integrins such as the LFA-1 / ICAM-1 adhesion pair contain several layers of activity, that can be up or down regulated by various combinations of divalent cations and other stimuli. The physiological relevance of divalent cation regulation of function and activity is not yet clear. However one would assume that changes in Mg^{2+} / Mn^{2+} / Ca^{2+} concentrations at sites of injury and tissue inflammation would facilitate and guide the following reaction of LFA-1 / ICAM-1 mediated adhesion of leukocytes.

Activation of LFA-1 is also achieved via the T cell receptor (TCR) / CD3 complex. It has been shown that T cell receptor crosslinking leads to a change in the conformation of the extra-cellular binding domain of LFA-1, through intracellular signalling, which in turn increases the strength of the adhesion between LFA-1 and ICAMs (Springer 1990, Mackay & Imhof 1993). The mechanism involved is very rapid and transient, leading to activation within minutes and returning back to a resting state within 30 minutes (Springer 1990). This transience provides a fluid mechanism for antigen specific LFA-1 mediated adhesion and de-adhesion, clearly of importance for example to cytotoxic T lymphocytes.

Mac-1

Mac-1 ($\alpha M\beta 2$) is present on monocytes, macrophages and granulocytes (Springer 1990, Pigott & Power 1993). Its ligands include ICAM-1 (though not ICAM-2 or ICAM-3), fibrinogen and also the C3bi component of complement (Imhof & Dunon 1995). Mac-1 mediates granulocyte and monocyte adherence to inflamed endothelium, and also has a role to play in the trans-endothelial migration of granulocytes (though LFA-1 is thought to have a more prominent role in this respect). Mac-1 is also involved in the phagocytosis of complement coated particles (Hogg 1993, Pigott & Power 1993). It is upregulated on the surface of neutrophils and monocytes by inflammatory stimuli, and is also increased following E-selectin binding to neutrophils as well (Springer 1990, Shimizu et al., 1992, Smith 1993). Mac-1 is stored in intracellular granules, which are swiftly mobilised to the cell surface following chemotactic stimulation by PAF (platelet activating factor) and IL-8 for example (both of which are produced by activated endothelial cells). Due to its ability to use fibrinogen as a ligand, as well as ICAM-1, it has been suggested that Mac-1 provides neutrophils with greater flexibility, allowing them to adhere to a wider variety of surfaces, then say LFA-1, which would appear to serve a more closely defined role (Smith 1993).

1.7.2.3 The β7 integrins

This subfamily consists of the $\alpha 4\beta 7$ and $\alpha E\beta 7$ integrins. These adhesion molecules are involved in the strong adhesion of lymphocytes to Peyer's patch endothelium, to endothelial venules within gut lamina propria and to gut epithelial tissue.

α4β7

Also known as LPAM-1 (lymphocyte Peyer's patch adhesion molecule) and α4βP, this heterodimer is expressed on T lymphocytes (on most or all naive and on certain memory T lymphocytes), B lymphocytes, and to a lesser extent on natural killer cells and eosinophils (Holzmann et al., 1989, Erle et al., 1994). α4β7 is a lymphocyte homing receptor, mediating adhesion to Peyer's patch HEV and to gut lamina propria endothelial venules (Erle et al., 1994, Imhof & Dunon 1995). The integrin α4β7 has several ligands, the most prominent being the mucosal vascular addressin MAdCAM-1 (Andrew et al., 1994). It can also use VCAM-1 and the CS-1 region of fibronectin as ligands, though these interactions require a higher level of activation, than for adhesion to MAdCAM-1, and interactions with VCAM-1 only become important in inflammatory reactions (Andrew et al., 1994, Erle et al., 1994, Imhof & Dunon 1995).

Though α4β7 mediates lymphocyte adhesion to gut lamina propria endothelial venules by adhering to MAdCAM-1, its main function is to act as a lymphocyte homing receptor to Peyer's patches, were it mediates adhesion to HEV again via the vascular addressin MAdCAM-1. This adhesion pairing, like the LFA-1 / ICAM-1, 2 pair mediating lymphocyte traffic to peripheral lymph nodes, is the only organ specific adhesion pair, mediating traffic to Peyer's patch HEV. Its role is crucial in directing the passage of naive, unactivated lymphocytes to Peyer's patches. Though α4β7 is involved in strong lymphocyte adhesion, it is also known to function early on, in the initial attachment and rolling phase of lymphocyte adherence under flow conditions (Berlin et al., 1995). This was demonstrated by the ability of mouse lymphoma cells,

expressing high levels of $\alpha4\beta7$ to interact with MAdCAM-1 coated surfaces under laminar flow conditions within the physiological range of venular shear stresses in vivo (Berlin et al., 1995). Binding was blocked by the anti-MAdCAM-1 monoclonal antibody Meca-367 and the anti-$\alpha4\beta7$ monoclonal antibody DATK32 (Berlin et al., 1995). Hence $\alpha4\beta7$ can initiate primary contact, between lymphocyte and endothelium, as well as mediating the subsequent attachment and rolling stages, eventually supporting strong adhesion and arrest. Thus selectins are not the only adhesion molecules capable of initiating lymphocyte - endothelium interactions. It is not surprising therefore to find that the expression of $\alpha4\beta7$ (like L-selectin) is concentrated on the tips of lymphocyte microvillous projections, which represent the initial site of cell to cell contact under flow (Berlin et al., 1995). Expression of $\alpha4\beta7$ is also present on the non-villous cell surface, to mediate the strong adhesion stage of lymphocyte adherence that follows the rolling stage.

MAdCAM-1, the ligand for $\alpha4\beta7$ is expressed at high levels on Peter's patch and mesenteric lymph node HEV, and to a lesser extent on non-lymphoid mucosal endothelial venules in the intestinal lamina propria and the lactating mammary gland (Sampaio et al., 1995). Recently MAdCAM-1 expression has also been found on a population of sinus lining cells surrounding the T cell dependent periarteriolar lymphocyte sheath (PALS) of the spleen (Kraal et al., 1995). This is quite interesting since the spleen is devoid of HEV, and yet expression is observed on sinus lining cells which morphologically resemble endothelial cells. The role MAdCAM-1 plays in the localisation of lymphocytes to the spleen needs further clarification.

The appearance of MAdCAM-1 expressing HEV in the inflamed pancreas of non-obese diabetic mice, and again the expression of MAdCAM-1 by endothelial venules in the brain of animals during relapsing episodes of chronic experimental allergic encephalomyelitis (O'Neill et al., 1991), has lead to the suggestion that MAdCAM-1 may also have a role to play in inflammation. Indeed it is known that MAdCAM-1 expression can be induced on endothelial cell lines by proinflammatory stimuli, such as IL-1 and TNF-α (Sikorski et al., 1993). However to date, the expression of MAdCAM-1 has not been reported in any other tissue site of inflammation. It would be of great interest to elucidate the role that this adhesion molecule (and its counter-receptor α4β7) may have in inflammatory diseases of mucosal origin. Due to its nature of acting as a vascular addressin for the homing receptor α4β7, and initial contact receptor for L-selectin, MAdCAM-1 can be considered to be a multifunctional adhesion molecule, capable of initiating the primary rolling and attachment phase (L-selectin and also α4β7 dependent), as well as strengthening and arresting lymphocyte adhesion in the secondary phase of the multi-step adhesion cascade.

Another ligand for α4β7, is VCAM-1. However adherence is only observed in vitro and the predominant counter receptor for VCAM-1 is VLA-4 (α4β1) of the β1 integrin subfamily (Chiu et al., 1995). It has been proposed that α4β7 has two distinct binding regions for adhesion to MAdCAM-1 or VCAM-1 based upon experiments using a panel of monoclonal antibodies directed against an α4β7 integrin expressing mouse lymphoma cell line. A unique region on α4β7 was identified for MAdCAM-1 adhesion, as was a different epitope for VCAM-1 adhesion (Andrew et al., 1994). It was concluded that α4β7 interactions with MAdCAM-1 and VCAM-1 can be modulated by monoclonal antibody

binding to distinct epitopes and hence functionally separate binding sites (Andrew et al., 1994). More recent work has suggested that the amino acid residue requirements for murine VCAM-1 adhesion to murine VLA-4 and $\alpha4\beta7$ are similar but not identical, based upon site-directed mutagenesis experiments, where amino acid residues of VCAM-1 important in adhesion to VLA-4 and $\alpha4\beta7$ were identified by testing the adherence of VLA-4 and $\alpha4\beta7$ positive cells to various site directed mutations of VCAM-1 (Chiu et al., 1995). It is also important to realise that although $\alpha4\beta7$ can bind to VCAM-1, VLA-4 cannot bind to MAdCAM-1 in the same manner. Hence the $\alpha4\beta7$ / MAdCAM-1 interaction is an organ specific selective adhesion pairing.

The ability of $\alpha4\beta7$ to use VCAM-1 as a ligand depends largely on its activity state. Studies have shown that the $\alpha4\beta7$ binding activities can be ranked in the order of increasing affinity; MAdCAM-1 > VCAM-1 > fibronectin (Andrew et al., 1994). Hence, the binding of VCAM-1 by $\alpha4\beta7$ would necessitate a greater state of activity, than it would to bind to MAdCAM-1. This increase in avidity is achievable by increased concentrations of Mn^{2+} and pro-inflammatory stimuli. Therefore $\alpha4\beta7$ is a unique adhesion molecule which modulates its adherence to VCAM-1 and MAdCAM-1 via distinct, differentially regulated epitopes. These function by selective binding capabilities for $\alpha4\beta7$; to VCAM-1 on mucosal non-lymphoid inflamed endothelium (where MAdCAM-1 is absent) and to MAdCAM-1 on HEV of mucosal lymphoid tissues such as Peyer's patches and mesenteric lymph node. This sustains a common adhesive framework by virtue of $\alpha4\beta7$ expression on cell surfaces for naive and subsequently activated lymphocytes that migrate throughout the mucosal tissues. This duality also raises the possibility of future immunomodulation treatment. By developing antibodies directed against

one epitope, it is conceivable to inhibit $\alpha 4\beta 7$ mediated adherence to inflamed endothelium, whilst not affecting $\alpha 4\beta 7$ mediated lymphocyte traffic to Peyer's patches for example.

$\alpha E\beta 7$

This integrin, also known as $\alpha_{M290}\beta 7$, $\alpha_{IEL}\beta 7$ (intraepithelial lymphocyte) and $\alpha_{HML}\beta 7$ (human mucosal lymphocyte) is expressed on 95% of intraepithelial lymphocytes and 40% of lamina propria lymphocytes, and mediates adhesion between lymphocytes and intestinal epithelium (Cerf-Bensussan et al., 1987, Hamann et al., 1994, Imhof & Dunon 1995). It has recently been demonstrated that lymphocytes which express $\alpha 4\beta 7$ also express $\alpha E\beta 7$, however $\alpha E\beta 7$ does not bind to MAdCAM-1 (Chao et al., 1994). It would seem therefore that $\alpha 4\beta 7$ bearing lymphocytes also have the capability of interacting with epithelial cells within non-lymphoid mucosal intestinal tissue.

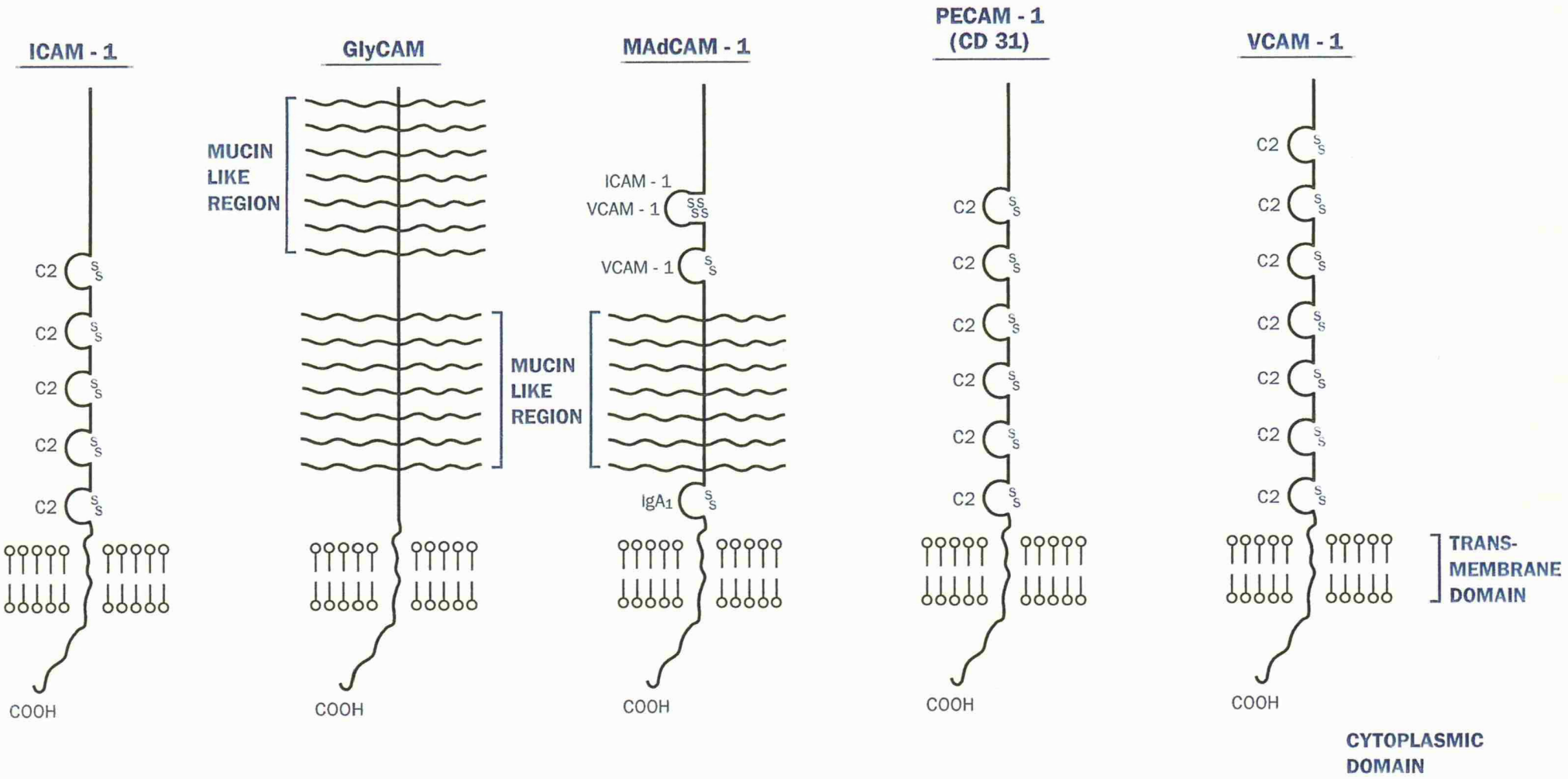
$\alpha E\beta 7$ is known to be upregulated on intraepithelial lymphocytes in vitro by TGF-β, a cytokine which is found to be concentrated within the gut area (Imhof & Dunon 1995). To date only E-Cadherin, which is expressed on epithelial tissue, has been found to act as a ligand for $\alpha E\beta 7$ (Cepek et al., 1994). E-Cadherin is linked to cytoskeletal actin, and is thought to be involved in the generation and maintenance of epithelial layers during development and possibly in adult tissue as well (Pigott & Power 1993).

1.7.3 The Immunoglobulin (Ig) superfamily

This is the largest family, with over 70 members. The Ig superfamily consists of molecules with multiple immunoglobulin like domains (Pigott & Power 1993, Imhof & Dunon 1995). The roles of these various molecules differ, but are generally concerned with the control of cell behaviour, acting as signal transducing receptors or as cell adhesion molecules for example. With the exception of a few molecules which exist as secreted proteins, most are found as cell surface structures. Five members of the Ig superfamily are important in leukocyte - endothelial cell interactions. These are ICAM-1, ICAM-2, ICAM-3, VCAM-1 and PECAM-1. With the exception of ICAM-3 all serve as endothelial ligands for leukocytes.

The vast array of structures found within the Ig superfamily is thought to have emerged from one single ancestral unit, namely the immunoglobulin fold. This basic structure is thought to have evolved, undergoing divergence and duplication resulting in a common domain composed of around 90 - 100 amino acids arranged in a sandwich of two sheets of anti-parallel β-strands, which is stabilised by a disulphide bond at its centre (Springer 1990). This basic motif is more or less the standard structure preserved by species as diverse as humans and insects. The evolutionary resistance to change in this particular case, is thought to have emerged in order to preserve a unit which is protease resistant and is able to survive in the extracellular environment (Pigott & Power 1993).

PART II : GENERAL OUTLINE OF THE STRUCTURES OF SOME OF THE IMMUNOGLOBULIN SUPERFAMILY ADHESION MOLECULES



C2 = CONSTANT IMMUNOGLOBULIN DOMAIN 2

S S = DISULPHIDE BRIDGES

IgA_1 = IMMUNOGLOBULIN-A LIKE DOMAIN

ICAM-1

ICAM-1 (intercellular adhesion molecule) is expressed on inflamed endothelium (and weakly expressed on resting endothelium), though cytokine inducible (IL-1, TNFα, IFN-γ) expression on thymocytes, dendritic cells, fibroblasts, keratinocytes, chondrocytes, B and T lymphocytes, epithelial cells and resting endothelial cells has been observed (Pigott & Power 1993). ICAM-1 is important in mediating inflammatory responses, monocytes, lymphocytes and neutrophils adhering to activated endothelium via its two complimentary receptors LFA-1 and Mac-1 (see section 1.7.2.2). Other than mediating leukocyte adhesion, ICAM-1 can also serve as a ligand for the adhesion of rhinovirus and some strains of coxsackie virus to endothelium (Cronstein & Weissman 1993). Expression of ICAM-1 has also been implicated in tumour metastasis (Johnson et al., 1989).

Leukocyte adhesion facilitated by ICAM-1 interaction with either LFA-1 or Mac-1 (both β2 integrins) is achieved following selectin mediated interactions, namely the initial attachment and rolling phase of adhesion. The integrin - ICAM-1 interactions facilitate the strong adhesion stage, which follow the initial rolling phase. ICAM-1 is also believed to play a role in the leukocyte transmigration across endothelial cell junctions, which subsequently follows the strong adhesion phase (Cronstein & Weissman 1993, Hogg & Berlin 1995). Although ICAM-1 is involved in adhesion under inflammatory conditions, it also has an important role in the strong adherence of lymphocytes to peripheral lymph node HEV by interacting with LFA-1 (Girard & Springer 1995).

ICAM-2 and ICAM-3

ICAM-2 is constitutively expressed on endothelial cells and its expression is not augmented by activation, unlike ICAM-1 (Shimizu et al., 1992). It serves as a ligand for LFA-1 and has an important role in the LFA-1 mediated strong adhesion of lymphocytes to peripheral lymph node HEV. Interestingly the affinity of ICAM-2 for LFA-1 seems to be weaker than that of ICAM-1 (de Fourgerolles & Springer 1992, de Fourgerolles et al., 1994), which would make sense, since constitutively expressed ICAM-2 mediates low rate lymphocyte traffic through peripheral lymph nodes, whereas cytokine upregulated ICAM-1 mediates the adhesion of leukocytes to inflammatory sites. This necessitates a higher degree of activity and leukocyte infiltration, not forgetting the role that ICAM-1 plays in transendothelial migration. To date, no function for ICAM-2 has been demonstrated in lymphocyte transendothelial migration. ICAM-2 mediated adhesion is also known to provide a co-stimulatory signal for T lymphocyte activation, hence providing an important function where ICAM-1 expression on antigen presenting cells is absent or low.

ICAM-3, a recently discovered Ig superfamily adhesion molecule, is strongly expressed on resting lymphocytes and monocytes and is believed to be important in initiating T lymphocyte mediated immune responses (Imhof & Dunon 1995). ICAM-3 acts as a ligand for LFA-1, and has also been suggested to interact with the recently identified β2 integrin αDβ2, which is present on tissue macrophages (Hogg & Berlin 1995). ICAM-3, though not expressed on endothelial cells, has nevertheless been suggested to be involved in competitively inhibiting

LFA-1 mediated adherence to ICAM-1 (de Fourgerolles & Springer 1992).

VCAM-1

VCAM-1 (vascular cell adhesion molecule) is present on activated endothelial cells, tissue macrophages, dendritic cells, and bone marrow fibroblasts (Pigott & Power 1993). It is a cytokine inducible adhesion molecule mediating leukocyte adherence to inflamed endothelium. VCAM-1 is important in the recruitment of leukocytes to sites of inflammation, mediating strong adhesion to inflamed endothelium by functioning as a ligand for VLA-4 ($\alpha 4\beta 1$), and to a lesser extent $\alpha 4\beta 7$ (Berlin et al., 1993, Sonnenberg 1993, Ager 1994, Andrew et al., 1994). VCAM-1 is absent from resting endothelial cells, but expression can be induced by IL-1, TNFα, and IFN-γ, (Cronstein & Weissmann 1993, Pigott & Power 1993). VCAM-1 is not expressed on HEV, and therefore is not expected to play a role in lymphocyte homing, however low levels of VCAM-1 have been detected on rat peripheral lymph node HEV (May et al., 1993). Other than mediating strong lymphocyte adherence to inflamed endothelium, the VLA-4 / VCAM-1 adhesion pairing also appears to play a small role in transendothelial migration (Smith 1993).

PECAM-1

PECAM-1 (platelet endothelial cell adhesion molecule, also known as CD31) is a Ig superfamily adhesion molecule mediating leukocyte and platelet / endothelial cell adhesion and transendothelial migration (Imhof

& Dunon 1995). PECAM-1 is expressed on platelets, endothelial cells, monocytes, granulocytes and naive T lymphocytes (Albelda et al., 1991, Pigott & Power 1993). Its exact function is not clear, however PECAM-1 has a vital role as a triggering molecule, activating β1 and β2 integrins prior to leukocyte adherence to endothelium, and more recently PECAM-1 has been shown to be involved in leukocyte transendothelial migration by mimicking homophilic interactions with PECAM-1 on the endothelial cell surface (Smith 1993, Ager 1994, Hogg & Berlin 1995). However the presence of PECAM-1 has not, so far been detected on transmigrating lymphocytes using the LFA-1 / ICAM-1 adhesion pair, suggesting that PECAM-1 is not required for the purpose of transendothelial migration in these lymphocytes (Ager 1994). A cytoskeletal role has also been suggested for PECAM-1, from the observation that PECAM-1 can move to endothelial cell-cell borders taking place in interactions limiting vascular permeability (Albelda et al., 1991).

1.7.4 Other adhesion molecules

VAP-1 and L-VAP-2

VAP-1 (vascular adhesion protein) is an endothelial adhesion molecule mediating lymphocyte adherence. It is preferentially expressed in peripheral lymph node type HEV under normal conditions, and not in mucosal lymphoid organ HEV, however expression can be induced during chronic inflammation in the mucosa, synovium, tonsils and skin endothelial venules (Salmi & Jalkanen 1995). Low expression can also be found in brain, kidney, liver and heart (Imhof & Dunon 1995). VAP-1 is

expressed both on the luminal surface and in cytoplasmic granules in HEV, which are distinct from Weibel-Palade bodies (which can carry Factor VIII related antigen and P-selectin), and preferential localisation on either surface or granule varies from vessel to vessel (Salmi et al., 1993). The regulation of VAP-1 expression is thought to be quite distinct from other adhesion molecules, yet a clear analysis of this unique regulatory system has not yet emerged. Also the exact nature of VAP-1 as an adhesion molecule, its counter-receptor, its role in inflammation and its possible function in lymphocyte adherence to peripheral lymph node HEV is still largely unknown. Clearly more work needs to be done to elucidate the functions of this interesting molecule.

L-VAP-2 (lymphocyte vascular adhesion protein) is involved in lymphocyte adhesion to vascular endothelium (Airas et al, 1993). Expression is limited to human umbilical vein endothelial cells (HUVEC) and around 20% of peripheral blood lymphocytes of the B and CD8 T lymphocyte subsets (Airas et al., 1993, Imhof & Dunon 1995). Expression of L-VAP-2 cannot be upregulated by cytokines or LPS on HUVEC. There has been some suggestion that L-VAP-2 may be another endothelial cell ligand for LFA-1, or that L-VAP-2 may share a common ligand with LFA-1 (Airas et al., 1993). Whatever the possible outcome, as with VAP-1 much more needs to be learned about the function of this molecule.

CD44

The CD44 transmembrane glycoprotein is widely expressed. It is found on B and T lymphocytes, monocytes, neutrophils, epithelial cells,

fibroblasts, glial cells and myocytes (Pigott & Power 1993). CD44 has been known for more than 10 years under various names and roles, such as lymphocyte homing receptor (gp90 Hermes), phagocytic glycoprotein (Pgp-1), extracellular matrix receptor (ECMRIII) and hyaluronate receptor (H-CAM) with monoclonal antibody studies revealing identity between these molecules (Gunthert 1993). As one would expect from such a widely distributed molecule CD44 has a large array of functions, from cell-cell to cell-matrix interactions, to hematopoiesis, lymphocyte and monocyte activation, cell migration and adhesion and finally metastasis (Haynes et al., 1989, Haynes et al., 1991). It is now known that the multitude of functions listed above are performed by different isoforms of CD44 generated by alternative splicing from a single gene (Imhof & Dunon 1995). Five different isoforms have been encountered in leukocytes.

CD44 was initially thought to be a homing receptor, mediating lymphocyte adherence to Peyer's patch HEV, but not to peripheral lymph node HEV, in frozen tissue section assays, based on experimental observations made with an anti-CD44 monoclonal Hermes-3, which inhibits human lymphocyte adherence to HEV in either mouse Peyer's patch or human appendix, but not to mouse or human peripheral lymph node HEV (Jalkanen et al., 1986, 1987, Berg et al., 1989). This view has however led to debate, since in vitro frozen tissue section assays do not necessarily reflect the in vivo situation, and indeed in vivo work would suggest that CD44 has a much more general role and is more likely to be involved in leukocyte extravasation into non-lymphoid inflammatory sites. Even if CD44 has a role in lymphocyte adhesion to HEV, it would appear to function only as an accessory molecule (Imhof & Dunon 1995). Similarly the wide distribution of CD44 on so many leukocytes and other

cell types would make it very difficult to suggest that CD44 is specific in its function as a homing receptor. The debate continues, and it is possible that CD44 may have different isoforms, which in certain in vitro experiments prove to display a more prominent role in organ specific lymphocyte adherence, due to the restricted conditions presented to it. It is known for example that CD44 mediates lymphocyte adherence with the involvement of hyaluronic acid (HA) to cultured endothelial cells, but not to endothelium in frozen section assays (Imhof & Dunon 1995). In general CD44 is believed to mediate lymphocyte adherence mainly by binding to HA, but also by interacting with extracellular matrix molecules such as collagen, laminin, and fibronectin (Peach et al., 1993, Imhof & Dunon 1995). This binding to HA also mediates cellular aggregation, cytokine release and lymphocyte activation (Haynes et al., 1989). The most plausible function for CD44 is that of an accessory adhesion molecule. It is known that CD44 can bind to and present chemotactic cytokines, such as MIP-1β (macrophage inflammatory protein) to leukocytes which are in contact with endothelium, leading to integrin activation and subsequent strong adhesion (Imhof & Dunon 1995). Therefore CD44 participates in many functions by means of its various isoforms, and has an important, though as yet not fully understood role in leukocyte adhesion. Once the ligands which interact with the various isoforms are identified, the overall functions and regulation of CD44 expression can be addressed.

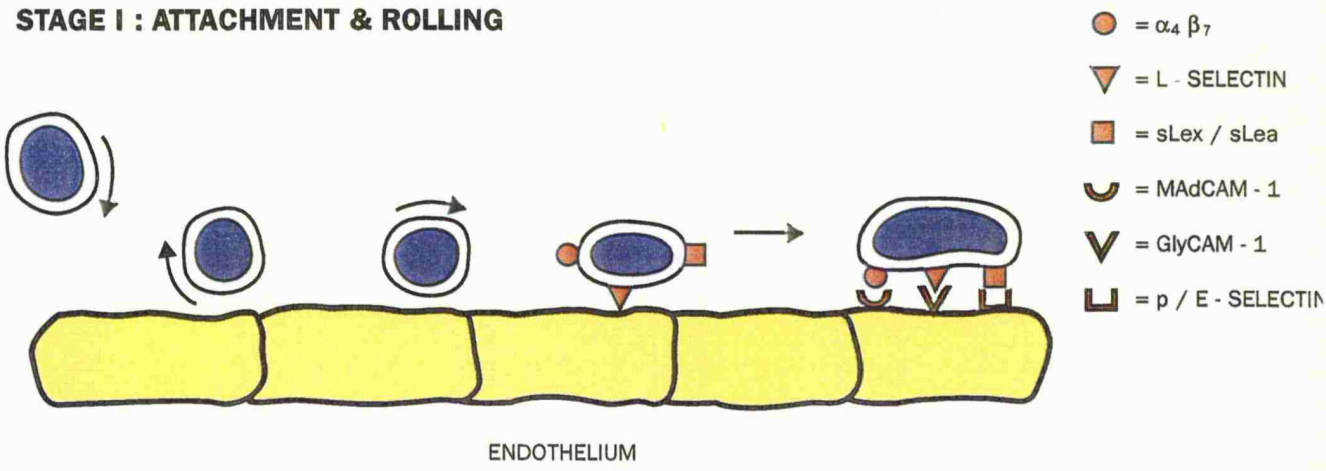
1.7.5 The adhesion cascade

All of the various adhesion molecules mentioned so far, have a part to play in leukocyte - endothelium interactions. Some are involved in the

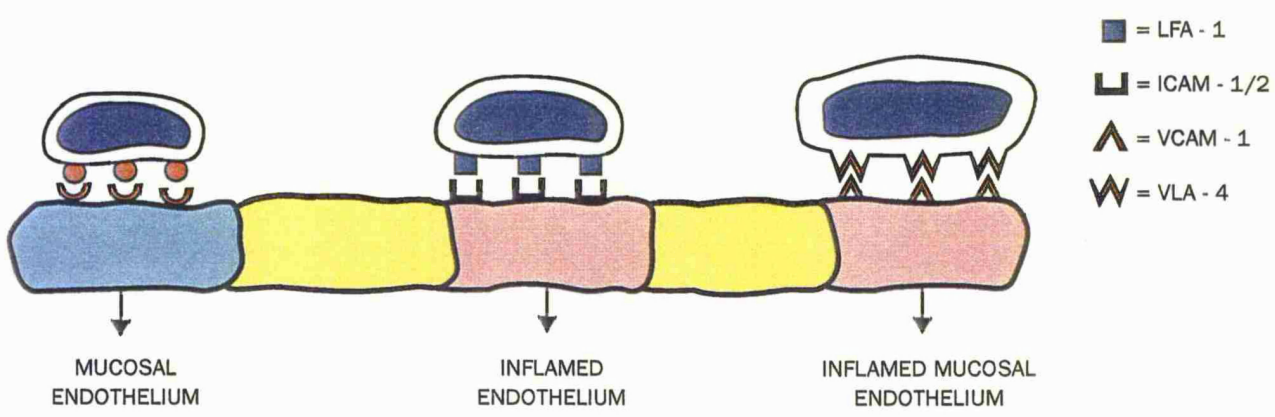
migration of lymphocytes to secondary lymphoid organs, such as peripheral lymph nodes and Peyer's patches, and mediate adhesion to HEV by functioning as homing receptors or vascular addressins, while others have more prominent functions in leukocyte adhesion to adhesion molecules expressed on inflamed endothelium and / or activated leukocytes. Some adhesion molecules will mediate adhesion in both inflammatory and non-inflammatory situations. A multi-step adhesion cascade has been developed (Butcher 1991, Springer 1994) to present a comprehensible picture of the interactions of adhesion molecules in leukocyte - endothelium interactions in vivo (see diagram-3). Briefly, the model states that in flowing blood the initial *attachment and rolling* of leukocytes on endothelium (see diagram-3, stage-1) is mediated by low-affinity, selectin carbohydrate interactions. This primary adhesion can involve separable contact formation (termed *tethering*) with loose rolling of the leukocyte along the endothelial vessel wall, and molecularly distinct mechanisms for slowing the rolling velocity or a transient immediate arrest without discernible rolling (Hogg 1993, Butcher & Picker 1996). The shear forces of the flowing blood cause the rolling of leukocytes along the endothelium surface, and this initial attachment phase slows the transit of these cells, allowing them enough time to sample the vessels for soluble or endothelial surface proadhesive factors (Butcher & Picker 1996). Adhesion in this stage is mediated by selectins (L, E and P-selectin) and their counter-receptors found on the endothelium or leukocyte cell surface (in some cases $\alpha4\beta7$ / MAdCAM-1 and, or L-selectin / MAdCAM-1 can be involved in this first stage as well). The participation of $\alpha4\beta7$ in this stage of adhesion is thought to mediate a slower rolling rate than L-selectin mediated adherence, which can be important in order to slow down selectin initiated rolling long enough for the engagement of activation dependent adhesion mechanisms. In the

DIAGRAM 3 : THE ADHESION CASCADE

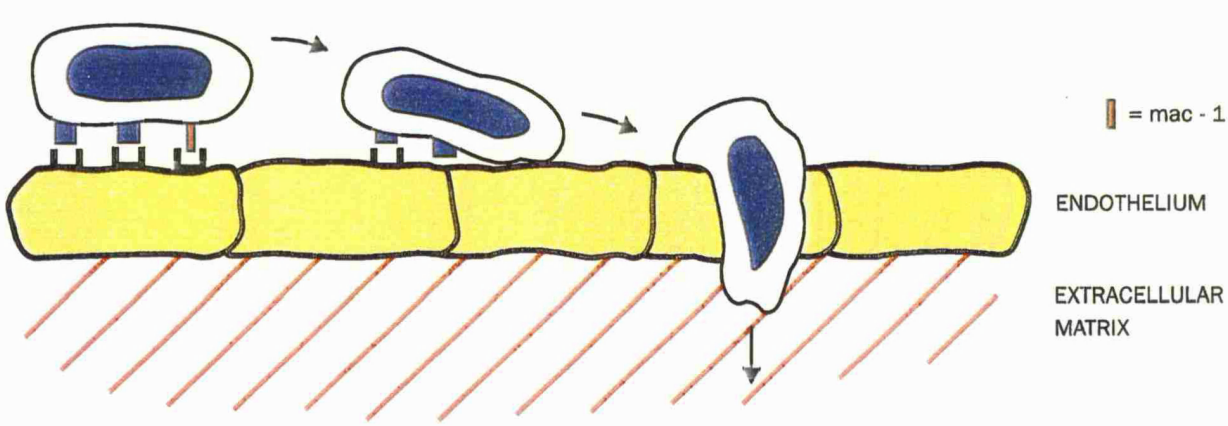
STAGE I : ATTACHMENT & ROLLING



STAGE II : STRONG ADHESION



STAGE III : TRANS - ENDOTHELIAL MIGRATION



second stage of the cascade, the rolling and initial attachment of leukocytes leads to *strong adhesion* (see diagram-3, stage-2). Adhesion is stabilised and leukocytes are arrested from the flowing blood, onto the endothelial surface. This stage is mediated by integrins and their ligands of the Ig superfamily (adhesion pairs such as $\alpha4\beta7$ / MAdCAM-1, LFA-1 / ICAM-1 and or ICAM-2, and VLA-4 / VCAM-1 are involved). The involvement of different adhesion pairs in this stage depends largely upon whether the tissue in question is inflamed, or if migration is taking place in secondary lymphoid organs, such as the Peyer's patches (where the $\alpha4\beta7$ / MAdCAM-1 pair would be involved). The final and last stage is called the *trans-endothelial migration* or *transmigration* stage (see diagram-3, stage-3). In this stage strongly adherent leukocytes from the previous stage, will travel from the luminal side of the endothelium to the sub-endothelial basal membrane within minutes. This travel is one way since transmigrating cells are trapped by the extracellular matrix of the basal membrane. It is thought that the adhesion pairs of LFA-1 / ICAM-1 and Mac-1 / ICAM-1 along with CD44 and VLA-4 / fibronectin are involved in leukocyte transmigration (Hogg 1993, Ager 1994, Hogg & Berlin, Imhof & Dunon 1995). This final stage of the adhesion cascade is arguably the least well known of all the three stages.

Our knowledge regarding the multi-step adhesion cascade is being continually renewed, since its inception over five years ago, from a clear cut, well defined system of leukocyte adhesion to a continuous, flowing and more often then not, overlapping picture of adhesion molecule interaction and behaviour. With the emergence of new roles and functions for the already incorporated adhesion molecules to the cascade, and the advent of completely new adhesion molecules over the last four to five years, the initial confines of the cascade have now lead to a bewildering

abundance of adhesion molecule permutations and possibilities. No doubt this will continue to increase as new roles are assigned to lesser known molecules, developing the picture further, and thereby demonstrating the complexity of such a system which has evolved to operate in different tissues and under different conditions.

The role of recirculating lymphocytes in the pathogenesis of inflammatory diseases has been known since the pioneering studies of the late seventies (Paulus et al., 1977, 1979) which showed that prolonged thoracic duct drainage (which preferentially depletes recirculating T cells) had a beneficial effect on the clinical course of inflammatory conditions such as rheumatoid arthritis and sytemic lupus erythematosus. Furthermore, reinfusion of viable thoracic duct lymphocytes exacerbated the disease, while reinfusion with killed thoracic duct lymphocytes had no such effect (Paulus et al., 1977). Direct studies of lymphocyte trafficking using intravenous injection of ^{51}chromium labelled cells demonstrated a small proportion of injected lymphocytes in the inflamed synovial membrane, the synovial fluid and thoracic duct, confirming the recirculating nature of lymphocytes in rheumatic disease (Schrieber 1994). In the following chapters rheumatoid arthritis will be discussed with emphasis on the immunology of the disease, and also the particular role that adhesion molecules play.

1.8 Rheumatoid arthritis and its clinical features

Rheumatoid arthritis (RA) is a disabling and painful inflammatory disease of multiple joints (typically involving the interphalangeal joints of the hands, the metacarpophalangeal joints and the wrists), affecting

similar joints bilaterally with extra-articular involvement of several organ systems a frequent secondary feature. In this respect RA can be regarded as a systemic disease, it is however also classified as an autoimmune disorder, due to the strong correlation between serum levels of autologous antiglobulins (rheumatoid factors), and severity of disease. It is characterised by chronic synovial inflammation, brought about by the invasion of the synovial membrane and fluid mainly by memory T cells, macrophages and plasma cells, accompanied by the maintenance of this cellular immune response by inflammatory cytokines (Harris 1990, Ziff 1990, Lawrence 1994). This leads in most cases to progressive destruction of cartilage and bone, which occurs after invasion of these tissues by the cellular synovial tissue and is believed to be mainly mediated by cytokine induction of destructive enzymes, chiefly matrix metalloproteinases (Feldmann et al., 1996). The disease is prevalent throughout the world, in with an estimated 1-2% of the general population being afflicted. It has been found in every ethnic or racial group that has been studied, with climate, geography and altitude no affecting the prevalence of the disesae, although climate has been reported to influence symptoms, with wet or humid climates leading to increased discomfort (Gordon & Hastings 1994). Rheumatoid arthritis is on average three times commoner amongst women than men, with the average age of disease onset around middle age, though no age is exempt. This has been noted in all populations studied. The severity of this disease is broad, ranging from mild to severe, however the disabling effects are cumulative and at the severe end of the spectrum can lead to premature death (Webb 1993, Maini et al., 1995). The aetiology of RA remains largely unknown. Most of the current thinking on aetiology is speculative, based on analogy with experimental models of arthritis in animals and other forms of human disease, particularly reactive arthritis. It remains to be seen how valid

such comparisons actually are. In recent years, considerable progress has been made in unraveling at least some of the genetic factors involved in RA. Since direct evidence of infection in the aetiology of RA has proved difficult to obtain, the emphasis has shifted from trying to identify a single trigger factor for the disease, to possibly a variety of factors that cause the disease to develope in susceptible individuals. For many years clinicians have noted familial aggregation of RA cases. These observations led to an array of genetic epidemiological studies aiming to define the contribution of inherited factors to the aetiology of RA. The association of the MHC class-II HLA-DR4 gene with RA was observed, with the demonstration of a relationship between increasing DR4 frequency and features of disease severity including levels of erosions, development of extra-articular disease and seropositivity (Ollier & MacGregor 1995).

The clinical course of RA generally falls into three catagories. In the majority of cases, periods of disease activity are interspersed with intervals of spontaneous remission, the duration of remission periods lasting between a few months to several years. This pattern offers the most favourable prognosis. The second course, which is also fairly common, is characterised by a progressive and unremitting synovitis, interrupted by sporadic flares typified by severe joint pains and effusions, marked inflammation, fever, general malaise, anaemia and debilitation. The third and far less frequent course of disease, is one of very aggressive and rapid polyarticular synovitis, accompanied by very high rheumatoid factor titers, nodules, and extensive extra-articular organ involvement (Webb 1992).

Subcutaneous nodules occur in 20% of RA patients with positive tests for blood rheumatoid factors and rarely in seronegative patients. The

nodules reflect the level of rheumatoid disease activity and accompany severe disease (Matteson et al., 1994). Nodules develope most commonly on pressure areas, including the elbows, finger joints, achilles tendon, occipital scalp and ischial and sacral prominences. Rheumatoid nodules are firm and frequently adherent to the underlying periosteum. Histologically there is a focal central fibrinoid necrosis with surrounding fibroblasts, which is thought to occur as a result of small vessel vasculitis with fibrinoid necrosis which forms the centre of the nodule, and surrounding fibroblastic proliferation (Matteson et al., 1994).

Small vessel vasculitis is intimately associated with many of the clinical manifestations seen in RA. Inflammation of the small and medium sized arteries in the extremities, peripheral nerves, and occasionally other organs may complicate RA. Patients with this form of rheumatoid vasculitis have a higher frequency of HLA-DR4 than those with uncomplicated RA, suggesting an inherited susceptibility to this complication (Scott et al., 1981). Study of the early lesions of vessels from patients with rheumatoid vasculitis, show fibrinoid necrosis of the vessel wall with an inflammatory cell infiltrate. Chronic changes with artery wall fibrosis, occlusion, and recanalisation may also be seen. The acute arterial lesions are immune complex mediated, as indicated by the finding of immunoglobulins and complement in the involved arteries (Conn et al., 1972). Systemic vasculitis that complicates RA is generally uncommon. It usually occurs in rheumatoid patients who have longstanding disease, usually of 10 years or more. Rarely is systemic vasculitis present at the onset of RA, which in such cases the outcome is poor. Rheumatoid synovitis may not be active when the features of the systemic vasculitis are present. Small vessel vasculitis commonly involves the skin and causes nailfold infarcts, digital gangrene and leg ulcers. In

some patients, the vasculitis may extend to involve mesenteric, coronary and cerebral arteries. Patients with rheumatoid vasculitis have high serum titres of rheumatoid factor, circulatory immune complexes and low serum complement, in addition to a elevated erythrocyte sedimentation rate, anemia and a diminished serum albumin.

Ocular, hepatic and pulmonary involvement in RA is also present, with keratoconjunctivitis affecting between 10 to 35% of patients, elevation of liver enzymes occasionally associated with active RA and interstitial pulmonary fibrosis also a feature in 15 to 25% of patients (Matteson et al., 1994).

1.8.1 Historical background of rheumatoid arthritis

The term "rheumatoid arthritis" was first introduced into medical literature by Sir Alfred Barring Garod, in 1859. However specific features of RA, such as hyperextension deformities (swan neck deformity) of the finger joints were identified as early as 1676, by the physician Thomas Sydenham. Another early example is madame de Sevigne, who died in 1696, and described her joint disorder in letters she wrote. According to reviewers, her disease had several features suggestive of RA, and would have even satisfied the ARA (American Rheumatism Association) criteria for the classification of RA (Halberg 1994). The first detailed description of RA is found in the doctoral thesis of Landre-Beauvais in 1800. He described nine patients, all female, with a polyarthritis associated with joint ankylosis and progressive deformity, which he termed primary asthenic gout (Symmons 1995). Sir Alfred Garrod's son, Sir Archibald Edward Garrod was the first to present a

clear and well defined clinical description of the disease, in his paper of 1890 titled "A treatise on rheumatism and rheumatoid arthritis". In his paper Sir Archibald Garrod also described osteoarthritis (which is now classified as a separate entity) and speculated on the origins of RA, tracing it back to ancient Egyptian and Roman times, based on paleopathological studies. Others have argued however that RA is a relatively new disease, with no convincing descriptions prior to the late 17th century (Snorrason 1952, Boyle & Buchanan 1971, Short 1974). The issue remains controversial and has been renewed since the findings of archaic Indian skeletal remains indicating RA, in Alabama, dating back 3000-5000 years (Halberg 1994). The British Ministry of Health adopted RA as an official term in 1922, with the American Rheumatism Association following suit in 1941 (Symmons 1995).

1.8.2 Immunology of rheumatoid arthritis

The earliest stage of rheumatoid arthritis, is clinically refered to as the "acute phase" and is characterised by immune hyperactivity. During this phase the synovium becomes heavily infiltrated by T lymphocytes (mainly memory helper cells), plasma cells, monocytes and macrophages, with neutrophil accumulation within the synovial fluid. These cells and the cytokines that they produce trigger synovial cell proliferation, activation of the vascular endothelium, generation of new blood vessels, and further accumulation of activated leukocytes (Issekutz & Issekutz 1991, Webb 1993, Mertens et al., 1994). Histological examination of rheumatoid synovium reveals synovial cell hyperplasia, infiltration by plasma cells, lymphocytes, macrophages and mast cells, and the overgrowth of the fibrovascular granulation tissue known as pannus. This

tissue progressively covers and erodes articular cartilage, and along with the secretion of metalloproteinases such as collagenase and proteoglycanase by inappropriately stimulated resident connective tissue cells (synoviocytes, chondrocytes and osteoclasts) irreversible joint destruction is achieved (Schrieber 1994).

A major aspect of pannus, is the formation of new blood vessels (angiogenesis). The factors that control angiogenesis in RA, are poorly understood however, although a variety of soluble factors such as phorbol myristic acetate, fibroblast growth factors α and β, transforming growth factor β (TGF–β), TNF-α, heparin, prostoglandin E2, and platelet activating factor are thought to be involved (Folkman & Klagsbrun, 1987). Several cell types, such as macrophages, lymphocytes, polymorphonuclear leukocytes, mast cells, and fibroblasts can stimulate angiogenesis by their secreted products (Schrieber 1994). Since many of these cells are present within the rheumatoid joint, it is conceivable that any combination of them could be responsible for the development of new blood vessels during pannus formation. The synovial fluid has also been suggested as a possible activator of angiogenesis, though experimental evidence is still scarce. Both TNF-α and TGF-β are present in rheumatoid synovial fluid, which would suggest involvement.

The synovium of RA often shows histological features similar to lymph nodes (Kurosaka & Ziff, 1983). Three regions have generally been described, these being the plasma cell rich zones, which consist of nearly 75% plasma cells, secondly the lymphocyte rich zone, which contains around 80% lymphocytes and finally the transitional zone, which consists of a high percentage of blast cells, antigen presenting cells, small lymphocytes and macrophages (Firestein 1994). A variety of immune

cells exist within the synovium, with T lymphocytes making up 30-50% of such cells. The predominant T cell subset is the CD4+ helper lymphocyte, with the CD4/CD8 ratio ranging from anything to 4:1 to 14:1 (Firestein 1994). This relative lack in the numbers of CD8+ T cells (hence suppression), allows the interaction of CD4+ cells with MHC class-II positive antigen presenting cells, driving the immune reactivity forward. The majority of the CD4+ population is known to express the CD45 RO marker characteristic of mature memory lymphocytes, and therefore suggesting previous exposure to antigen. Although naive (CD45 RA) cells are also present within the synovium, they represent a clear minority. T lymphocytes are also abundant within the synovial fluid, however the numbers of CD4+ and CD8+ cells are approximately equal to each other, in contrast to synovial membrane. Although the reasons for this discrepancy are not clear, it may represent the preferential migration of CD8+ cells from membrane to fluid, rather than CD4+ cells. Furthermore the high levels of IL-6 found within the synovial fluid may favour such expansion, since IL-6 is known to induce CD8+ T cell proliferation.

Other events take place within the synovium as well, the most important being the proliferation of B lymphocytes, with subsequent rheumatoid factor (RF) synthesis, and formation of immune complexes with autologous IgG antibodies. During the clearance of such immune complexes from the synovial fluid, neutrophils release proteolytic enzymes such as collagenase and elastase, pro-inflammatory prostaglandins, leukotrienes and free oxygen radicals which sustain the inflammatory state within the synovium and also contribute to the eventual destruction of the joint. Non-phagocytosed immune complexes on the other hand, lead to the activation of the complement system

(complement proteins are found within the synovial fluid), which in turn mediates further inflammatory systems. The liver is the major source of complement synthesis in humans and passive transfer of serum proteins into synovial fluid can account for some of the complement proteins found there. On the other hand, these proteins are also known to be produced locally in the joint, with macrophages and / or fibroblasts being the most likely sources (Firestein 1994).

T cells, especially CD4(+) helper cells of the memory phenotype are thought to be central to the whole process of RA joint inflammation, orchestrating the immune response through cytokines and activating effects on other cells, perpetuating joint inflammation (Panayi et al., 1992, Sewell & Trentham 1993). There is debate however about this central role, with the observation that cytokine levels produced by such T lymphocytes (such as IL-2 and IFN-γ) are low within RA synovial membrane and fluid, and that monocyte / macrophage derived products, such as TNFα, IL-1 and IL-6 are plentiful, suggesting a more prominent role for such cells (FitzGerald & Bresnihan 1995). However, evidence to support the role of T cells in the development and progression of RA is substantial. CD4 T cells are the dominant T cell phenotype in the synovial membrane, the disease improves with T cell targeted interventions (such as thoracic duct drainage, total lymphoid irradiation and cyclosporin treatment) and active arthritis is less severe with patients with AIDS, who have CD4 cytopenia (Sewell & Trentham 1993). Due to the abundant presence of monocyte / macrophage derived cytokines and the low levels of T cell derived cytokines within the rheumatoid joint, it would seem more likely that the former rather than the latter type of cells have a dominant role in the perpetuation of RA. However it has been recently suggested that the biochemical evidence favouring monocytes /

macrophages and the histological evidence favouring memory T lymphocytes, is compatible. It has been put forward that the mature memory T cell population is in a dysfunctional or downregulated state, hence explaining the lack of T cell derived cytokines within the rheumatoid joint. This theory is held together by two major points. First, although memory T cells produce low levels of IL-2, this is enough to support T cell function, if high affinity IL-2 receptors are expressed and second, low detectable IL-2 is the consequence of rapid turnover (Sewell & Trentham 1993).

Whatever the various roles lymphocytes, neutrophils, monocytes or macrophages have to play in joint inflammation, the essential factor is one of migration and eventual accumulation within the synovial environment. The migration of such cells is controlled and mediated by adhesion molecules. The expression of adhesion molecules on inflamed synovial membrane endothelial cells, and the recognition of these molecules by their complementary counter-receptors on leukocyte cell surfaces is the essential first step in synovial infiltration.

1.8.3 Adhesion molecules involved in rheumatoid arthritis

Inflammatory cytokines stimulate the expression of certain adhesion molecules on synovial endothelial cells, and on circulating leukocytes in cases of acute and chronic inflammation of the rheumatoid joint. PECAM-1 (CD31), VCAM-1, CD44, P-selectin, L-selectin, $\alpha 4\beta 7$, VLA-4, and fibronectin are all involved in mediating leukocyte adherence to inflamed rheumatoid synovial membrane endothelium. It would seem that the VLA-4 / VCAM-1 adhesion pair is the most prominent of all these

adhesion molecules, functioning as an inflammation specific homing receptor / vascular addressin system for lymphocytes within rheumatoid synovium (van Dinther-Janssen et al., 1991, Morales-Ducret et al., 1992, Cronstein & Weissmann 1993). Adhesion of synovial membrane T lymphocytes to synovial membrane endothelium was shown to be inhibited by anti-VLA-4 and anti-VCAM-1 monoclonal antibodies, whereas antibodies to other adhesion molecules such as CD44, L-selectin and LFA-1 did not exhibit similar levels of inhibition and also synovial membrane T lymphocytes were found to show poor adherence to peripheral lymph node HEV as compared to non-rheumatoid peripheral blood T lymphocytes adherence (van Dinther-Janssen et al., 1991). Others have found high expression levels of $\alpha 4\beta 7$ on T lymphocytes derived from rheumatoid synovium, as analysed by flow cytometry using an anti-$\alpha 4\beta 7$ murine monoclonal antibody (Lazarovits & Karsh 1993), the implication being that $\alpha 4\beta 7$ expressing T lymphocytes adhere to VCAM-1 and fibronectin expressing synovial endothelium, providing an alternative to the VLA-4 / VCAM-1 adhesion pair. This does raise questions however about the adherence of $\alpha 4\beta 7$ and VLA-4 expressing lymphocytes to the same endothelial adhesion molecule. Further experimental evidence is required to substantiate the role $\alpha 4\beta 7$ plays in lymphocyte adhesion to synovial endothelium, and also whether VCAM-1 is indeed the first choice ligand for $\alpha 4\beta 7$, or whether another as yet unidentified ligand present on endothelium is involved. More recent research has suggested the involvement of CD44 as a homing receptor of lymphocytes to rheumatoid synovium, with expression of CD44 being upregulated on rheumatoid synovial fluid T lymphocytes relative to peripheral blood lymphocytes from the same individuals (Kelleher et al., 1995). However the relative contribution of CD44 as a homing receptor is debatable, since the majority of T lymphocyte adherence to synovial

endothelium occurs through the VCAM-1 pathway, which is not a ligand for CD44. In fact the ligand involved in CD44 mediated adherence to synovial endothelium is not yet clear, however hyaluronic acid, which is expressed in synovium and which can act as a ligand for CD44, has been implicated (Kelleher et al., 1995). It would seem more likely that CD44 functions as a signalling molecule within the synovium, rather than a homing receptor, signalling the production of cytokines such as TNF and the upregulation of other adhesion molecules involved in synovial adhesion.

The involvement of fibronectin, in the retention of T lymphocytes within the rheumatoid joint has also been suggested, with VLA-5 acting as the counter-receptor on T lymphocytes (Rodriguez et al., 1992). The abundance of fibronectin in rheumatoid synovium would make it a likely candidate for T lymphocyte interactions, however whether this role is important in lymphocyte adhesion to synovial endothelium remains to be seen.

Adhesion molecules such as P and L-selectin have also been implicated in adhesion to synovium. L-selectin is however expressed to a limited degree on all synovial tissue components, suggesting a more general role in adhesive interactions (Johnson et al., 1993). P-selectin on the other hand is expressed by synovial endothelium, and therefore seems likely to play a role in leukocyte adhesion. Indeed it has been shown to play a role in the in vitro adherence of monocytes to frozen RA synovial tissue (Grober et al., 1991). PECAM-1 on the other hand, which is expressed on synovial endothelium, synovial lining cells and subsynovial macrophages in RA, is not thought to play a role in adhesion. Instead it has been suggested this occurrence may be the result of the chemotaxis of

PECAM-1 expressing monocytes and neutrophils into the rheumatoid joint (Johnson et al., 1993).

Adhesion molecules are also found in soluble (circulating) forms in the circulation, and raised levels have been reported in a number of conditions, including septic shock, certain cancers, impaired renal function, haemodialysis, hypertension, systemic lupus erythematosus, Wegener's granulomatosis and rheumatoid arthritis (Blann et al., 1995). Although different groups have found slightly fluctuating and in some cases different results, overall in RA, a raised level of circulating VCAM-1 and circulating ICAM-1 has been found in synovial fluid, as compared to plasma levels (Mason et al., 1993). Raised levels of circulating VCAM-1 correlated with the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels in RA patients, whereas circulating ICAM-1 levels did not. The importance of circulating adhesion molecules in the pathogenesis of RA is unknown, what is known however is that circulating adhesion molecules act as good indicators of inflammatory disease. What is perhaps more interesting is to determine how these molecules are released from cells and whether they perform a function following shedding, in terms of modulating leukocyte action, or if they are just shed for clearance and degradation purposes. It is known for example that circulating VCAM-1 and ICAM-1 can support leukocyte adherence when immobilised to plastic (Wellicome et al., 1993), therefore it is not unreasonable to assume that they could have some role to play in the continuation of inflammation in RA.

1.8.4 Evidence for gut involvement in the pathogenesis of rheumatoid arthritis

In general, there have been two approaches regarding the aetiology of rheumatoid arthritis. One has been to search for the synovial acquisition of a novel antigen, while the other has been the possibility of a primary genetic abnormality, culminating in an immunoregulatory dysfunction. Despite longstanding research into a possible aetiological role in the onset of rheumatoid arthritis, implicating a connection between several organisms from viruses (parvovirus, slow acting viruses, oncornoviruses, Epstein-Barr virus) to bacteria (*Proteus mirabilis*, *Clostridium perfringens, Streptococcus pyogenes, Mycobacteria* species, to name a few), none have really produced conclusive evidence. Possibly the most promising work has come from research concerned with *Proteus mirabilis*. Increased levels of antibodies to this bacteria in the sera of patients with RA, but not in those with ankylosing spondylitis or control subjects have been found, with the suggestion that RA might be the end stage of recurrent episodes of *Proteus* induced reactive arthritis (Ebringer et al., 1985, 1988). More recently the same authors have also demonstrated amino acid homology between the outer membrane haemolysin protein of *Proteus mirabilis* and the HLA-DR1 and HLA-DR4 subtypes associated with RA, and that patients with RA have increased titres of antibodies against a synthetic peptide common to HLA-DR4 derived from *Proteus mirabilis* membrane haemolysin (Ebringer et al., 1992, Wilson et al., 1995). These studies though interesting, do not sufficiently deliver answers as to how the *Proteus* antigens recognised by RA patient antibodies might be involved in the pathogenesis of RA. Clearly such immunochemical data is important, but in itself not enough

to prove conclusively the role of this bacteria in the onset and subsequent development of RA.

Another recent approach has been to focus on the relationship between diseases of the gastrointestinal tract and RA, and the connection between the gut and joints. There has been long unresolved debate as to whether the gut is abnormal in RA. There is no doubt that certain arthritides such as Reiter's disease and reactive arthritis are associated with enteric bacteria (Isdale & Wright, 1989), the most obvious being enteric reactive arthritis, where organisms such as *Salmonella* species, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Shigella flexneri* have been implicated in the onset of this disease. It has been demonstrated that such organisms (or their antigenic material) in cases of enteric reactive arthritis can be absorbed systematically and reach the joints (Granfors et al., 1989). It is also well established that diseases of the gastrointestinal tract such as chronic inflammatory bowel disease (which include Crohn's disease, Whipple's disease and ulcerative colitis) are associated with a peripheral arthritis with some features in common with RA, namely, the development of a synovitis affecting the larger peripheral joints such as the knees and ankles (Segal et al., 1986, Webb 1993). Indeed, the suggested causative agent of Whipple's disease (*Tropheryma whippleii*, a bacterium closely related to the actinobacillus group) has been identified in the synovium of patients suffering from Whipple's disease using electron microscopy (Gaston 1995). It is also established that serum IgA rheumatoid factor (RF) correlates with RA disease activity (March et al., 1987), as does IgA immune complexes (Westedt et al., 1986) implicating the gut in RA, since lamina propria lymphocytes from inflamed intestines, secrete increased amounts of IgA (MacDermott et al., 1986). Further evidence for such a link is provided by the observation that polymeric

(IgA-RF), rather than the monomeric form of IgA, predominates in RA synovial fluid (Elkon et al., 1982). Also IgA-RF has been detected in synovial membrane plasma cells, although synovial RF is chiefly of the IgG and IgM classes (Koopman et al., 1983).

The remission of RA in many women during and several months after pregnancy is also a well recognised fact in RA. It has been shown that during lactation the number of IgA plasma cells within the breast increases 900-fold. This increased homing has been suggested to be hormone dependent (Weisz-Carrington et al., 1978). It has also been proposed that at the time of lactation, gut derived blast cells in the circulation are diverted to the connective tissue of the mammary gland for IgA antibody secretion into the milk, with these plasma cells within breast tissue producing antibodies specific for gut associated antigens, presumably to confer passive immunity to the intestinal tract of the newborn (Roux et al., 1977). Blast cells isolated from mesenteric lymph nodes were shown to exhibit significantly better specificity for localising within the lactating breast, than peripheral lymph node blasts cells, when the migration of these two populations were compared following labelling and intravenous injection into mice. Mesenteric lymph node blast cells localised within this tissue 3 to 10 fold better than peripheral lymph node blast cells, while both cell populations localised poorly to the non-lactating breast (Roux et al., 1977). Also, mesenteric lymph node blast cells localised within the small intestine 3 to 4 fold better than peripheral node blast cells. Indeed, the inference that lymphocyte homing to the lactating breast involves an endothelial binding specificity similar to that of the small intestine has been supported by studies with the anti-MAdCAM-1 monoclonal antibody Meca-367 which has identified MAdCAM-1 expressing endothelium within Peyer's patches, mesenteric

lymph nodes, gut lamina propria and mammary gland (Streeter et al., 1988).

Concomitant with this, it has also been suggested that the breast tissues of a pregnant patient with RA would compete with other mucosa associated tissues, diverting some of the cells which would pathologically have migrated to the rheumatoid joint (Sheldon 1988). It has been postulated that an abnormality in the migratory behaviour is exhibited by lymphocytes operating within the mucosal immune system, whereby lymphocytes which normally "home" to gut associated tissues may, in RA, exhibit a pathological tendency to home to the rheumatoid synovium instead (Sheldon 1988). The so called "Iteropathy hypothesis" has suggested that these cells may encounter, in the gut, an antigen cross-reactive with a self epitope present within the joint (Sheldon 1988). Such an antigen (e.g., a bacterial cell wall component autoreactive with articular cartilage) would render certain lymphocyte clones abnormal in the sense that they would home to the synovium, expanding therein, and initiating inflammation (Webb 1993). Such a migratory behaviour would imply that gut lymphocytes would need to recognise adhesion molecules within the synovium, which share the same characteristics with those found in the gut. In other words, synovial endothelium would have to present adhesion molecules of a complimentary nature to the homing receptors expressed on such circulating lymphocytes. Although theoretical at the time, recent data has provided interesting new insights into the gut-synovium homing relationship.

It was demonstrated for example that human IL-2 stimulated T cell lines in vitro, usually bound most efficiently to the same type of tissue from which they were derived. Lamina propria lymphocytes (from

inflammatory bowel disease patients) adhered best to mucosal HEV, synovial lymphocytes (isolated from inflamed synovectomy specimens from arthritic patients) adhered best to synovial HEV and peripheral lymph node lymphocytes (from surgically resected nodes later determined to be histopathologically normal) adhered best to lymph node HEV. T cell lines derived from peripheral blood lymphocytes bound relatively well both to mucosal and synovial HEV. Moreover, T cell lines from lamina propria and peripheral blood were shown to adhere to mucosal and synovial HEV in significantly greater numbers than to peripheral lymph node HEV (with lamina propria lymphocytes adhering better than peripheral blood lymphocytes), and T cell lines from synovial membranes were shown to adhere to mucosal HEV equally as well as to synovial HEV, and both in significantly greater numbers than to peripheral lymph node HEV (Salmi et al., 1992). Furthermore, it has been shown that freshly isolated human mucosal immunoblasts adhere significantly better than small mucosal lymphocytes to mucosal HEV and that the immunoblasts also adhere to synovial HEV as efficiently as to mucosal HEV, while not adhering to peripheral lymph node HEV (Salmi et al., 1995). It was found that the adherence of mucosal immunoblasts to mucosal HEV was mediated by $\alpha 4\beta 7$ and to a lesser extent CD44, while mucosal immunoblast adherence to synovial HEV was mediated largely by VLA-4 (Salmi et al., 1995). More importantly perhaps, it was shown that mucosal immunoblasts did not adhere to human peripheral lymph nodes that were severely inflamed any better than to non-inflamed lymph nodes, just as activated T cell lines from peripheral lymph showed only marginal binding to inflamed synovial HEV, thus indicating that synovial HEV adherence is not merely due to the inflammatory status of the tissue (Salmi et al., 1995).

An alternative study has shown high expression levels of $\alpha4\beta7$ on rheumatoid synovium derived T cells (Lazarovits & Karsh 1993). The known capacity of $\alpha4\beta7$ to act as a mucosal homing receptor, binding to its ligand MAdCAM-1 (which is selectively expressed on mucosal lymphoid organ HEV and on gut lamina propria endothelium) may suggest an important role for this integrin in the recirculation of activated lymphocytes between the gut mucosa and the synovial membrane, although the expression of MAdCAM-1 on synovial endothelium has not yet been reported, possibly suggesting the involvement of VCAM-1, which can mediate the adherence of $\alpha4\beta7$ in inflammatory conditions (Andrew et al., 1994). Another recent study has demonstrated the presence of antigen specific B lymphocytes in the synovial tissue of rheumatoid patients, both after oral and parenteral immunisation with an influenza virus vaccine, with the magnitude of the synovial B cell response comparable to that seen in peripheral blood following oral immunisation (Trollmo et al., 1994). The authors suggest three possible pathways to induce a synovial B cell reactivity in response to mucosal immunisation, the first being the entrance of the mucosally deposited influenza vaccine into the blood and then subsequent entry into the synovium followed by the activation of synovial resting B lymphocytes. This argument is countered by the suggestion that only minute amounts (nanograms) of antigenic material would pass the intestinal barrier, with only trace amounts entering the joints, which would be unable to evoke a synovial B cell response. A second suggestion is that intestinal antigen specific B cells could be activated in situ by oral vaccination, and then migrate to the synovium. The final third suggestion put forward is that oral immunisation may activate antigen specific mucosal T lymphocytes, which in turn would interact with antigen committed B cells to induce antibody production, following migration to synovial tissue. The

demonstration of αEβ7 (also known as HML-1, which is restricted to intestinal intraepithelial lymphocytes) on some synovial lymphocytes would also seem to add some support to the theory of immunological "cross-talk" between the gut lymphoid tissue and the synovium (Trollmo et al., 1994).

These studies and their findings would seem to implicate the involvement of the gut in synovial inflamation and possibly rheumatoid arthritis. Although research into such a possible connection has increased over the years it is still relatively new and needs further attention and time. The general outlook in rheumatoid arthritis research has generally been to focus on events that take place within the synovium once the disease process has fully taken place and inflammation has irrevocably set in, be it research on cytokine expression, B cells and rheumatoid factors, or the role of T cells in perpetuating this process. Of course most of such research has justifiably focused on converting acquired knowledge into possible new therapeutic strategies (such as the case with anti-cytokine and anti-T cell therapies currently under study), without addressing the essential question, which should be the unknown aetiology of the disease. It is felt that any such research that tries to address this problem (be it studies on the possible genetic, hormonal or gut associated origins of disease) should be regarded equally important and noteworthy of attention.

1.8.5 Corticosteroids in rheumatoid arthritis

The treatment of rheumatoid arthritis requires a multidisciplinary approach, with combinations of non-steroidal anti-inflammatory drugs

(NSAIDs), corticosteroids and drugs which suppress the rheumatic disease process, providing the basis for treatment alongside physical therapies including general and specific exercises, attention to patient psychology and in some circumstances careful diet control.

NSAIDs are one of the most commonly prescribed group of drugs, with up to 20% of people admitted to hospital taking them. They include drugs such as the short half-life aspirin, ibuprofen, indomethacin, ketoprofen and diclofenac, along with the long half-life fenbufen, naproxen, salicylate, and piroxicam to name a few. The principle action of NSAIDs is to interfere with prostaglandin production, though other cellular processes such as cell membrane functions, superoxide generation, rheumatoid factor production, cartilage metabolism and leukotriene synthesis are also modified by these drugs (Sewell & Trentham 1993). NSAIDs are one of the most common causes of adverse reactions, the most frequent being gastrointestinal effects, followed by renal damge, skin reactions, headaches and nausea (O'Brien & Bagby 1985). Drugs which suppress the rheumatic disease process, such as gold, penicillamine, hydroxychloroquine, sulphasalazine, and immunosuppressants such as azathioprine, methotrexate, chlorambucil and cyclophosphamide, do not produce an immediate therapeutic effect like NSAIDs, but require 2 to 6 months of treatment for a full response. However these drugs may not only improve the symptoms and signs of inflammatory joint disease, but also extra-articular manifestations such as vasculitis (British National Formulary, 1995). They have a wide range of side effects from rashes and nausea to conditions of renal impairment, hypertension, hepatic fibrosis and oncogenicity.

Corticosteroids are the only drugs in routine use which reliably, effectively and rapidly suppress synovial inflammation in rheumatoid arthritis (Kirwan 1994). They are superior in effectiveness, when compared to non-steroidal anti-inflammatory drugs (NSAIDs) and given in low doses may have fewer disadvantages than immunosuppressants. They have been used in the treatment of RA since 1949 (Hench et al., 1950) and despite initial enthusiasm for the treatment, the plethora of adverse effects that generally followed high dosages, led to a re-evaluation of their role. Recent interest in chronic low dose oral corticosteroid treatment in RA has led to more widespread use, with possibly less toxicity then previously thought (Smith et al., 1990). Intra-articular corticosteroids, to relieve pain and increase mobility, still have an important place in RA treatment. In recent years pulse therapy, involving the intravenous (i.v.) infusion of a large dose of corticosteroid (usually 500-1000 mg of methylprednisolone) up to three times over a seven day period has been used to induce remission prior to the ameliorating effects of disease modifying drugs.

Pulse therapy was initially used for renal transplant patients, but spread to the treatment of other renal disorders, such as lupus nephritis. The observation that synovial inflammation responded rapidly, and for prolonged periods with i.v. therapy led to controlled studies throughout the 1980s, which suggested beneficial effects lasting up to 6-12 weeks, with fewer side effects (Kirwan 1994). Today, methylprednisolone acetate (a synthetic corticosteroid with predominantly glucocorticoid activity) is one of the most frequently used corticosteroids in pulse therapy, with a favourable risk / benefit ratio, and relatively minor side effects. It is known to cause a temporary lymphocytopenia (of T lymphocytes in particular), decreased IL-6, IL-8 and soluble IL-2 receptor levels in

serum, diminished synthesis of IL-1 by mononuclear cells, decreased CRP, ESR and immune complex (IC) levels and also results in fluctuations in the levels of synovial fluid and peripheral blood neutrophil levels (Smith et al., 1988b, Smith et al., 1988c, van den Brink et al., 1994, Kirwan et al., 1994). On the other hand very little is known about the effects of pulse therapy on adhesion molecules and lymphocyte migration. It would seem reasonable to assume that a therapy which leads to the suppression of joint inflammation, and causes fluctuations in circulating leukocyte levels, may also exert an inhibitory effect on adhesion molecules. It would also be of great interest to know if such an effect were a general, overall inhibitory effect or a specific one, confined to any particular lymphocyte subsets.

Recently many clinical studies have concentrated on monoclonal antibody treatment against TNFα and CD4 lymphocytes as a possible therapy of rheumatoid disease and also as an alternative to conventional drugs (Maini et al., 1995). Monoclonal antibodies directed against human cell surface antigens such as the Campath-1 antigen (CD52) have also been studied with apparent efficacy (Isaacs et al., 1996). It was found that patients with active rheumatoid arthritis who had been treated over a ten day period with a small dose (60 mg in total) of Campath-1 improved clinically with response durations of up to seven months (Isaacs et al., 1992). However subsequent studies have demonstrated adverse effects such as fever, nausea, increased incidence of hypotension, rash and incidence of serious opportunistic infections with increased doses of up to 400mg over ten days (Isaacs et al., 1996). These findings are perhaps not surprising as Campath-1 therapy has been reported to cause prolonged peripheral blood CD4+ lymphopenia along with short term monocyte and natural killer cell depletion (Isaacs et al., 1996).

Although interesting and promising for the future of RA treatment, currently such therapies are short lived, with symptomatic improvement rarely exceeding a few months, with several side effects, the most prominent being low tolerance against administered monoclonal antibodies. There are also conflicting reports as to the benefit of such therapies, especially in the case of anti-CD4 treatment. It would seem that combinations of such agents at low doses, with already established drugs in RA therapy should be used in future clinical studies to evaluate the benefit of such potential treatments in rheumatic disease.

1.9 Objectives of study

The main objective of the work carried out was to examine the possible connection between gut homing mononuclear cells (MCs) and those that infiltrate the joint in rheumatoid arthritis, within the light of previously published work. Experiments were carried out to compare the adhesion characteristics of different MC populations. To this end, porcine MC populations of distinct circulatory behaviour (mucosal and peripheral lymphoid tissue homing) were used and compared with human control and rheumatoid MCs in their adhesion properties. For this pupose an in vitro adherence assay was used, and a considerable effort was made to validate this assay and to address possible shortcomings. A series of monoclonal antibody experiments was carried out to try to elucidate the likely adhesion molecules involved. Analysis of temperature and cation sensitivity experiments along with a series of assays determining MC-endothelium interaction characteristics were also done. Mononuclear cell adhesion in other arthritic conditions was studied and compared with RA.

The second part of the study was concerned with the effects of a commonly used corticosteroid (methylprednisolone) on the adhesion characteristics of rheumatoid MCs. In vitro and ex vivo experiments were carried out. Mononuclear cell adhesion as well as circulating MC subsets were studied before and after steroid therapy over a 24 hour period. Finally, the effects of steroid therapy on adherent MC subsets was analysed as well as its effect on circulating adhesion molecule VCAM-1 over a 24 hour period.

The overall aim of this study therefore, was to establish a dependable, reproducible in vitro adhesion assay system, to address the problems associated with such an assay and then to study the overall adhesion characteristics of several MC populations (including those from rheumatoid arthritis patients) and by inference their possible homing properties. Once this was achieved, possible factors and agents that may effect MC adhesion were analysed, with special attention paid to the effects of a commonly used corticosteroid in rheumatoid arthritis patients.

Table-1 The Integrin Family And Their Ligands

Name of Integrin	Other names	Ligands
β1 Integrins		
α1β1	VLA-1	Coll (I, IV), LN
α2β1	VLA-2	Coll (I, IV), LN, FN
α3β1	VLA-3	Coll (I), LN, FN, Ep
α4β1	VLA-4, LPAM-1	VCAM-1, FN
α5β1	VLA-5	FN
α6β1	VLA-6	LN
α7β1	VLA-7	LN
α8β1		?
α9β1		?
αvβ1		FN
β2 Integrins		
αLβ2	LFA-1	ICAM-1, 2, 3
αMβ2	Mac-1	ICAM-1, FIB
αXβ2	p150, 95	FIB
αDβ2		ICAM-3 ?
β3 Integrins		
αIIbβ3		FIB, FN, VN
αVβ3		FIB, FN, VN, LN
β7 Integrins		
αEβ7	M290 IEL, HML-1	E-cadherin
α4β7	LPAM-1	MAdCAM-1, VCAM-1
Other β integrins		
α6β4		LN
αVβ5		VN
αVβ6		FN
αVβ8		?

LN (laminin), FN (fibronectin), VN (vitronectin), FIB (fibronectin) Coll (collagen), Ep (epiligrin).

CHAPTER 2

Methods

2.1. Animals

Porcine Peyer's patch, peripheral blood and peripheral lymph node samples were obtained from local abattoirs. In all, 34 porcine samples; 15 Peyer's patch, 12 peripheral blood and 7 peripheral lymph nodes were obtained. All samples came from healthy, disease free animals, which had been approved for slaughter and human consumption by the abattoir health inspector.

2.2. Patients and human controls

A total of 92 patients were studied, of which 58 were patients with active disease, who all satisfied at least four of the revised ARA criteria (Arnett et al, 1988, see appendix-2) for Rheumatoid Arthritis (RA) and had at least three clinically inflamed joints, a raised plasma viscosity, and an increased C-reactive protein level. None had been on steroids or immunosuppresive agents prior to inclusion into study.

Of those with RA, paired samples of peripheral blood (PB) and knee synovial fluid (SF) were obtained from 25 patients (15 female and 10 male). Synovial membrane (SM) samples were obtained from 7 patients (5 female and 2 male) undergoing synovectomy operations. The disease duration of all 32 patients ranged from five months to 12 years, and their age range was 50 to 80 years old. None of the patients were on steroids or immunosuppresive agents.

Samples of PB were obtained from another 26 RA patients (18 female and 8 male) prior to and 24 hours following 500mg intravenous

glucocorticoid (methylprednisolone acetate) pulse therapy. Their age range was 55 to 75 years old. This therapy is generally intended to induce benefit prior to the effect of their new Disease Modifying Anti-Rheumatic Drug (DMARD), or as an intercurrent treatment of patients already established on their existing DMARD, but whose disease had flared. None of the patients studied were on immunosuppressive drugs.

Peripheral blood samples were also obtained from 30 volunteers who acted as control samples (23 female and 7 male) with an age range of 50 to 65 years old. These volunteers were patients who had come to hospital with complaints of back pain and were not on any medication except for symptomatic treatment with topical non-steroidal anti-inflammatory drugs (NSAIDs) to relieve back pain and stiffness. Four additional peripheral blood samples were obtained from healthy laboratory personnel, of age range 23 to 34 years old.

2.3 Preparation of Porcine and human tissue for cryostat sectioning

Small intestine and peripheral lymph nodes were obtained from freshly slaughtered pigs. The small intestine and lymph nodes were excised and transported to the laboratory immediately, in PBS. All samples were transported in ice. Porcine Peyer's patches (PP) were identified as raised, spherical dome like areas on the outer epithelium of the jejunal small intestine. They were excised from the rest of the tissue and cut into pieces of 0.5 -1cm^2 in size. A total of 20-25 Peyer's patch pieces were obtained in this manner from each sample. All PP pieces were

washed twice in 100ml of PBS for 15 minutes each, on a stirrer with a magnetic flea. The supernatant was aspirated and discarded each time.

Cylindrical moulds were made by wrapping aluminium foil around 4ml Bijoux tubes, while the PP pieces were being washed. Once ready these moulds were filled up to an depth of 1cm with Tissue Tek OCT tissue embedding compound. The washed PP pieces were embedded in this compound one at a time and frozen in liquid nitrogen using an iso-pentane heat buffer. The moulds were then wrapped and stored at -70°C. Each mould contained two horizontally embedded pieces of PP tissue. The same procedure was used for the preparation of porcine peripheral lymph nodes (LN) except that the node was cut into pieces of 0.5 - 1cm^2 and frozen.

Human Synovial Membrane (SM) samples from RA patients and human gut tissue from 1 patient undergoing gut resection for carcinoma, were processed in the same manner as Porcine PP and LN tissue. As was the case with LN tissue, all of the synovial membrane was cut and frozen.

2.4 Procedure for isolating mononuclear cells (MCs) from porcine and human samples

2.4.1 Isolating mononuclear cells from porcine tissue (enzymic method)

Porcine PP tissue was obtained and prepared as previously discussed (sections 2.1 and 2.4 respectively) with the exception of freezing the PP tissue pieces. Instead, up to 40 pieces of PP tissue were

added to 100ml of Hank's Balanced salt Solution (HBSS), to which was added 150mg of Dispase (neutral protease from *Bacillus polymyxa* grade 2) and stirred at 37°C for 45 minutes. The supernatant, containing the PP cells dissociated from the tissue, was aspirated and centrifuged at 1000 rpm (150g) for 10 minutes at 20°C, and the remaining enzymatically disrupted PP tissue pieces discarded. The cell pellet was washed twice more with HBSS, and finally resuspended in 5 ml of sICM. This final suspension of PP cells was overlayered onto an equal volume of Histopaque (mononuclear cell separation medium) and centrifuged at 1800 rpm (400g) for 30 minutes at 20°C. PP mononuclear cells (MCs) were aspirated from the interface layer, washed twice and finally resuspended in 1ml sICM. The same procedure was used for obtaining MCs from porcine peripheral lymph node.

It should be added that mononuclear cells aspirated from the interface following Histopaque separation, would normally yield a population of cells consisting largely of B and T lymphocytes, as well as the non-B, non-T lymphoid cells called Natural killer (NK) cells in smaller numbers (NK cells make up less than 10% of the total percentage of lymphocytes in peripheral blood, lymph node and spleen), along with a small percentage of monocytes (from 2-11 % depending upon the source of sample), while erythrocytes, granulocytes (or polymorphonuclear cells) and platelets would sediment to the bottom of the tube (Boyum 1968, Janeway & Travers 1996). The implications of using mononuclear cells rather than B and T lymphocytes shall be analysed in section 4.2 of the discussion.

2.4.2 Isolating mononuclear cells from human tissue (enzymic method)

Synovial membrane samples were obtained as discussed earlier (section 2.2). According to the size of the synovium, 10 to 15 pieces were excised and added directly to 50ml HBSS with 150mg dispase, and stirred at 37°C for 60 minutes. The supernatant was treated in the same manner as PP tissue supernatant was following enzymatic dissociation, for the isolation of MCs (section 2.5.1).

2.4.3 Alternative method used for isolation of mononuclear cells from porcine and human tissue

An alternative procedure to dispase treatment was used in order to isolate porcine PP, LN and human SM MCs. This additional method of extraction was used to see if MCs isolated by this method were comparable to the same cells extracted by dispase treatment, in terms of their adherence properties. The following procedure was identical for all three types of tissue. This consisted of cutting the tissue samples into small pieces (0.5 - 1 cm^2) and transferring them to a sterile, stainless steel, mesh strainer, rinsed several times with HBSS, and gently teased through the mesh, using the barrel of a sterile 2ml syringe untill the tissue pieces were disrupted. Cells were washed three times through the mesh, and the suspension collected in an petri dish. This suspension was later aspirated and discharged several times more through the mesh, using a sterile 5ml Gilson tip, and finally transferred to a sterile 15ml conical tube. This was allowed to stand on ice for 10 minutes to enable debris to sediment. The cell suspension was centrifuged at 1000rpm (150g) for 10 minutes at

20°C. the cell pellet was washed twice more and resuspended sICM. This final suspension was overlayered onto an equal volume of Histopaque, centrifuged at 1800rpm (400g) for 30 minutes at 20°C. The MCs were aspirated from the interface layer, washed twice and finally resuspended in 1ml of sICM.

2.4.4 Procedure for isolating mononuclear cells from peripheral blood and synovial fluid samples

Mononuclear cells were isolated from porcine PB, human control PB and human RA SF and RA PB. In all cases except for human RA SF, 4ml DSM was added per 20ml of blood. This was done in order to sediment erythrocytes and to prevent the blood from coagulating. Once DSM was added, the blood was left to settle for 15-20 minutes. Following the sedimentation of erythrocytes, the white cell rich supernatant was collected, centrifuged at 1000rpm (150g) for 10 minutes at 20°C. The pellet was resuspended in 5ml of sICM and carefully layered over 5ml of Histopaque used for separation of MCs by density gradient centrifugation. Samples were spun for 30 minutes at 1800rpm (400g) at 20°C. The interface layer was aspirated, spun down at 1000rpm (150g) for 10 minutes at 20°C, resuspended in 5ml of sICM, washed twice more and finally resuspended in 1ml of sICM.

Mononuclear cells were obtained from SF samples by density gradient centrifugation as described above. No DSM was added to SF samples.

2.5 Mononuclear cell enumeration, viability, the in vitro adherence assay, quantification and freezing/recovery

2.5.1 Cell enumeration and measurement of mononuclear cell viability

Mononuclear cells were enumerated using a Biological model Z_{BI} Coulter counter (Coulter Electronics), with threshold levels, aperture current, amplification settings and orifice diameter set for white blood cell counting. All counts were performed in triplicate, and the mean value taken as the final count. All cell suspensions used were adjusted to a final concentration of 5 x 10^6/ml.

The viability of each cell suspension used was ascertained by trypan blue staining. Viable MCs exclude the stain and appear colourless, dead cells are permeable to trypan, and appear blue. A total of over 250 cells were counted from each cell suspension to be tested, by bright field microscopy at x 250 magnification. All counts were done in duplicate, and the mean percentage viability quoted.

A Leishmann's differential white blood cell stain was also carried out on every MC suspension, to detect contamination, by polymorphonuclear cells.

2.5.2 In vitro adherence assay

Frozen tissue samples which were kept at -70°C following processing (section 2.4) were moved to -20°C, 24 hours before cryostat

sectioning. On the day of sectioning, 10 micron sections were taken at -18°C to -25°C on a Bright microtome. A total of five sections were used for each assay. Sections were then allowed to dry at room temperature for 20 minutes, and the embedding compound surrounding the tissue was peeled away, and discarded. A wax circle was drawn using a Chinagraph wax pen, around each tissue section, of no larger then 1.5 cm in diameter. Fifty microlitres of cell suspension of 5 x 10^6/ml (250000 cells), was layered onto the tissue section and incubated on a shaking platform at 70 rpm for 30 minutes at 4°C. After incubation the non-adherent cells were decanted from the tissue by gentle tapping, and with the aid of a Q-tip applicator, by means of aspirating the volume of suspension covering the tissue section. The sections were then washed gently in PBS, and fixed in 2% glutaraldehyde for 10 minutes at room temperature and again washed several times with PBS. Once dried, the sections were stained with Haematoxylin and Eosin. Adherent cells appeared dark blue to purple, and on a slightly different focal plane than the underlying tissue, which appeared pink/red. After staining, the sections were fixed with DPX mountant for permanent storage.

2.5.3 Quantification of adherent cells

The counting of adherent cells was carried out using bright field microscopy, x 250 magnification, with a graticule equipped eyepiece. On each section a total of ten 1 mm^2 areas were quantified. In the case of PP tissue, adherent MCs were counted in five randomly chosen areas within the lamina propria and five such areas in the follicular region of the tissue section (see diagram-2). The number of MCs adherent within the lamina propria were counted and subtracted from the number of MCs adherent

within the follicular areas, to give the net number of MCs adherent within the lamina propria areas independent of follicular adherence. The intra-observer coefficient of variation was 3.9% based upon the quantification of adherent MCs in ten sections with a total of 100 areas counted.

The final result was calculated as follows;

No. MCs in lamina propria - No. MCs in follicular areas = Net no. MCs

$$\frac{\text{Net no. MCs} \times 250 \text{ (magnification factor ; x10 eyepeices and x25 lens)}}{100 \text{ mm}^2 \text{ (area of graticule ; 10mm x 10mm)}} = X$$

$$\frac{X}{\text{No.of areas counted}} = \text{MCs adherent per mm}^2$$

2.5.4 Mononuclear cell freezing and recovery

Mononuclear cells were suspended in sICM, and adjusted to 10-20 x 10^6/ml cells. They were then centrifuged for 10 minutes at 1000rpm (150g) at room temperature, and resuspended in an equal volume of

lymphocyte freezing medium (LFM). They were then stored at -70°C in a Nalgene cryofreezing container (gradual temperature cooling of -1°C/minute) overnight, and then transferred to long term storage containers. Cells were kept at -70°C for up to two to three weeks at the most.

Once cells were ready to be used, they were thawed out quickly in hot (55°C) water and resuspended in 3 times the original volume, this time in cell suspension medium (CSM). They were then centrifuged at 1000rpm (150g) for 10 minutes at room temperature and finally resuspended in sICM. MCs were enumerated and concentration was adjusted again, and cell viability determined before use.

Frozen MCs were only used initially for some porcine PB samples, all human samples were used fresh. No adverse effect of freezing was observed on lymphocyte populations in terms of cell viability.

2.6 General adhesion studies

The following experiments were performed in order to validate certain aspects of the in vitro adherence assay used. Since it was of importance to develope a reproducible assay system reflecting certain aspects of in vivo adherence, it was sought to examine the adherence of human control PB MCs to gut lamina propria (GLP) endothelial cells under the effects of different temperatures, two different cations, an inhibitor of metabolic activity, two phosphorylated monosaccharides, a mucus remover and different anatomic origins of cells used, most of

which have been shown to effect cell adhesion in vivo, to which the in vitro adherence assay results were to be compared.

2.6.1 Manganese sulphate, EGTA, sodium azide, and phosphorylated monosaccharide experiments

The effects of Mn^{2+} (at concentrations of 0.5mM, 1mM, 2mM, 4mM), the Ca^{2+} chelator EGTA (50mM), the metabolic inhibitor sodium azide (20mM), galactose 6-phosphate (20mM) and mannose 6-phosphate (20mM) on the adherence of human control PB MCs to Peyer's patch tissue was studied. Mn^{2+}, EGTA and sodium azide were incubated with 5 x 10^6/ml of MCs for 30 minutes at 4°C in sICM. The cells were then washed and resuspended in fresh sICM and the in vitro adherence assay performed. Mannose 6-phosphate and galactose 6-phosphate were incubated with 5 x 10^6/ml of MCs for 30 minutes at 4°C and were then used directly, with no wash-out phase, for the in vitro adherence assay.

2.6.2 Temperature experiments

Using the in vitro adherence assay as described before (section 2.6.2), the adherence of human control PB MCs to lamina propria endothelial cells was studied at 4°C, 20°C and 37°C.

2.7.3 Dithiothreitol (DTT) experiments

DTT was used as a mucus remover and Peyer's patch tissue pieces were incubated in 2mM DTT containing PBS, on a shaking platform for 10 minutes at room temperature. Following this incubation period, the PP tissue pieces were washed with PBS, frozen and stored for sectioning as previously described (section 2.4) ready for the adherence assay.

2.6.4 Variation of Peyer's patch tissue segments from small intestine

Porcine Peyer's patch segments were identified and excised from the duodenum, jejunum, and ileum of the small intestine, and processed as described previously (section 2.4) for future sectioning.

2.6.5 Monoclonal antibody experiments

The effects of monoclonal antibodies Meca-367 (anti-mouse, directed against the mucosal vascular addressin MAdCAM-1), Mel-14 (anti-mouse, directed against the L-selectin adhesion molecule) and Hermes-3 (mouse anti-human, directed against CD44) upon the adherence of human control PB MCs and paired RA SF and PB MCs, to GLP endothelial cells was studied. Meca-367 and Mel-14 were kind donations from Prof. Eugene Butcher (Stanford University, USA), and Hermes-3 was generously supplied by Dr. Sirpa Jalkanen (Turku University, Finland).

All three monoclonal antibodies were diluted in Nanopure water and stored at -70°C for long term storage, and at -20°C for short term use. MCs (50µl of 5 x 10^6/ml) were incubated with 50 µl of Hermes-3 or Mel-14 at 4°C for 60 minutes on a rotating platform. This was followed by the in vitro adherence assay, as described previously (section 2.6.2).

Meca-367 (100µl) was preincubated on 10 µm PP tissue sections, at 4°C on a shaking platform for 30 minutes, excess antibody suspension was gently tapped off after incubation and this was followed by the in vitro adherence assay. In all monoclonal antibody adherence inhibition experiments, a non-specific monoclonal antibody was used as a control.

Meca-367 was also used in an indirect two-step immunoperoxidase staining method for the localisation of mucosal endothelial cells. Acetone fixed (10 minutes at 4°C) 10 µm PP tissue sections were incubated with 100µl of normal rabbit serum (diluted 1:20) at room temperature for 30 minutes. Serum was then tapped off and excess wiped away. Following this, 100µl of Meca-367 was layered onto sections and incubated overnight at 4°C in a humid chamber (to avoid evaporation of antibody suspension). The next morning the sections were washed in PBS for 20 minutes. Then, 50µl of peroxidase conjugated rat anti-mouse monoclonal antibody (diluted in Nanopure water 1:100) was added onto sections. They were incubated for 30 minutes at room temperature, and following this were washed in PBS for 20 minutes. The substrate-chromogen solution was prepared as follows: 6mg of three-diaminobenzidine tetrahydrochloride (DAB) was added to 10ml 0.05M Tris buffer, pH 7.6. After DAB had fully dissolved, 100µl 3% hydrogen peroxide was also added. The substrate solution was used within 20 minutes. It was added freely to the sections, and incubated for 5-10 minutes (until desired colour

intensity had developed), and then washed gently off with distilled water. Finally the sections were washed in tap water for 15 minutes. Sections were counterstained for 30 seconds with haematoxylin and fixed in DPX mountant. They were then examined for positively stained endothelial cells. The same staining procedure was used for monoclonal antibodies anti-factor VIII (anti-porcine), Meca-325 (anti-mouse) and anti-CD31 (anti-human), all three were used to identify endothelial cells within porcine gut lamina propria.

In all experiments three negative controls were used. These consisted of excluding the primary antibody, or the secondary peroxidase conjugated antibody or by not incubating with the substrate-chromogen solution.

2.7 Effects on adhesion of corticosteroids In vitro and ex vivo

The effects of the corticosteroid methylprednisolone, on MC adhesion to GLP endothelial cells, was studied in vitro, using porcine PB MCs, and ex vivo, using human RA PB MCs.

2.7.1 In vitro experiments

The corticosteroid methylprednisolone, was supplied as Solu-medrone (Upjohn Pharmaceuticals), sodium succinate powder (6-methylprednisolone 21-hemisuccinate).

A total of 125mg methylprednisolone was dissolved in 20ml of sICM, giving final concentration of 6.25mg/ml. This solution was then divided into 1ml aliquots and stored at -20°C.

Porcine PB MCs were obtained by density gradient centrifugation as previously described (section 2.5.4). MCs were resuspended in 2ml of sICM at a concentration of 10x 10^6/ml. To this was added a mixture of 1ml methylprednisolone stock at 12.6mM, and 7ml of sICM. This gave a ten-fold dilution which contained a concentration of 1.26mM methylprednisolone. Methylprednisolone at a concentration of 1.26mM was chosen because this was the calculated concentration of MP in the blood stream of an RA patient following treatment with intravenous MP. It was important to carry out the in vitro experiments with MP concentrations close to those attained in vivo. Therefore the minimum likely in vivo concentration was chosen to be used in vitro as well. It was hoped that this concentration, would have an effect in vitro. Control samples consisted of 2ml cell suspensions of 10 x 10^6/ml MCs added to 8ml sICM.

Both control and test samples were incubated at room temperature for five different time periods; 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours. The 24 hour incubations were done in 5% CO_2 incubation chamber, with 130µl penicillin/streptomycin and 130µl L-glutamine added to each suspension. After incubation at different time intervals, the suspensions were centrifuged at 1000rpm (150g) for 10 minutes. The pellet was resuspended in HBSS and centrifuged as above again. Finally the cells were resuspended in sICM and adjusted to an concentration of 10 x 10^6/ml. These cells were then used in the in vitro adherence assay, with the exception that 25µl was added per PP tissue section rather then 50µl

(though the number of cells layered onto each section did not change). In all cases MC viability was tested and cell suspensions with less then 85% viability were discarded.

2.7.2 Ex vivo experiments

A total of 26 RA patients undergoing pulse methylprednisolone therapy was used (section 2.2). All patients received an intravenous infusion of 500mg methylprednisolone acetate. Peripheral blood samples were taken into a 25ml syringe 1 hour prior, 1 hour and 24 hours after pulse therapy for 14 patients. For 4 patients sampling was done over a 48 hour period, with samples taken 24 hours, and 1 hour prior and 1 hour, and 24 hours after pulse therapy, one patient was followed up over a two week period. MCs were obtained from all samples as previously described (section 2.5.4) and viability, enumeration, the in vitro adherence assay and quantification was performed (sections 2.6 - 2.6.3). All experiments were performed coded and read double blind.

2.7.2.1 Flow cytometric analysis

Blood samples from 8 patients were taken 1 hour prior and 24 hours after pulse therapy. Phenotypic analysis of MCs from whole blood and separated blood samples were performed by flow cytometry using an FACscan (Becton Dickinson). Monoclonal antibodies directed against CD2, CD3, CD4, CD8, CD19, CD44, CD45RA, CD45RO and HLA-DR were used. Monoclonal antibodies were conjugated with either

fluorescein-isothiocyanate or phycoerythrin. Results were expressed as the percentage of positively stained lymphocytes.

In conjunction with flow cytometric analysis, the in vitro adherence assay was performed on each sample.

2.7.2.2 Immuno-histochemistry

The phenotype of adherent MCs was determined by immuno-histochemistry, using the Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) method. Blood samples were obtained from 8 RA patients treated with pulse methylprednisolone, as previously described (additionally 4 blood samples from healthy volunters were also obtained for APAAP staining). Samples were taken 1 hour prior and 24 hours after pulse therapy. MCs were separated (section 2.5.4), enumerated, tested for viability and the in vitro adherence assay performed (sections 2.6 - 2.6.3) on 10μm PP tissue sections which were previously desiccated over silica gel, overnight at 4°C. Following the adherence assay, the sections were fixed with acetone for 10 minutes at 4°C, instead of glutaraldehyde, to prevent cross-linking of the MC surface antigens.

The sections were then air dried, and washed in PBS for 5 minutes. Normal rabbit serum (diluted 1:5 in PBS) was overlayed onto each section following washing, for 5 minutes at room temperature. Excess serum was tapped off, and 50μl of monoclonal mouse anti-human CD3, CD4, CD8, CD19 and HLA-DR (all diluted 1:50 in PBS) was layered onto the sections (in all experiments, 3 sections were used for each CD molecule to be tested for, hence a total of 15 sections were used, along with 3 sections for

negative controls), and incubated at room temperature for 30 minutes. This was followed by another 5 minute PBS wash, and incubation with 50µl of rabbit anti-mouse secondary monoclonal antibody (diluted 1:20 in PBS), for 30 minutes at room temperature. After a further 5 minute PBS wash, the section was incubated with 50µl of mouse APAAP monoclonal antibody (diluted 1:40 in PBS), for 30 minutes at room temperature. A final 5 minute PBS wash was done, during which time the APAAP substrate was made by dissolving 12.5mg of Naphtol phosphate sodium salt in 25ml Veronal acetate buffer, and by adding 12.5mg of fast red TR salt and 6mg of levamisole.

The sections were rinsed briefly in 0.9% saline, and then incubated with the APAAP substrate for 20 minutes at room temperature, followed by another rinse in saline. The sections were then briefly counterstained in haematoxylin and washed in tap water. They were mounted in aqueous mounting medium (Glycergel). All MCs adherent to mucosal endothelial cells, were counted, and expressed as percentage positive or negative cells.

2.7.2.3 Enzyme Linked Immuno-sorbent Assay (ELISA)

Whole blood samples from 8 RA patients were collected in 4ml EDTA tubes, 1 hour prior, and 24 hours after pulse steroid therapy. Samples were centrifuged at 1000rpm (150g) for 10 minutes at room temperature. Plasma was aspirated, aliquoted and stored at -20°C until samples from all 8 patients had been gathered.

Human soluble VCAM-1 (sVCAM-1) was assayed by a commercially available immunoassay kit (R & D systems). A 96 well

microtitre plate was used. Briefly; 100µl of anti-sVCAM-1 horseradish-peroxidase conjugate was added to each well, followed by 100µl of RA plasma (diluted 1:50), or 100 µl of sVCAM-1 control standard. Wells were mixed gently and allowed to incubate for 1.5 hours at room temperature. After incubation the contents of each well were aspirated and washed with 300µl of wash buffer. This process was repeated 5 times for a total of 6 washes. After the last wash, the contents of each well were decanted and any remaining wash buffer was removed by tapping the inverted plate firmly on a clean paper towel. Immediately after decanting, 100µl of substrate solution (tetramethlybenzidine) was added, and incubated at room temperature for 30 minutes. Finally 100µl of acid solution was added to each well to stop the reactions, and the optical density of each well was read within 30 minutes, using a microtitre plate reader (Dynatech MR600) set at 450nm with a correction wavelength of 620nm. All samples were assayed in duplicate, and by analysing standards of known sVCAM-1 concentration and plotting a curve of absorbance versus concentration, the concentrations of unknown samples were determined and expressed as ng/ml of sVCAM-1.

2.8 Latex particles and statistical analysis

Latex particles of similar diameter (10µm) to MC populations were used in the in vitro adherence assay, as an internal control for background adhesion. In general for each experiment, two slides were used with latex particles (25 µl of 10 x 10^6/ml) incorporated with MCs. Latex particles were enumerated and quantified in the same manner as MCs were, and latex adherence results were also expressed in the same fashion.

All statistical analysis unless stated otherwise, was evaluated by the Mann-Whitney U test, with the level of significance (α) set at 5 % ($P = 0.05$).

CHAPTER 3

Results

3.1 General adhesion studies

3.1.1 The effect of temperature on adhesion

The adhesion assay was performed at 4^0C, 20^0C and at 37^0C, to see whether higher (i.e. physiological temperatures) had an effect upon the adherence of human control PB mononuclear cells (MCs) to GLP endothelial cells. 4^0C temperature assays were taken as control. At 20^0C adherence was decreased by 24% and at 37^0C by 14% as compared to control. These results would suggest that different temperatures effect the adherence characteristics of PB MCs to GLP endothelial cells. See figure-1 and table-1.

3.1.2 The effects of EGTA, phosphorylated monosaccharides and sodium azide on adhesion

Calcium dependency, endothelial/epithelial adherence inhibition and the metabolic dependancy of MC-endothelial cell adhesion was studied, by means of EGTA, mannose/galactose 6-phosphate and sodium azide respectively (control experiments were performed without the addition of these substances). The adherence of human control PB MCs to GLP endothelial cells under these conditions was examined.

Table-2 shows their effects upon human control PB MCs adherence. As can be seen, adherence was almost totally inhibited by sodium azide (adherence down by 98% as compared to controls), implying the requirement for metabolic activity. The Ca^{2+} chelator EGTA and the endothelial cell adhesion inhibitor mannose 6-phosphate

also inhibited adherence by 75% and 65% respectively as compared to controls. The epithelial cell adhesion inhibitor galactose 6-phosphate had no significant effect on PB MCs adhesion. Figure-2 represents the mean percentage adherence values.

3.1.3 The effect of manganese sulphate on adhesion

The effect of increasing concentrations (0.5mM, 1mM, 2mM, 4mM) of manganese sulphate on human control PB MC adherence to GLP endothelial cells was studied. As can be seen from table-3, 0.5mM and 1mM manganese sulphate had no substantial effect upon MC adherence as compared to the control sample (which was not incubated with manganese sulphate), with 0.5mM increasing adhesion by nearly 6% and 1mM decreasing adhesion by 6%. There was however a significant increase in PB MC adherence, with the addition of 2mM and 4mM manganese sulphate, with 2mM increasing adhesion by 39% and 4mM by 52%. Figure-3 represents the mean percentage adherence values.

3.1.4 The effect of mucus on adhesion

The effect of mucus, (present within the gut lamina propria) on MC adhesion was studied, lest it have an enhancing effect upon adhesion to endothelial cells within the lamina propria. Therefore dithiothreitol (DTT) was used to remove mucus from gut lamina propria tissue, before porcine PP MCs were used in the adherence assay. As can be seen from table-4, there was no appreciable difference in adherence to endothelial

cells following the procedure. Therefore the possible presence of residual mucus would not appear to lead to non-specific MC adherence.

3.1.5 Adherence of PP mononuclear cells from different regions of small intestine

Porcine Peyer's patches from three different regions of the small intestine were obtained (duodenal, jejunal and ileal) and PP MCs extracted from these regions to study the possible differences in adhesion to GLP endothelial cells. MCs from the jejunal region of the small intestine, were used in the standard adhesion assay.

In table-5 it can be seen that PP MCs extracted from Peyer's patches of different anatomical regions did not show any significant difference in adhesion to GLP endothelial cells. Therefore difference in anatomical origin of PP MCs would not appear to have an appreciable effect upon their adhesion characteristics. By contrast, adhesion to proteased lamina propria endothelial cells did inhibit PP MCs adherence on average by 60%, again irrespective of tissue originality. Overall results are represented in figure-4.

3.2 Adherence characteristics of porcine mononuclear cells (MCs)

The adherence of porcine Peyer's patch (PP), peripheral blood (PB) and peripheral lymph node (LN) MCs to porcine gut lamina propria (GLP) endothelial cells and to perpherial LN endothelium was studied

(unless stated otherwise all GLP is of porcine tissue). PP MCs exhibited significantly greater levels of adherence to GLP endothelial cells, as compared to PB (P= 0.0001) and compared to LN MCs (P= 0.0002). There was no significant difference in adherence between PB and LN MCs (P= 1). In table-6 the individual results for each experiment are presented, figure-5 represents the mean experimental results as a histogram.

PP MCs also exhibited significantly greater adherence to GLP endothelial cells as compared to peripheral LN endothelium (P= 0.03). Similarly LN MCs adhered significantly better to LN endothelium than to GLP endothelial cells (P= 0.01). See table-7 and figure-6 for individual and mean results respectively.

3.3 Adherence characteristics of human RA and control mononuclear cells

3.3.1 Adherence to gut lamina propria (GLP) endothelial cells

The adherence of human control peripheral blood (PB) MCs, human RA PB, synovial fluid (SF) and synovial membrane (SM) MCs to GLP endothelial cells was studied. The adherence of SF MCs was significantly greater than the adherence of RA and control PB MCs (at P= 0.001 and P= 0.001 respectively, with no significant difference between RA PB and control PB MCs adherence; P= 0.07).

This difference in adhesion was most apparent when compared to RA SM MCs, which showed poor levels of adherence to GLP endothelial

cells. Both the adherence of RA SF, PB and control PB MCs was greater than RA SM MCs (statistical significance at P= 0.0001, P= 0.0035 and P= 0.0001 respectively).

In table-8, the individual results for each experiment are presented. Control PB MCs adherence results are presented in table-9, and the mean results of all experiments with RA and control MCs are represented in figure-7 as histograms. Figure-8 represents the paired RA SF and PB results as a scattergram (values for paired samples in table-8), with pairs of results from each patient connected with a line. The median values are also included. It can be seen that in 22 out of the 25 paired samples, the adherence of RA SF MCs is greater than RA PB MCs. Photo-1 and 2 show SF MCs adherence to gut lamina propria.

3.3.2 Adherence to peripheral lymph node endothelium

The adherence of four paired RA SF and PB MCs to peripheral lymph node endothelium was studied, and compared with adherence to GLP endothelial cells. The adherence of both RA SF and PB MCs to lymph node endothelium was low. RA PB MC adherence was slightly greater than that of RA SF, but this difference in adhesion was of no statistical significance (P= 0.47). The adherence of RA SF MCs to GLP endothelial cells was greater than that of RA PB MCs (statistical significance at P= 0.001), and both RA PB and SF MCs showed higher levels of adherence to gut endothelial cells than they did to lymph node endothelium. This difference in adhesion to lymph node endothelium as compared to GLP endothelial cells for both RA SF and PB was statistically significant (P= 0.0017 for RA SF and P= 0.0033 for RA PB).

In table-10, the individual results for each experiment are presented, and in figure-9, the mean results of all experiments are represented as histograms.

3.3.3 Monoclonal antibody experiments

The effects of monoclonal antibodies Mel-14, Meca-367 and Hermes-3, on the adhesion of paired RA SF and PB MCs to GLP endothelial cells was studied. Neither RA SF or RA PB MC adherence were significantly inhibited by Mel-14 (P= 0.77 and P= 0.66 respectively). However both Meca-367 and Hermes-3 were markedly inhibitory on both RA PB (adherence inhibited by 74% with Meca-367 and by 57% with Hermes-3) and RA SF (adherence inhibited by 69% with Meca-367 and by 79% with Hermes-3) MCs adherence to GLP endothelial cells. Statistical significance at P= 0.03, as compared to Mel-14 and control results, for both RA SF and PB MCs.

Though RA SF MCs showed greater adherence than RA PB MCs, in the control and Mel-14 experiments (difference was statistically significant at P= 0.03), when treated with Meca-367 or Hermes-3 this difference in adherence levels was reduced to a similar extent for both RA SF and RA PB MCs. The individual results for each experiment is presented in table-11, and the mean percentage of adherence is represented as a histogram in figure-10.

Meca-367 was also used in a two-step immunoperoxidase staining method on porcine gut lamina propria. Positive staining was observed

within the lamina propria of jejunal small intestine, between the crypts of Lieberkuhn closer to the muscularis externa. No staining was observed in the villus regions. Areas of Meca-367 positive staining corresponded to those of MC adhesion observed in the adherence assay (see photos 4 and 5). Three additional Mabs were also used for staining of gut lamina propria tissue using the same method as for Meca-367 staining. No endothelial staining was observed with Meca-325, or anti-CD31, either in the villi or between the crypt regions. Anti-factor-VIII stained regions within the lamina propria (though not villi) in a manner similar to that of Meca-367 staining (see photos 6 and 7).

3.3.4 Adherence to human gut endothelium

The adherence of paired RA SF, PB and human control PB MCs to human GLP endothelial cells was examined and compared to their adherence to porcine GLP endothelial cells. The adherence of RA SF MCs to both human and porcine GLP endothelial cells (202+/-14 and 221+/-12 MCs per mm^2 respectively) was appreciably higher than RA PB (118+/-9 and 131+/-8 MCs per mm^2 respectively) and control PB (124+/-11 and 134 +/-7 MCs per mm^2 respectively) adherence to the same tissues. Adherence to human gut endothelium closely resembled adherence to porcine gut endothelium (see photo-3). This was the case for all three MC populations studied, and the substantial differences observed in adherence to gut endothelium between RA SF and PB MCs was maintained when applied to human gut as well. In figure-11 the results are expressed as a histogram.

3.3.5 Comparison of enzymic and mechanical methods of mononuclear cell extraction

Possible differences in adhesion of MC extracted from tissue by two separate methods (enzymic or mechanical extraction) was studied. Human RA SM MCs showed very low levels of adherence to gut endothelial cells as compared to other MC populations (see section 3.3.1). Since SM MCs were extracted by enzymic method (protease treatment), another alternative extraction method (mechanical) was used to see whether or not the low levels could be attributed to enzymic extraction. The same procedure of both enzymic and mechanical extraction was used also for porcine PP MCs as a control. As can be seen from table-12, no appreciable difference was observed in RA SM or porcine PP MCs adhesion, when extracted by enzymic or mechanical method. Hence the low levels of RA SM MCs adherence to gut lamina propria endothelial cells would not seem be attributed to the fact that they were obtained by enzymic dissociation from synovial membrane.

3.3.6 Other arthritic diseases

The adherence characteristics of SF and PB MCs from other diseases including osteoarthritis, reactive arthritis, Paget's disease and Reiter's syndrome were studied. Adherence was compared with that of SF and PB MCs from rheumatoid arthritis.

Reactive arthritis was closely similar to rheumatoid arthritis, in terms of adhesion of SF and PB MCs to GLP endothelial cells. The adherence of SF MCs was significantly greater than that of PB MCs (P=

0.03), as with rheumatoid arthritis SF MCs (section 3.3.1). Osteoarthritis and Paget's disease showed markedly different results. The adhesion of their SF lymphocytes was much lower than RA SF MCs, and interestingly their levels of PB MC adhesion were higher than their SF MCs, the opposite being observed in reactive arthritis and Reiter's syndrome.

The paired results are presented in table-13, and these results are also represented as a scattergram in figure-12.

3.4 Effects of methylprednisolone (MP) pulse steroid (PS) therapy on mononuclear cell adherence, in vitro and ex-vivo

3.4.1 In vitro experiments

The effects of the corticosteroid methylprednisolone on porcine PB MC adherence to GLP endothelial cells was studied after 0.5, 1, 2, 4 and 24 hour incubation. Four separate experiments were done for each incubation period, and the results can be seen in table-14, with the overall mean results expressed as percentage adherence, in figure-13.

Maximal inhibition of adherence (34%) was achieved after 4 hours of incubation with steroid. After 24 hours of incubation the levels of adherence was seen to return to the control samples adherence level. Though there were appreciable differences in PB MCs adherence to GLP endothelial cells over 2 and 4 hours after steroid incubation, none were statistically significant as compared to control samples.

3.4.2 Ex-vivo experiments

Twenty-six RA patients undergoing intravenous (iv) pulse methylprednisolone therapy patients were studied. The effects on MC adhesion to porcine gut lamina propria endothelial cells, the effects on circulating MC subsets, and on adherent MC subsets, and finally the effects on circulating soluble adhesion molecules were studied.

3.4.2.1 Effects of pulse steroid therapy on mononuclear cell adhesion

The adhesion characteristics of human RA PB MCs, 24 and 1 hour prior to, 1 and 24 hours after MP PS therapy was studied. One patient was also studied 2 weeks after pulse steroid (PS) therapy.

Table-15 presents individual results for each patient at each time point. There was a statistically significant difference in adhesion to GLP endothelial cells 24 hours after PS therapy as compared to the 24 hour period prior to and 1 hour after PS therapy (P= 0.03 and P= 0.001 respectively). By 24 hours after PS therapy, adhesion was reduced on average by over 58% as compared to pre-pulse levels. This reduction in adhesion was not apparent by 1 hour after PS therapy. Indeed there was no significant difference in adhesion between 1 hour prior or 1 hour after PS therapy results (P= 0.59). Sampling of patients 24 hours prior to PS therapy, was primarily done in order to see if the results observed were an artifact of hospitalisation stress on patients. Again, however there was no significant difference in adhesion as compared to 1 hour prior to, or 1 hour after PS therapy (P= 0.66 and P= 0.88 respectively). There was no

significant difference therefore, in adhesion to gut lamina propria endothelial cells, 24 and 1 hour prior to, and 1 hour after PS therapy. As for the one patient sampled 2 weeks after pulse therapy, there did not seem to be any difference in adhesion as compared to the mean adhesion values of the 24 hour post pulse samples, suggesting that for this particular patient at least, the in vitro effects of the steroid was still apparent. This however was only one patient's sample, hence no overall conclusion can be drawn.

In figure-13.1, the mean percentage adherence values are represented for the patients undergoing PS therapy over 24 hours, in figure-13.2, the mean percentage values are represented for patients undergoing PS therapy over 48 hours, and finally in figure 13.3, the total mean perecntage results are given for all time periods, before and after PS therapy.

3.4.2.2 Effects of pulse steroid therapy on circulating mononuclear cell subsets

Phenotypic analysis of circulating MCs was carried out in whole blood and from MCs separated from whole blood, 1 hour prior to and 24 hours after pulse steroid therapy. Analysis was done by flow cytometry. The RA patients used in this study were the same patients used for the study on the effects of PS therapy on adhesion. All samples were taken in conjunction and experiments carried out within the same day (see section 3.4.2.1).

Results of the phenotypic analysis of circulating whole blood MCs can be seen in table-16 and figure-14, and for circulating MCs from separated blood samples in table-16.1 and figure-14.1.

Overall for circulating MCs, obtained after separation from whole blood, no statistically significant difference was observed in the circulating population of any of the subsets studied, before or after PS therapy. There was no significant difference between observed fluctuations in subsets and the effects of PS therapy on adhesion as analysed by percentage change or by change in the numbers of MCs / litre.

As for circulating MCs analysed as whole blood samples, a slightly different picture emerged. When the number of circulating MCs per litre was analysed, both the CD4+ and CD45RA+ subsets of MCs showed a significant decrease in circulation levels. CD4+, from pre pulse levels of 0.67 x 10^9/litre to 24 hour post pulse level of 0.35 x 10^9/litre (P= 0.05) and CD45RA+ from pre pulse level of 0.87 x 10^9/litre to post pulse level of 0.63 x 10^9/litre (P= 0.02). When however, the percentage change in circulating MC subsets are analysed (rather than the number of MCs / litre) before and after PS therapy, then only the CD3+ subset showed a significant decrease (P= 0.03) from pre pulse levels of 74% (+/- 3) to post pulse levels of 63% (+/- 3). The difference in statistical significance depends upon whether the percentage change or the actual numbers of MCs / litre are analysed before and after PS therapy.

These results would suggest that the 24 hour post pulse PS therapy inhibition of PB MCs adherence by over 58% (section 3.4.2.1, figure-

13.3), was only in part related to the observed decreases in circulating MC subsets.

3.4.2.3 Effects of pulse steroid therapy on adherent mononuclear cell subsets

In conjunction with the adhesion experiments and the phenotypic analysis of circulating MCs, the phenotypic analysis of PB MCs adherent to gut lamina propria endothelial cells was also studied, 1 hour prior to and 24 hours after pulse steroid therapy. The RA patients used in this study were the same patients used for the previous two studies (sections 3.4.2.1 - 3.4.2.2). Analysis was done by Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) immuno-histochemistry.

As can be seen from table-16.2, besides the HLA-DR subset there was no significant difference in adherence to GLP endothelial cells in any of the subsets of MCs, following PS therapy. Although there was a small decrease in the CD3+, CD4+ and CD8+ subsets following PS therapy, and an increase in CD19+ these were not statistically significant. However the HLA-DR+ subset was increased by 15% and this proved to be statistically significant (P= 0.01). In terms of overall MC adherence, before and after PS therapy, the small fluctuations observed in the adherence of the subsets of MCs analysed, would not seem to be the underlying cause behind the inhibition of adherence observed 24 hours after PS therapy (section 3.4.2.1, figure-13.3).

Phenotypic analysis of PB MCs adherent to GLP endothelial cells was also done (with the exception of the HLA-DR subset) with blood

samples from 4 healthy volunteers, in order to have some comparison with RA results. There were no appreciable differences in the levels of adherent CD3+, CD4+, CD8+ and CD19+ MCs to GLP endothelial cells, as compared to pre-pulse or post-pulse RA PB MCs subsets adherence levels (see table 16.2).

3.4.2.4 Effects of pulse steroid therapy on circulating soluble VCAM-1 (sVCAM-1) levels

The levels of circulating peripheral blood sVCAM-1 in 8 RA patients undergoing PS therapy over a 24 hour period was studied. Quantification of sVCAM-1 levels in plasma samples was done by ELISA and results expressed as ng/ml. As can be seen from table-17, there was no significant difference in the concentrations of circulating sVCAM-1, before or after PS therapy in any of the patients studied. Therefore PS therapy does not appear to have an effect on the levels of sVCAM-1 in circulation. However the mean value of sVCAM-1 concentration for RA patients (842 ng/ml +/- 50 for pre pulse and 813 ng/ml +/- 81 for post pulse samples) was substantially higher than previously reported levels of sVCAM-1 in control donors (553 ng/ml +/- 159 from 105 patients studied).

3.5 The use of latex beads as internal control

Latex beads were used in all adhesion assay experiments, as internal controls for background adherence to gut lamina propria tissue. Latex beads adhered randomly throughout lamina propria and follicular tissue

in small numbers, very different to MC adherence patterns. In all cases, the non-specific adherence levels of latex was 15 - 30 beads per mm^2 of tissue. Any experiment which showed significantly higher latex adherence levels, was discarded, and the experiment repeated with fresh tissue.

Table 1

Adherence of human control peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria endothelial cells, after incubation at 4°C, 20°C and 37°C. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Temperature (°C)		No. adherent MCs
4	=	283 (+/- 19)
20	=	216 (+/- 26)
37	=	242 (+/- 25)

Table 2

Adherence of human control peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria endothelial cells, following incubation with EGTA (50 mM), mannose 6-phosphate (20 mM), galactose 6-phosphate (20 mM) and sodium azide (20 mM). Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Treatment		No. of adherent PB MCs	% inhibition
Control	=	190 (+/- 7)	0 %
EGTA	=	47 (+/- 19)	75 %
Mannose 6-phosphate	=	66 (+/- 2)	65 %
Galactose 6-phosphate	=	187 (+/- 23)	2 %
Sodium azide	=	3 (+/- 1)	98 %

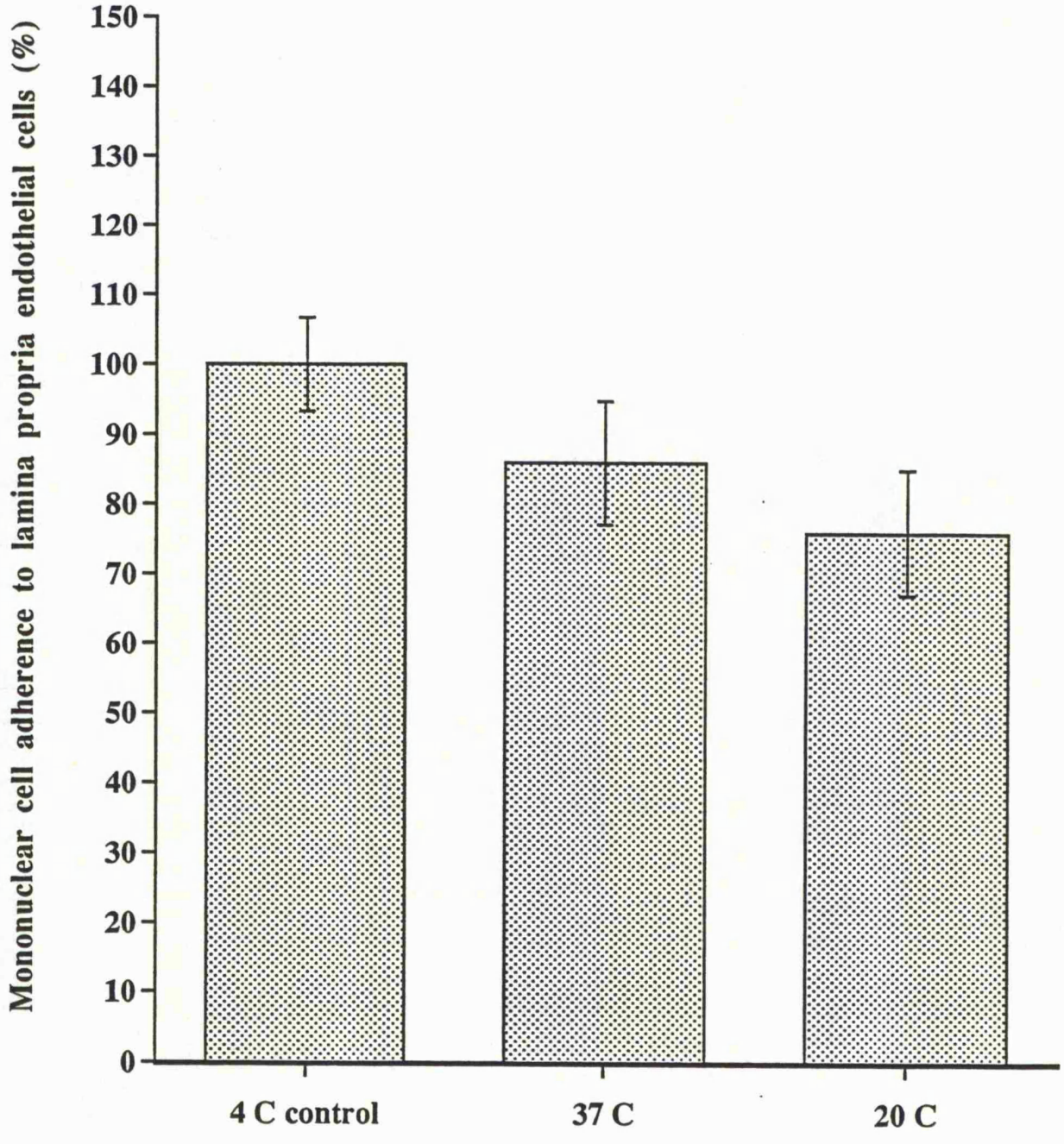


Figure-1

Results represent the mean % adhesion of human control PB mononuclear cells to gut lamina propria endothelial cells under different temperatures. Vertical lines represent +/- SEM.

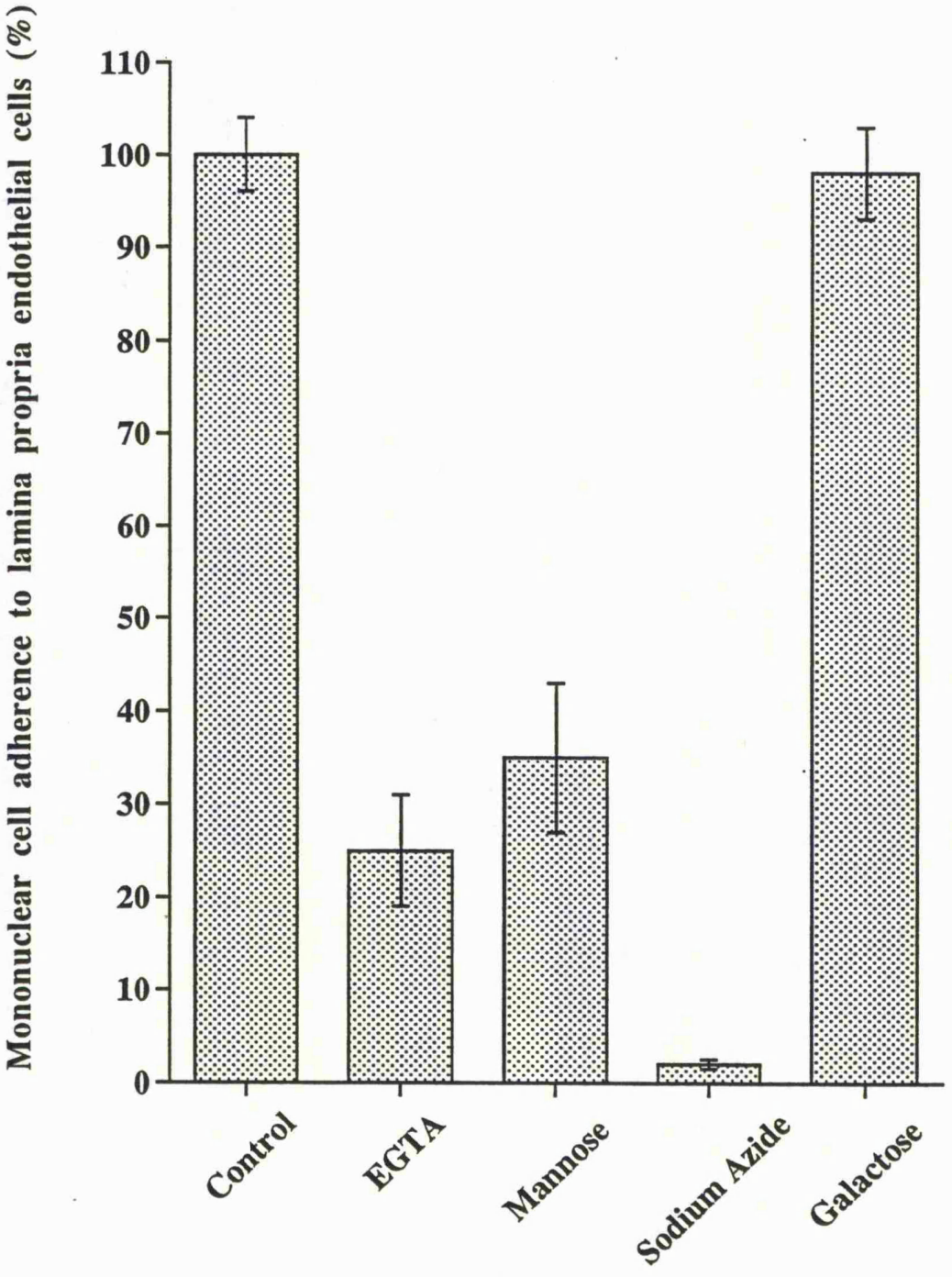


Figure-2

Results represent the mean % adhesion of human control PB mononuclear cells to gut lamina propria endothelial cells, following treatment with EGTA (20 mM), mannose 6-phosphate (20 mM), sodium azide (20 mM) and galactose 6-phosphate (20 mM). Vertical lines represent +/- SEM.

Table 3

Adhesion of human control peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria endothelial cells, with no added manganese sulphate, and with 0.5 mM, 1 mM, 2 mM and 4 mM added. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Manganese sulphate (mM)		No. adherent MCs
None added	=	163 (+/- 20)
0.5	=	172 (+/- 34)
1	=	153 (+/- 14)
2	=	226 (+/- 8)
4	=	248 (+/- 7)

Table 4

Adherence of porcine Peyer's patch (PP) mononuclear cells (MCs) to gut lamina propria endothelial cells, following treatment of PP tissue with 2 mM Dithiothreitol (DTT). Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Treatment of PP tissue		No. of adherent PP MCs
Control	=	272 (+/- 11)
DTT treated	=	274 (+/- 45)

Table 5

Adherence of porcine Peyer's patch (PP) mononuclear cells (MCs), extracted from ileal, jejunal and duodenal PP tissue, to jejunal lamina propria endothelial cells. The adherence to proteased ileal PP tissue is also included. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Tissue origin of PP MCs		Jejunal PP tissue	proteased jejunal PP tissue
Duodenal PP	=	212 (+/- 25)	91 (+/- 15)
Jejunal PP	=	220 (+/- 23)	78 (+/- 9)
Ileal PP	=	253 (+/- 31)	81 (+/- 13)

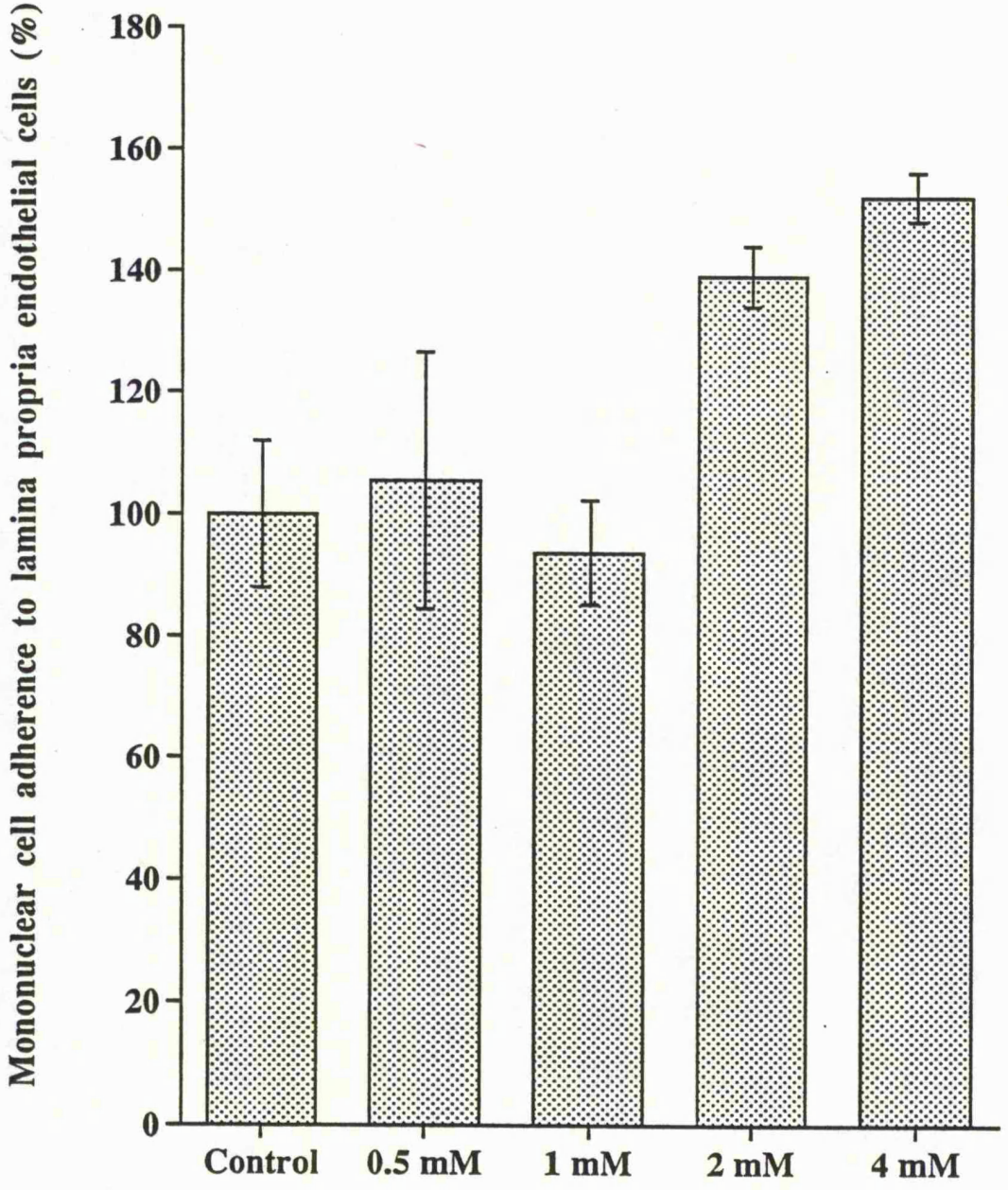


Figure-3

Results represent the mean % adhesion of human control PB mononuclear cells to gut lamina propria endothelial cell, with no added manganese sulphate (control sample) and with 0.5mM, 1mM, 2mM and 4mM manganese sulphate. Vertical lines represent +/- SEM.

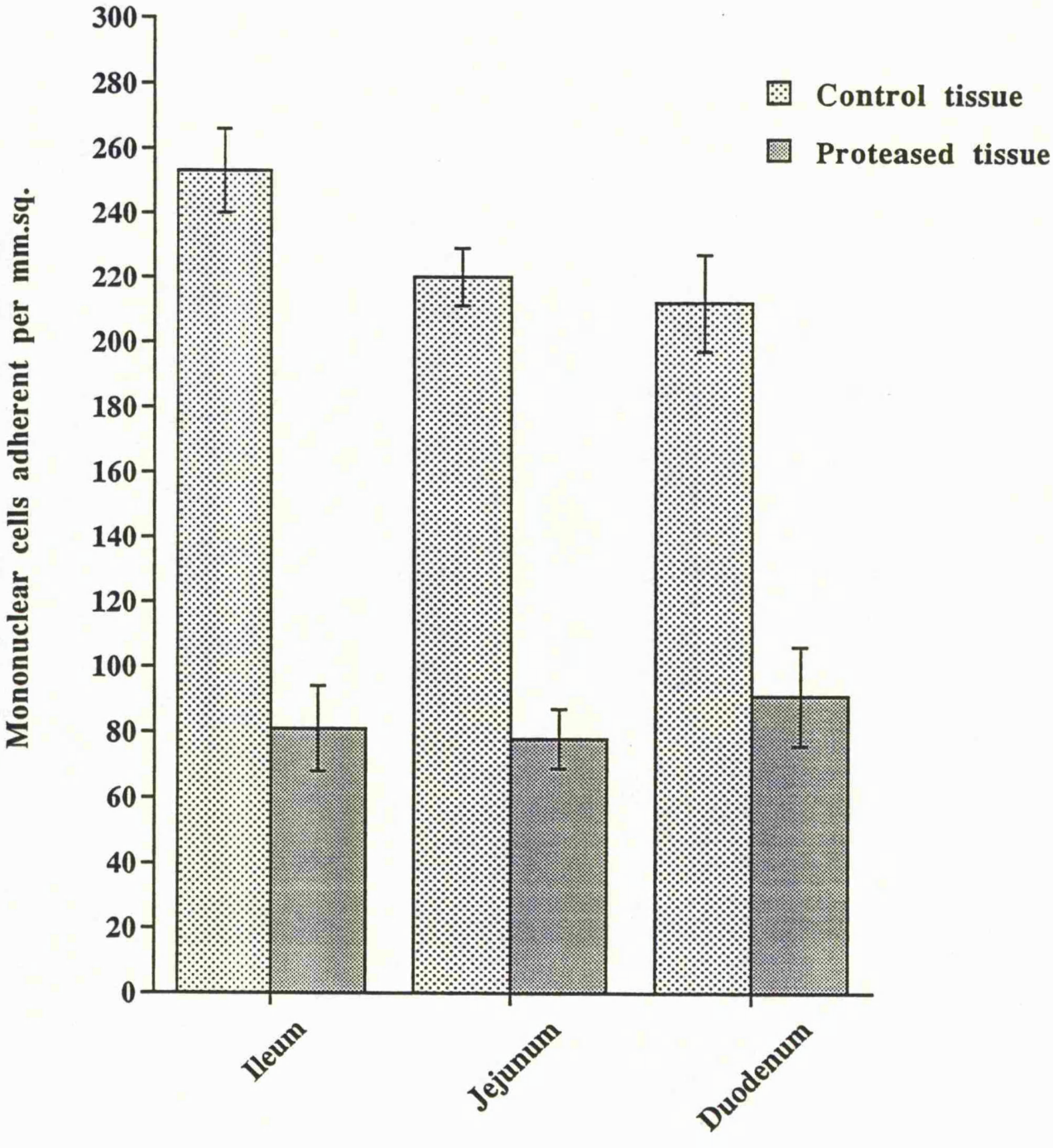


Figure-4

Results represent the mean adhesion of PP mononuclear cells (MCs), extracted from ileal,jejunal and duodenal PP tissue, to ileal gut lamina propria endothelial cells. The adhesion of ileal, jejunal and duodenal PP MCs to proteased gut lamina propria endothelial cells is also included. Vertical lines represent +/- SEM.

Table 6

Adherence of porcine Peyer's patch (PP) mononuclear cells (MCs), peripheral blood (PB) MCs and peripheral lymph node (LN) MCs to gut lamina propria endothelial cells. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

PP	PB	LN
385 (+/- 81)	88 (+/- 25)	67 (+/- 10)
486 (+/- 83)	99 (+/- 26)	52 (+/- 12)
244 (+/- 25)	75 (+/- 16)	161 (+/- 14)
466 (+/- 77)	62 (+/- 19)	109 (+/- 33)
343 (+/- 49)	115 (+/- 22)	192 (+/- 39)
269 (+/- 44)	67 (+/- 8)	150 (+/- 4)
445 (+/- 60)	135 (+/- 23)	161 (+/- 22)
257 (+/- 21)	213 (+/- 18)	
325 (+/- 32)	162 (+/- 22)	
279 (+/- 63)	64 (+/- 6)	
495 (+/- 46)	280 (+/- 12)	
200 (+/- 19)	259 (+/- 29)	
348 (+/- 20)		
384 (+/- 25)		
253 (+/- 31)		
mean PP (n = 15)	mean PB (n = 12)	mean LN (n = 7)
345 (+/- 25)	135 (+/- 22)	127 (+/- 20)

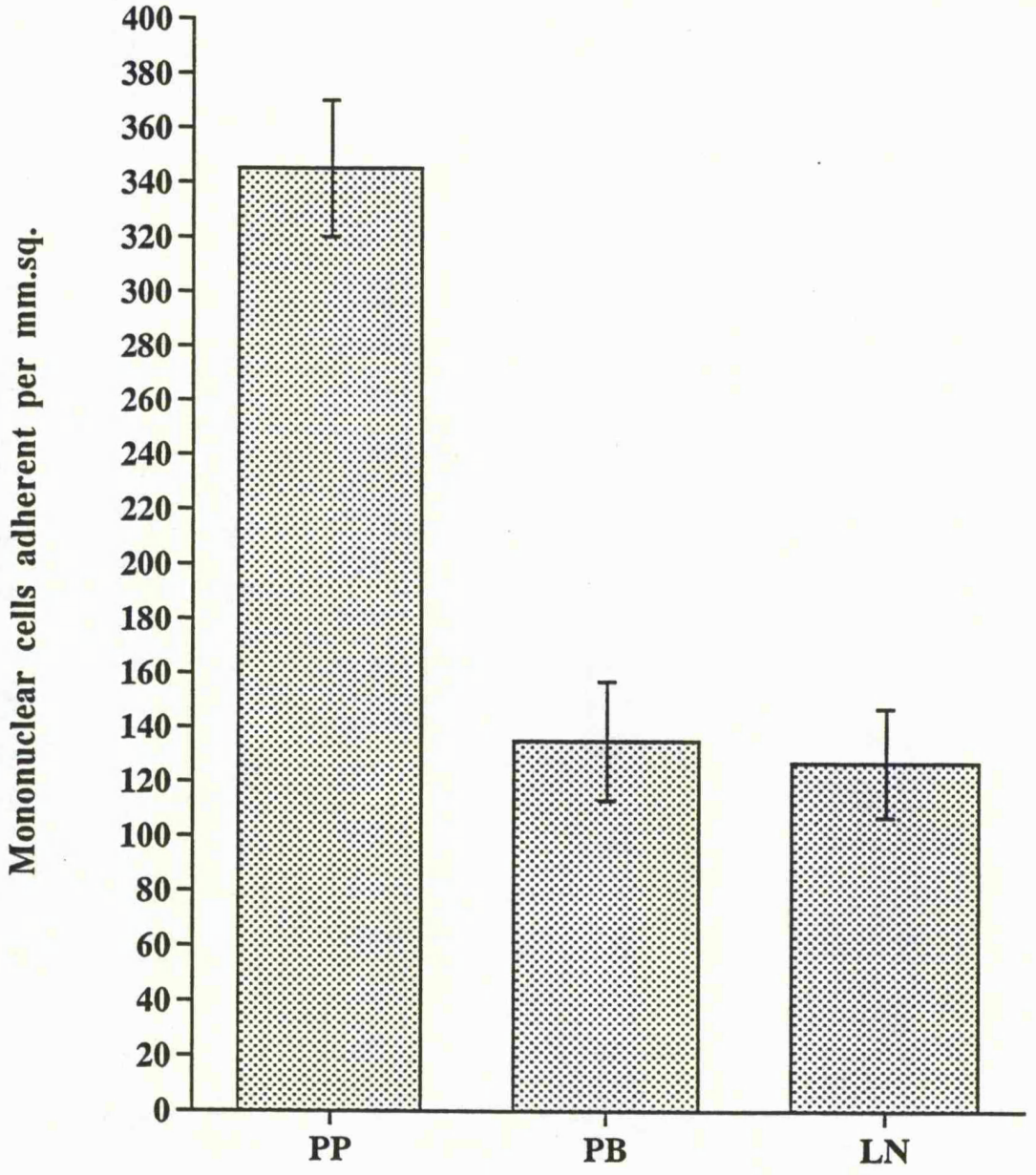


Figure-5

Results represent the mean adhesion of porcine PP (n=15), PB (n=12) and LN (n=7) mononuclear cells, to gut lamina propria endothelial cells. Vertical lines represent +/- SEM.

Table 7

Adherence of porcine Peyer's patch (PP) and peripheral lymph node (LN) mononuclear cells (MCs) to peripheral lymph node endothelium. Results expressed as adherent MCs per mm^2 of tissue. The standard error of the mean is given in parentheses (+/- SEM).

PP MCs	LN MCs
99 (+/- 8)	201 (+/- 23)
112 (+/- 14)	253 (+/- 40)
131 (+/- 10)	264 (+/- 29)
81 (+/- 6)	310 (+/- 24)
mean (n = 4)	mean (n = 4)
106 (+/- 11)	257 (+/- 22)

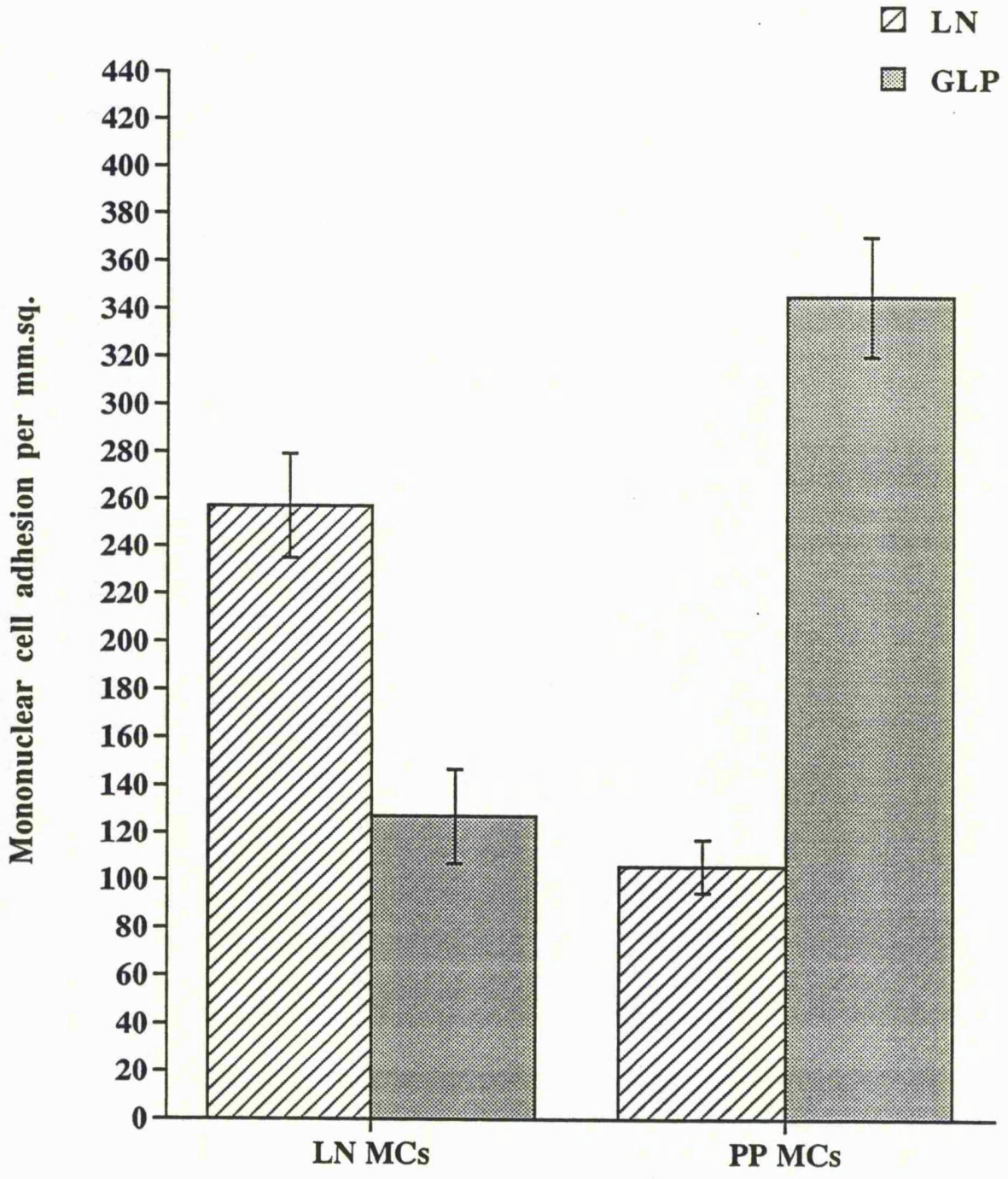


Figure-6

Adherence of porcine lymph node (LN, n=4) and Peyer's patch (PP, n=4) mononuclear cells (MCs) to porcine peripheral LN and gut lamina propria (GLP) tissue. Vertical lines represent +/- SEM.

Table 8

Adherence of paired RA peripheral blood (PB) and synovial fluid (SF), and RA synovial membrane (SM) mononuclear cells (MCs), to gut lamina propria endothelial cells. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM)

PB	SF	SM
83 (+/- 7)	217 (+/- 19)	21 (+/- 1)
110 (+/- 3)	276 (+/- 5)	45 (+/- 6)
136 (+/- 1)	215 (+/- 9)	35 (+/- 4)
142 (+/- 15)	156 (+/- 10)	41 (+/- 8)
95 (+/- 10)	124 (+/- 9)	20 (+/- 6)
104 (+/- 8)	160 (+/- 30)	80 (+/- 13)
85 (+/- 3)	187 (+/- 13)	112 (+/- 19)
122 (+/- 18)	246 (+/- 31)	
63 (+/- 12)	237 (+/- 28)	
24 (+/- 14)	134 (+/- 11)	
78 (+/- 3)	216 (+/- 34)	
97 (+/- 21)	223 (+/- 12)	
70 (+/- 8)	245 (+/- 25)	
62 (+/- 10)	176 (+/- 36)	
152 (+/- 9)	396 (+/- 37)	
137 (+/- 23)	366 (+/- 48)	
131 (+/- 18)	394 (+/- 31)	
216 (+/- 15)	242 (+/- 18)	
182 (+/- 21)	292 (+/- 19)	
104 (+/- 11)	191 (+/- 16)	
96 (+/- 9)	227 (+/- 17)	
201 (+/- 15)	177 (+/- 12)	
319 (+/- 43)	241 (+/- 15)	
67 (+/- 22)	78 (+/- 9)	
239 (+/- 98)	174 (+/- 5)	
mean PB (n = 25)	mean SF (n = 25)	mean SM (n = 7)
125 (+/- 13)	224 (+/- 16)	51 (+/- 13)

Table 9

Adherence of human control peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria endothelial cells. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

PB	
186 (+/- 38)	75 (+/- 23)
128 (+/- 10)	118 (+/- 20)
187 (+/- 36)	62 (+/- 16)
150 (+/- 21)	120 (+/- 24)
137 (+/- 14)	118 (+/- 15)
163 (+/- 20)	162 (+/- 23)
101 (+/- 16)	124 (+/- 28)
190 (+/- 16)	140 (+/- 17)
82 (+/- 26)	149 (+/- 31)
179 (+/- 18)	152 (+/- 18)
153 (+/- 11)	112 (+/- 9)
154 (+/- 18)	198 (+/- 29)
132 (+/- 8)	131 (+/- 13)
129 (+/- 11)	171 (+/- 17)
134 (+/- 12)	124 (+/- 28)

mean PB (n = 30) = 139 (+/- 6)

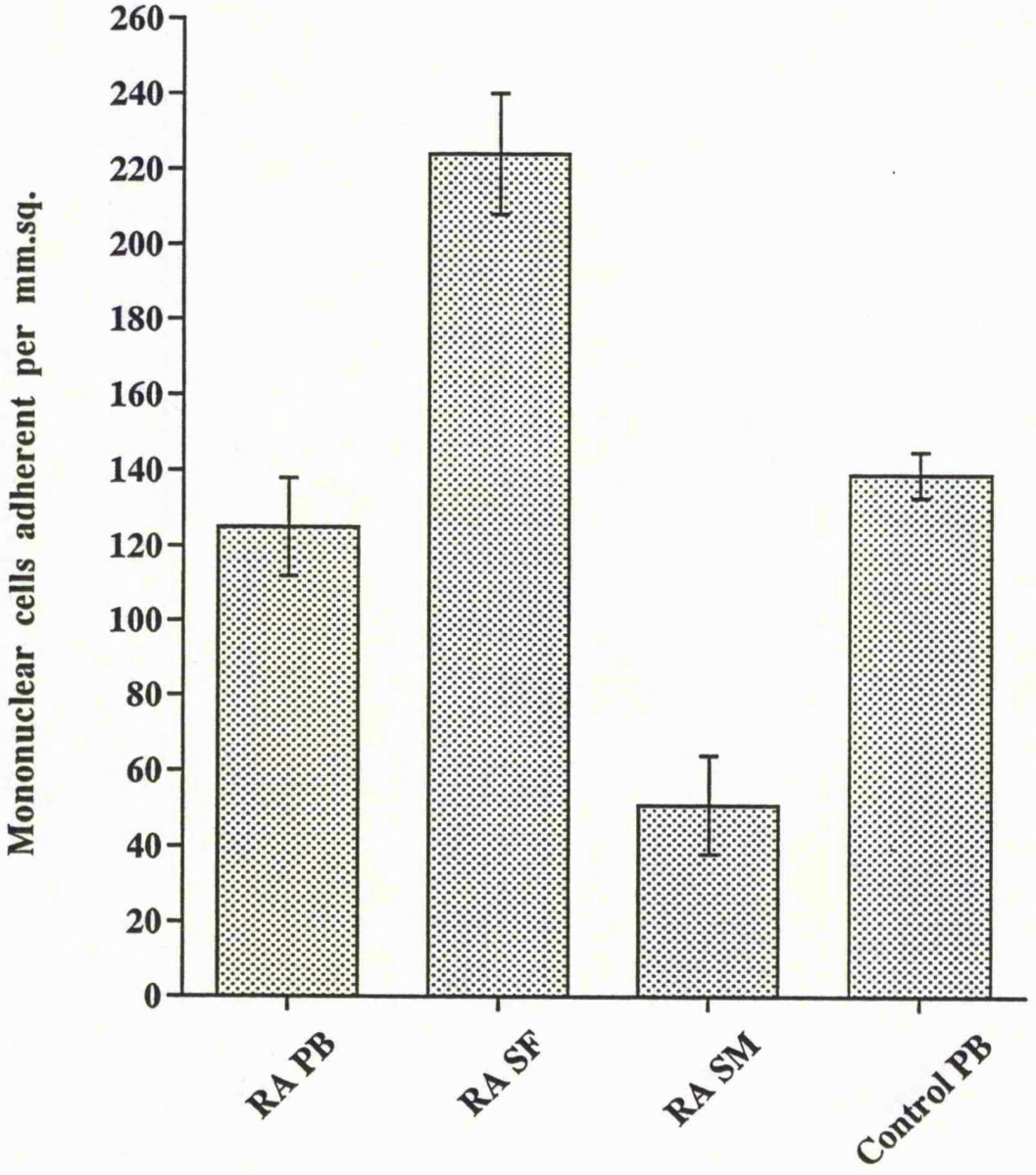


Figure-7

Results represent the mean adhesion of paired RA PB and SF (n=25), RA SM (n=7) and human control PB (n=30) mononuclear cells, to gut lamina propria endothelial cells. Vertical lines represent +/- SEM.

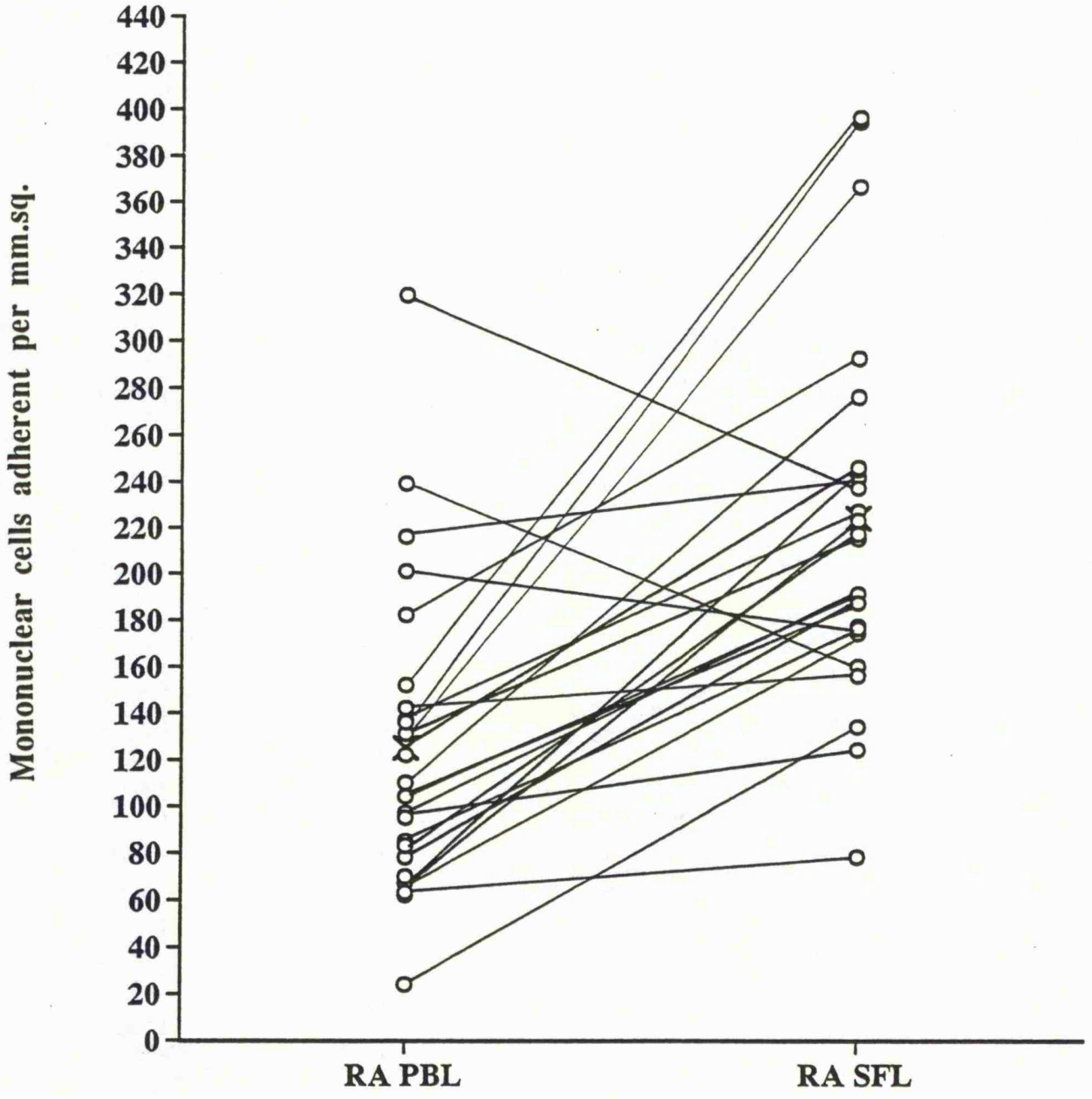


Figure-8

Results representing the adhesion of paired RA PB and SF (n=25) mononuclear cells, to gut lamina propria endothelial cells. Each pair of connected circles represent one paired sample. Cross represents median value.

Table 10

Adherence of paired RA peripheral blood (PB) and synovial fluid (SF) mononuclear cells (MCs) to gut lamina propria endothelial cells and peripheral lymph node endothelium. Results expressed as MCs adherent per mm^2 of tissue. The standard error of mean is given in parentheses (+/- SEM).

Tissue		RA PB (n = 4)	RA SF (n = 4)
Lymph node endothelium	=	40 (+/- 8)	29 (+/- 14)
Lamina propria endothelial cells	=	115 (+/- 7)	214 (+/- 10)

Table 11

Adherence of paired RA peripheral blood (PB) and synovial fluid (SF) mononuclear cells (MCs) to gut lamina propria endothelial cells, following incubation with monoclonal antibodies; Hermes-3, Meca-367 and Mel-14. Isotype matched IgG 2a class, used as control monoclonal. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

		RA PB (n = 4)	RA SF (n = 4)
Control	=	118 (+/- 23)	268 (+/- 19)
Mel-14	=	124 (+/- 11)	253 (+/- 29)
Meca-367	=	31 (+/- 4)	84 (+/- 3)
Hermes-3	=	51 (+/- 4)	56 (+/- 9)

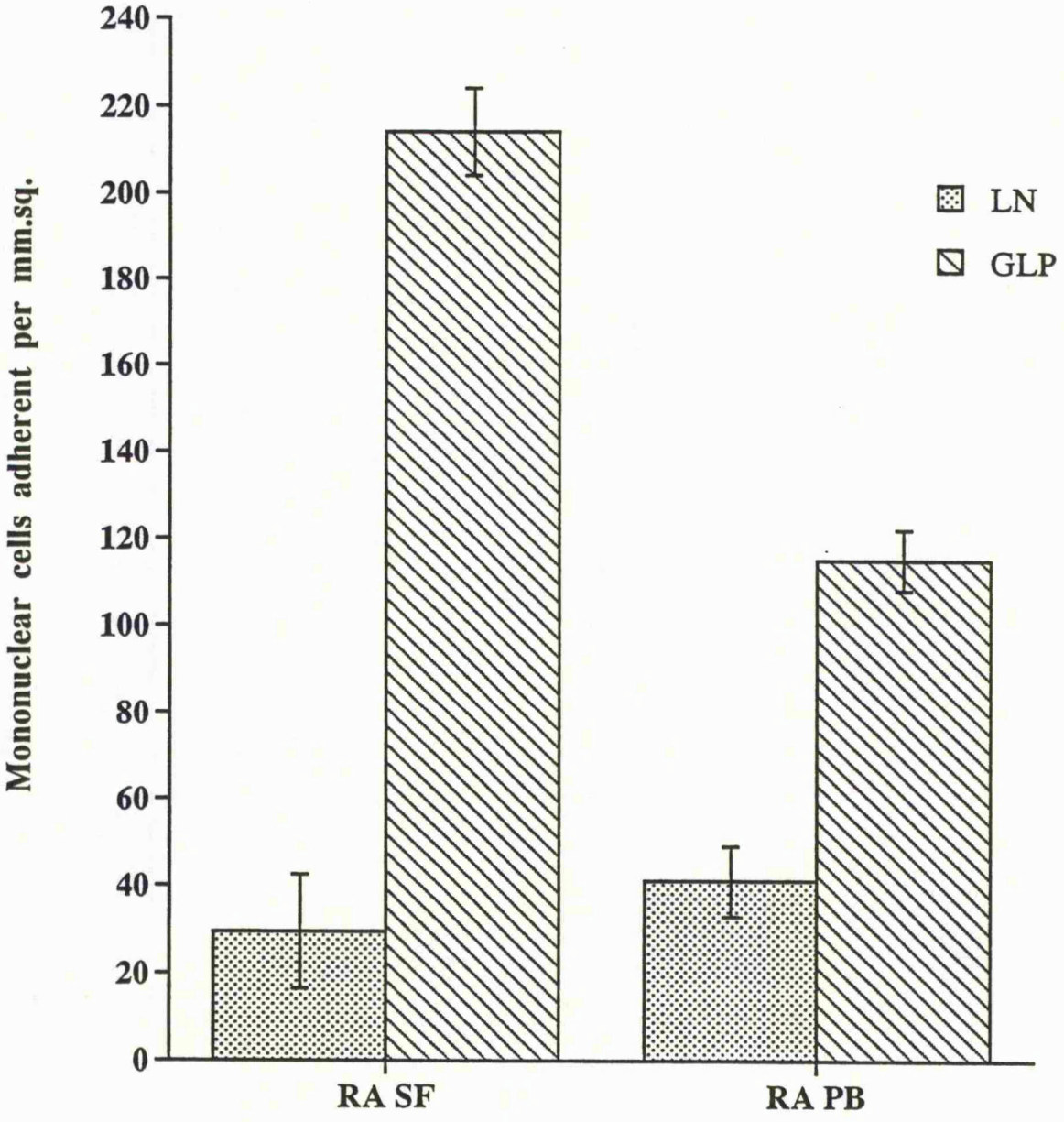


Figure-9

Results represent the mean adhesion of paired RA PB and SF (n=4) mononuclear cells, to gut lamina propria (GLP) endothelial cells, and also to lymph node endothelium. Vertical lines represent the +/- SEM.

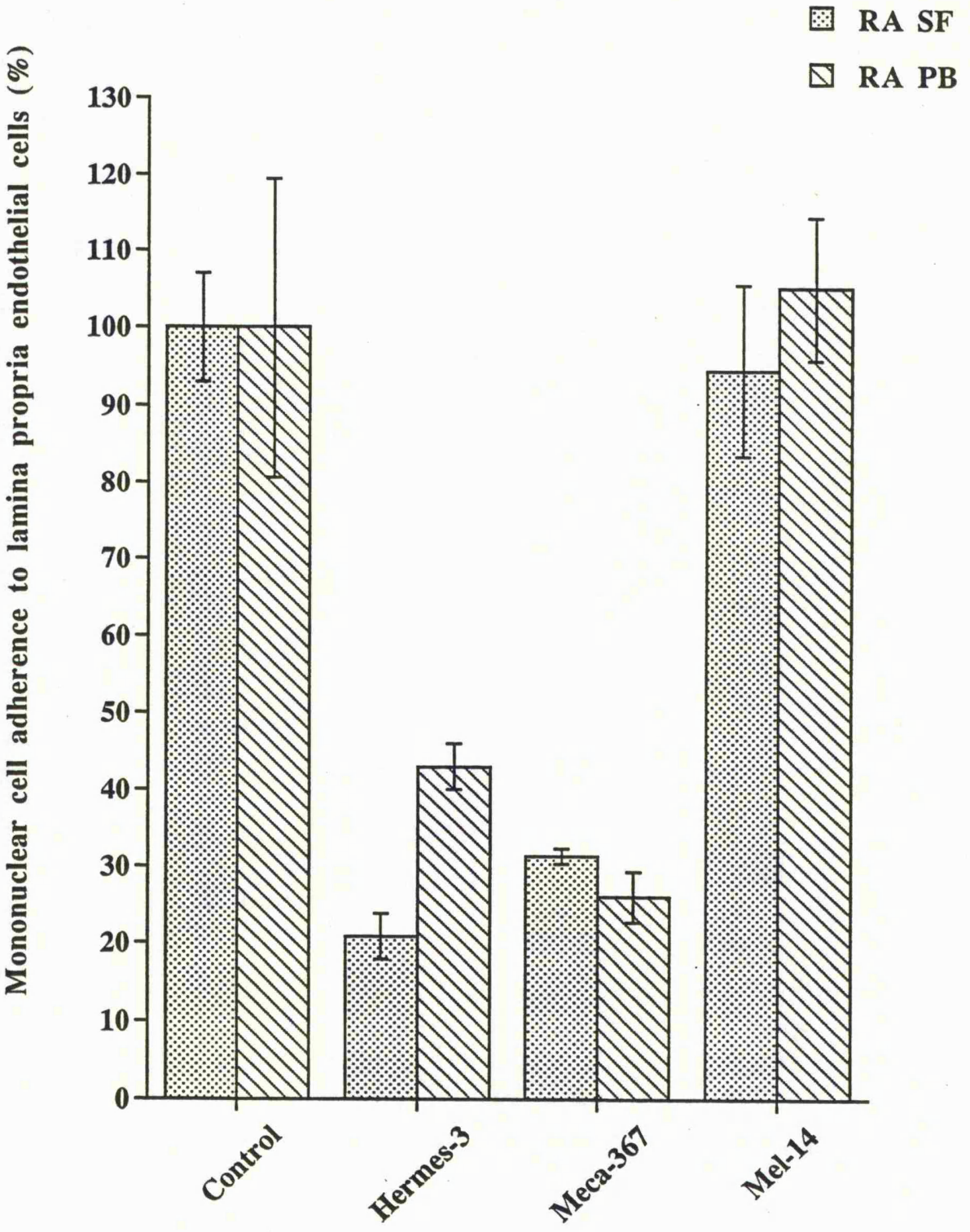


Figure-10

Results represent the mean % adhesion of RA PB and SF (n=4) mononuclear cells to gut lamina propria endothelial cells, following monoclonal antibody treatment. Control was a isotype matched monoclonal antibody (IgG 2a class). Vertical lines represent the +/- SEM.

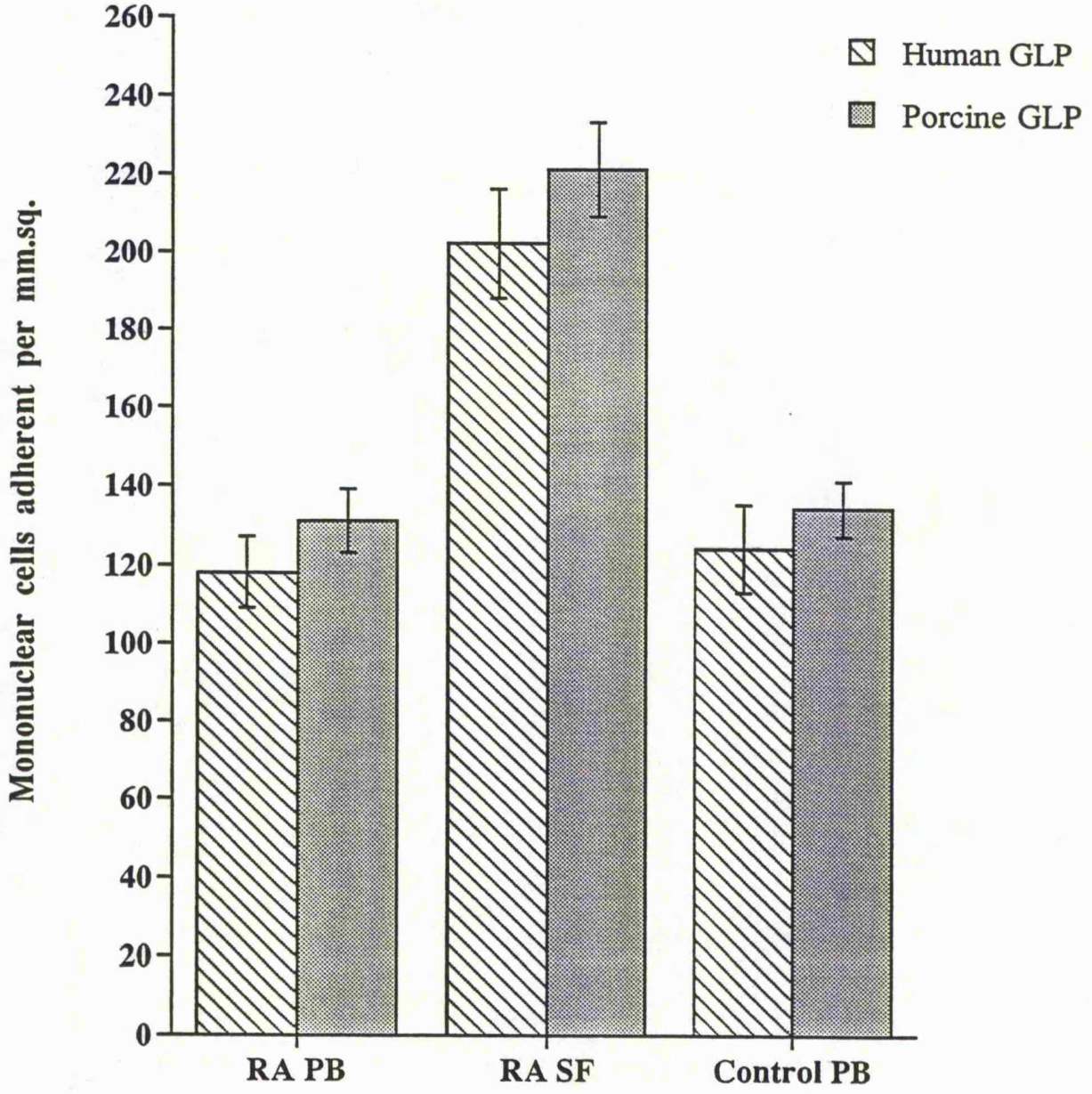


Figure-11

Results represent the mean adhesion of paired RA PB and SF (n=2) and human control PB (n=2) mononuclear cell adherence, to human and porcine gut lamina propria (GLP) endothelial cells. Vertical lines represent +/- SEM.

Table 12

Adherence of porcine Peyer's patch (PP) and human RA synovial membrane (SM) mononuclear cells (MCs) to gut lamina propria endothelial cells, following enzymic or mechanical extraction from PP and SM tissue respectively. Results expressed as adherent MCs per mm^2 of lamina propria. The standard error of mean is given in parentheses (+/- SEM).

Method of PP MC extraction	No. adherent PP MCs	No. adherent SM MCs
Enzymic extraction =	281 (+/- 20)	35 (+/- 4)
Mechanical extraction =	266 (+/- 16)	41 (+/- 8)

Table 13

Adherence of paired human peripheral blood (PB) and synovial fluid (SF) mononuclear cells to gut lamina propria endothelial cells, from reactive arthritis (n = 4), osteoarthritis (n = 2), Paget's disease (n = 1), and Reiter's syndrome (n = 1). The standard error of mean is given in parentheses (+/- SEM).

Arthritic disease		PB	SF
Reactive arthritis	=	137 (+/- 16)	266 (+/- 58)
		122 (+/- 8)	246 (+/- 22)
		151 (+/- 18)	241 (+/- 33)
		65 (+/- 4)	227 (+/- 41)
Osteoarthritis	=	138 (+/- 13)	13 (+/- 1)
		121 (+/- 9)	38 (+/- 4)
Paget's disease	=	42 (+/- 13)	25 (+/- 8)
Reiter's syndrome	=	30 (+/- 3)	72 (+/- 14)

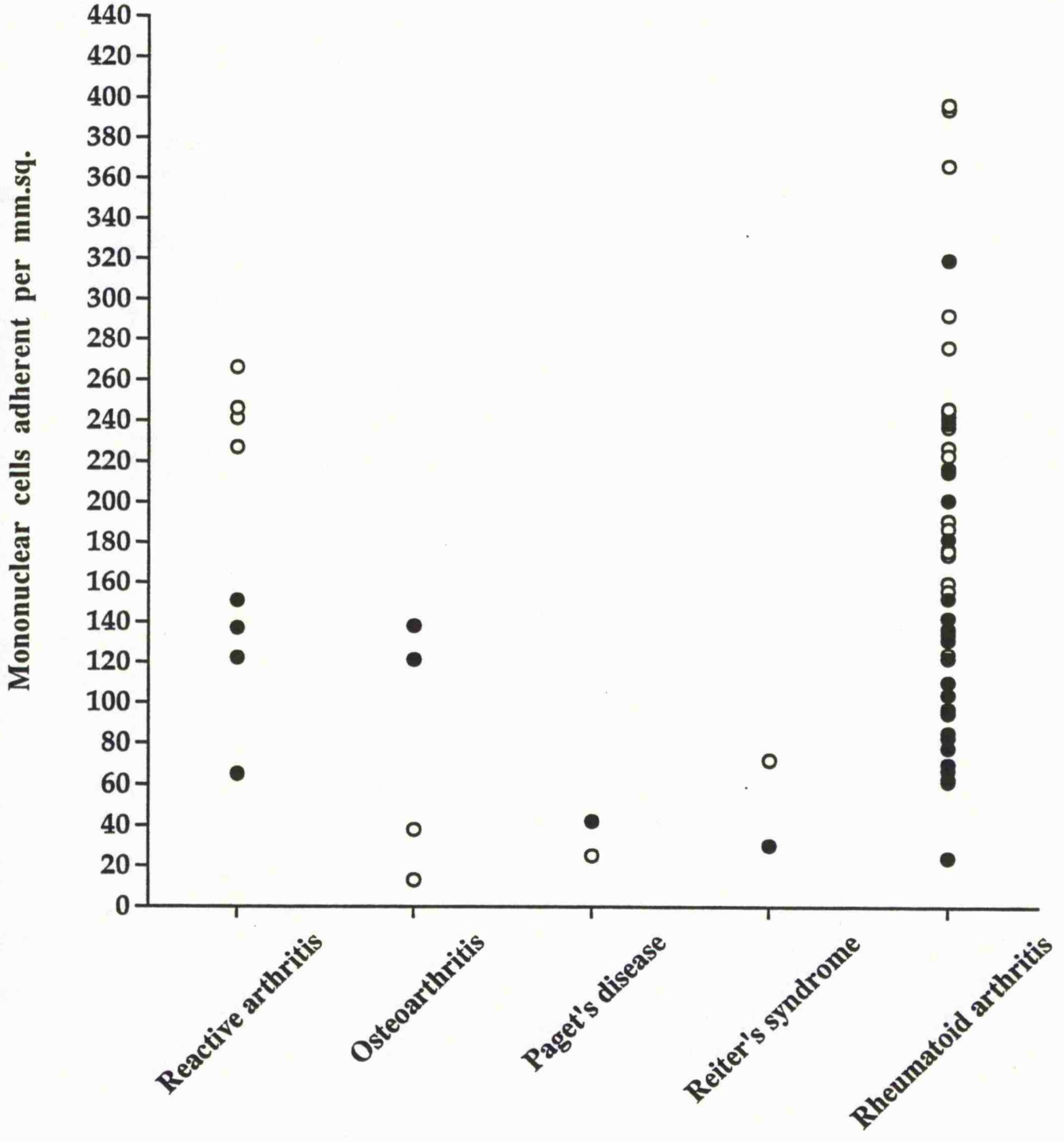


Figure-12

Results represent the adhesion of paired PB and SF mononuclear cells (MCs) to gut lamina propria endothelial cells, from rheumatoid arthritis (n= 25) reactive arthritis (n= 4), osteoarthritis (n= 2), Paget's disease (n= 1) and Reiter's syndrome (n= 1). o indicates SF MC,and o indicates PB MC adherence.

Table 14

Adherence of porcine peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria, following incubation with 1.26 mM methylprednisolone over a 24 hour period. Results expressed as adherent MCs per mm^2 of lamina propria tissue. The standard error of mean is given in parentheses (+/- SEM).

Time (hours)		No. of adherent MCs
0.5 hr steroid	=	199 (+/- 11)
0.5 hr control	=	197 (+/- 10)
1 hr steroid	=	164 (+/- 33)
1 hr control	=	194 (+/- 32)
2 hr steroid	=	129 (+/- 22)
2 hr control	=	190 (+/- 20)
4 hr steroid	=	121 (+/- 7)
4 hr control	=	183 (+/- 18)
24 hr steroid	=	163 (+/- 22)
24 hr control	=	180 (+/- 12)

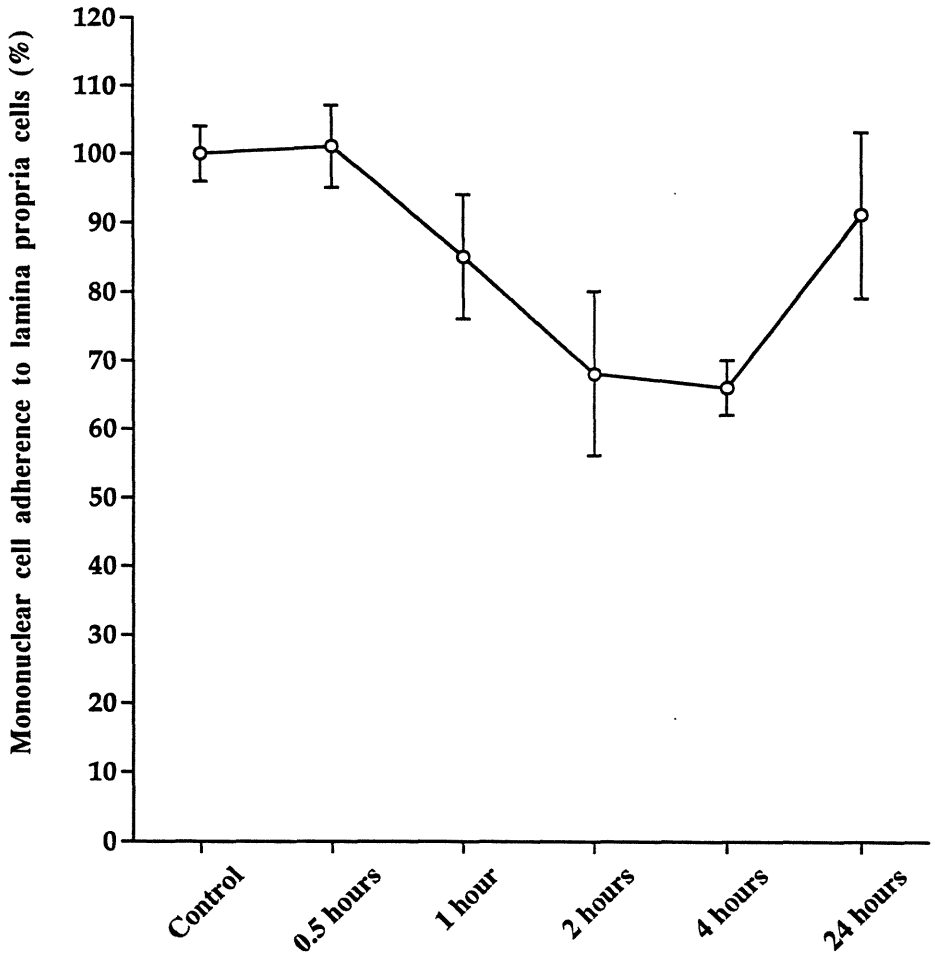


Figure-13

Results represent the mean % adhesion of porcine PB mononuclear cells to gut lamina propria endothelial cells, following incubation with 1.26mM methylprednisolone over a 24 hour incubation period. Vertical lines represent the +/- SEM.

Table 15

Adherence of RA peripheral blood (PB) mononuclear cells (MCs) to gut lamina propria endothelial cells, 1 hour prior, 1 hour and 24 hours after intravenous pulse methylprednisolone (500mg) therapy. A total of 18 patients were studied over 24 hours, (4 over a 48 hour period, and 1 over 2 weeks). Results expressed as adherent MCs per mm^2 of lamina propria. Mean results are given as μ, and the standard error of mean is given in parentheses, (+/- SEM).

Patient no	- 24 hours	- 1 hour	*pulse*	+ 1 hour	+ 24 hours	+ 2 weeks
1 =	85 (+/- 3)	105 (+/- 7)	^	76 (+/- 9)	42 (+/- 1)	74 (+/- 12)
2 =	131 (+/- 12)	124 (+/- 27)		122 (+/- 11)	7 (+/- 3)	
3 =	227 (+/- 16)	252 (+/- 25)		213 (+/- 12)	99 (+/- 16)	
4 =	201 (+/- 36)	223 (+/- 50)		197 (+/- 48)	93 (+/- 19)	
5 =		386 (+/- 74)		352 (+/- 79)	97 (+/- 6)	
6 =		148 (+/- 17)		152 (+/- 29)	78 (+/- 14)	
7 =		107 (+/- 24)		39 (+/- 6)	29 (+/- 7)	
8 =		191 (+/- 11)		213 (+/- 12)	121 (+/- 12)	
9 =		159 (+/- 24)		148 (+/- 13)	80 (+/- 12)	
10 =		161 (+/- 4)			143 (+/- 9)	
11 =		150 (+/- 25)			60 (+/- 10)	
12 =		130 (+/- 21)			52 (+/- 17)	
13 =		147 (+/- 13)			33 (+/- 7)	
14 =		79 (+/- 21)			20 (+/- 3)	
15 =		130 (+/- 11)			52 (+/- 10)	
16 =		178 (+/- 19)			89 (+/- 9)	
17 =		104 (+/- 9)			43 (+/- 8)	
18 =		131 (+/- 14)			51 (+/- 9)	
μ =	161 (+/- 32)	161 (+/- 17)		168 (+/- 30)	66 (+/- 9)	

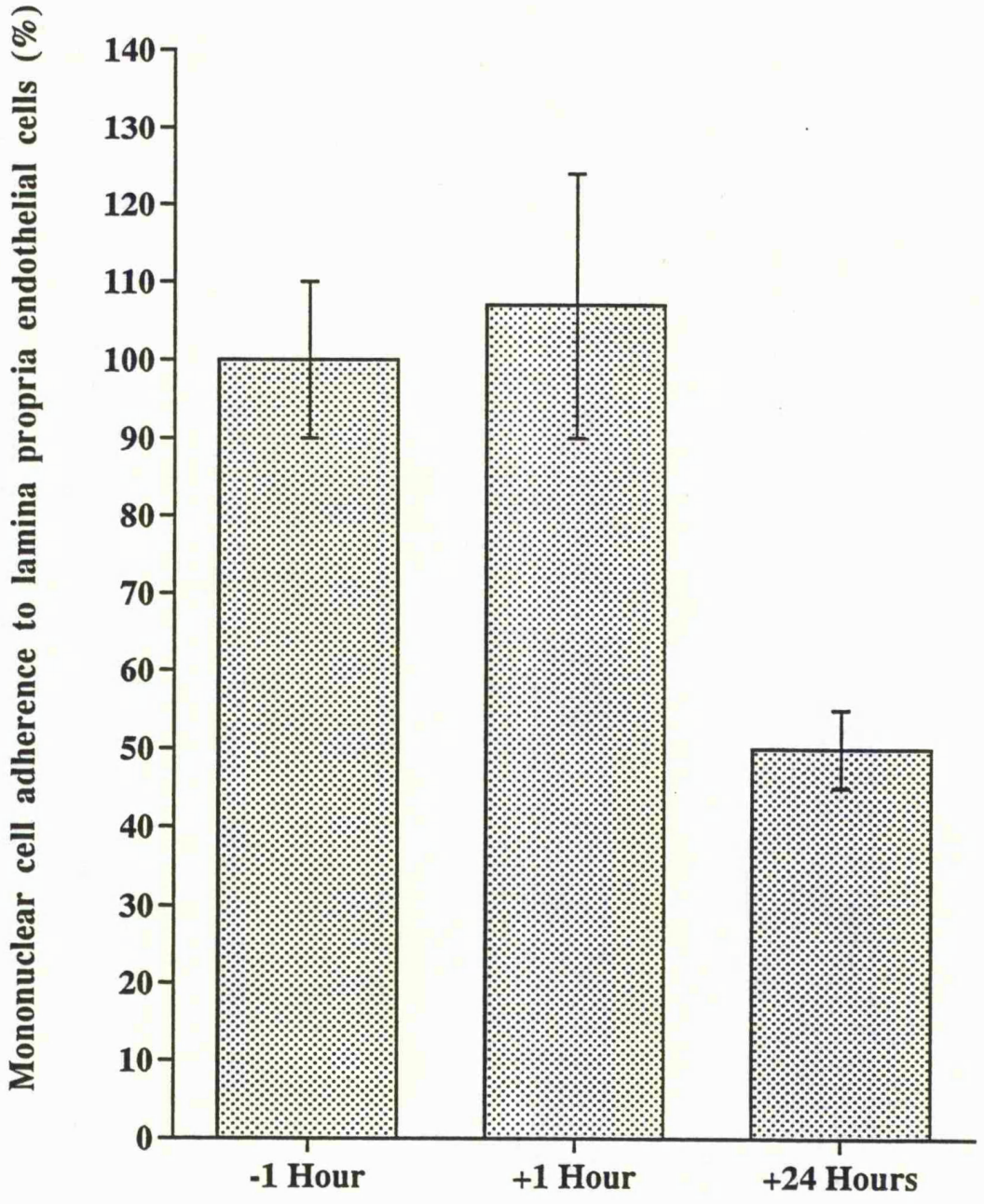


Figure-13.1

Results represent the mean % adhesion of RA PB mononuclear cells (n=14) to gut lamina propria endothelial cells, 1 hour prior, 1 hour and 24 hours after 500mg intravenous methylprednisolone pulse steroid therapy. Vertical lines represent +/- SEM.

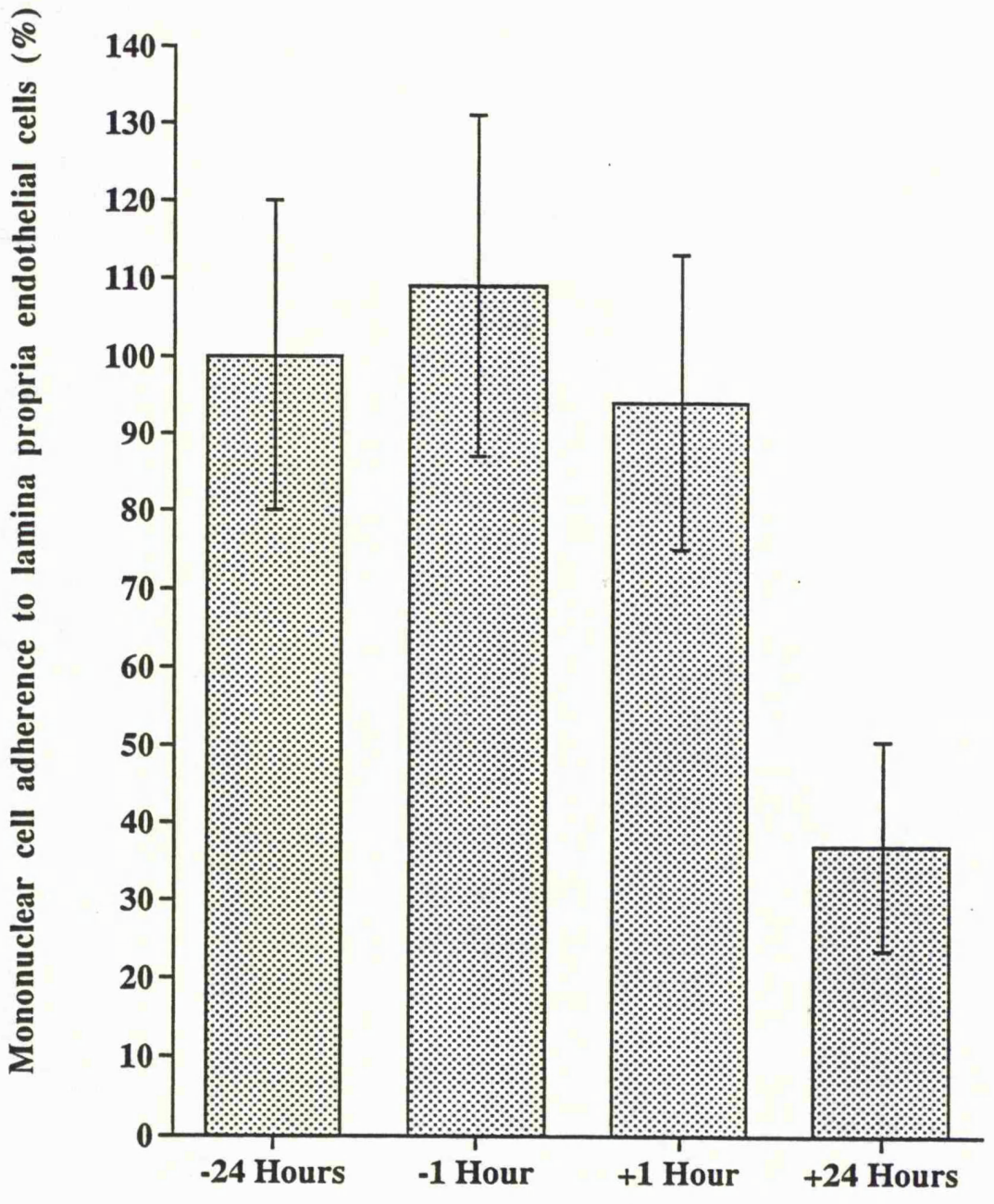


Figure-13.2

Results represent the mean % adhesion of RA PB mononuclear cells (n=4), to gut lamina propria endothelial cells, 24 and 1 hour prior, 1 and 24 hours after 500mg intravenous methylprednisolone pulse steroid therapy. Vertical lines represent the +/- SEM.

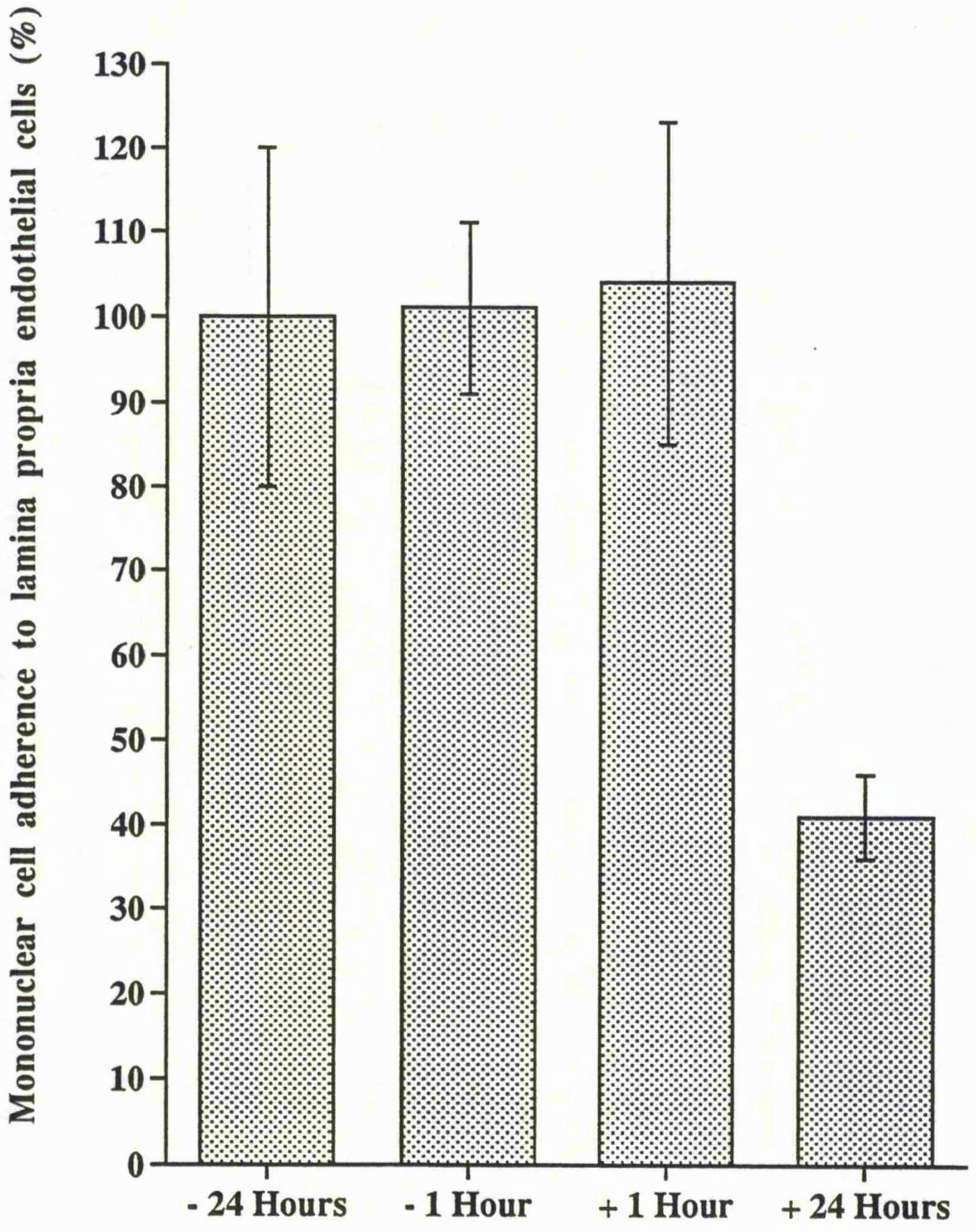


Figure-13.3

Results represent the total mean % adhesion of RA PB (n= 18) mononuclear cells adherence to gut lamina propria endothelial cells, 24 and 1 hour prior, 1 and 24 hours after 500 mg intravenous methylprednisolone pulse steroid therapy. Vertical lines represent the +/- SEM.

Table 16

Phenotypic analysis of circulating human RA peripheral blood mononuclear cells (MCs) from whole blood samples, over a 24 hour period, from 8 patients undergoing intravenous pulse (500 mg) methylprednisolone therapy. Analysis of circulating MCs was done by flow cytometry. Results are expressed as percentage positively stained MCs for each monoclonal concerned. Mean results are given as (μ), along with standard error of mean (+/- SEM).

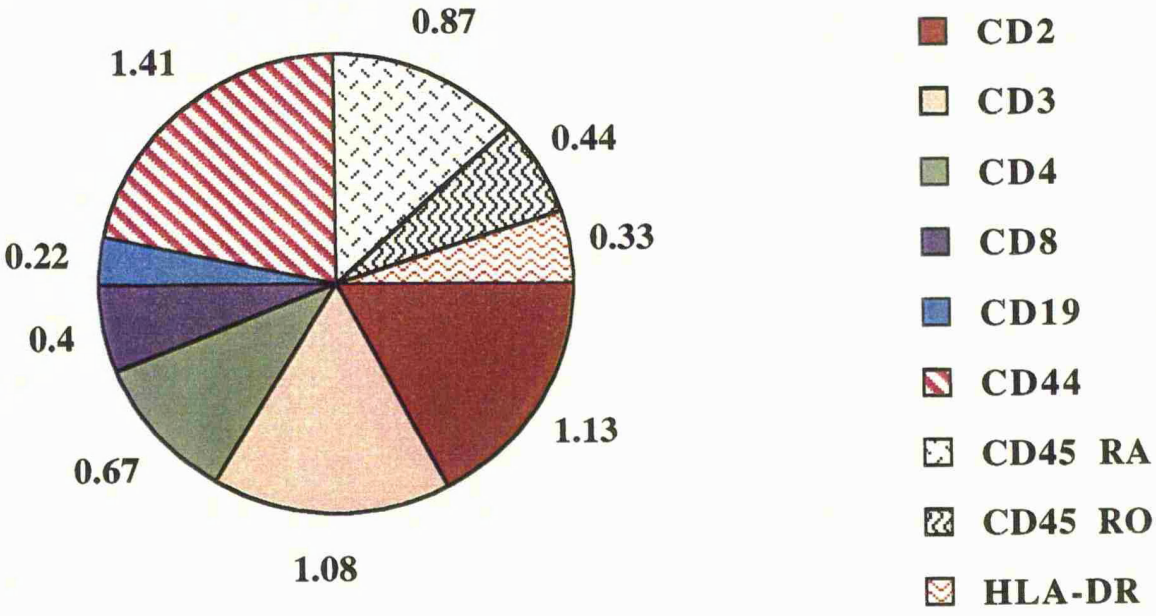
1) Pre pulse (- 1 hour) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD2	=	73	88	88	74	83	86	67	81	80(+/- 3)
CD3	=	66	67	86	68	82	72	69	78	74(+/- 3)
CD4	=	43	36	64	64	50	37	54	28	47(+/- 5)
CD8	=	25	40	20	9	29	35	15	47	28(+/- 5)
CD19	=	19	1	9	20	14	1	25	13	13(+/- 3)
HLA-DR	=	29	22	17	24	14	31	31	20	24(+/- 2)
CD44	=	98	99	98	96	99	92	97	98	97(+/- 1)
CD45 RA	=	53	49	61	70	75	36	50	67	58(+/- 5)
CD45 RO	=	39	37	33	25	19	57	37	24	34(+/- 4)

2) Post pulse (+ 24 hour) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD2	=	61	90	65	79	79	86	57	74	74(+/- 4)
CD3	=	52	55	65	62	74	67	51	75	63(+/- 3)
CD4	=	34	26	46	57	35	36	31	20	36(+/- 4)
CD8	=	20	45	22	13	36	36	19	56	31(+/- 5)
CD19	=	33	2	29	16	19	3	33	15	19(+/- 4)
HLA-DR	=	39	24	39	20	26	34	42	25	31(+/- 3)
CD44	=	97	98	96	98	97	96	92	96	96(+/- 1)
CD45 RA	=	65	63	61	73	65	34	61	76	62(+/- 4)
CD45 RO	=	30	30	24	22	27	58	31	20	30(+/- 4)

Pre pulse (- 1 hour) results



Post pulse (+ 24 hour) results

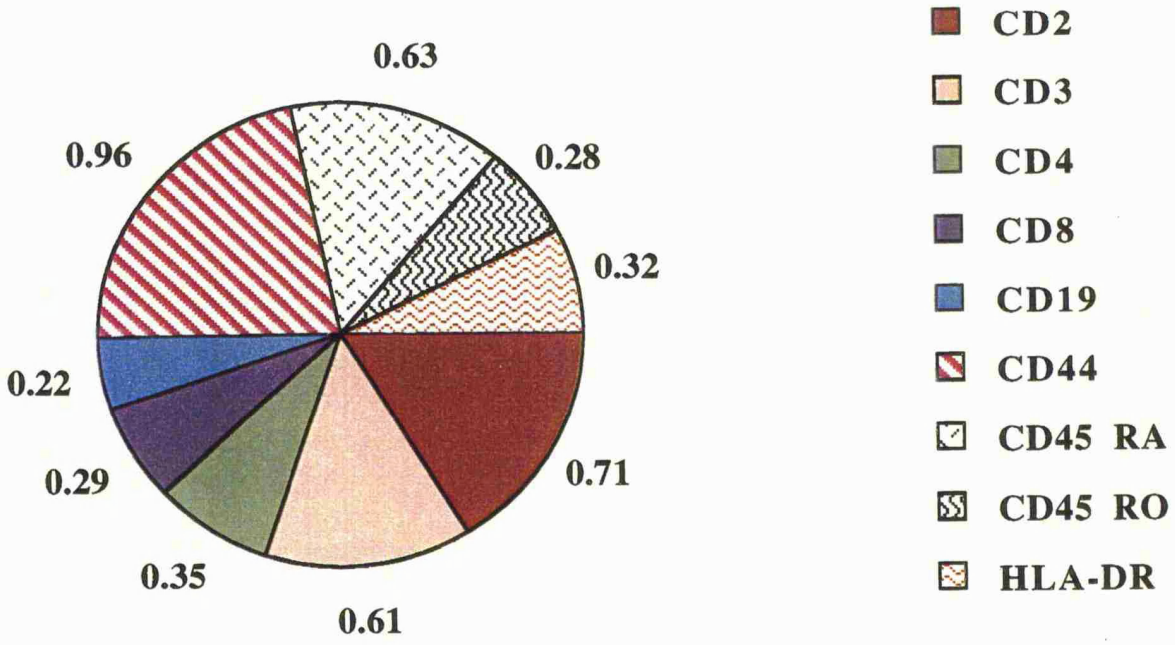


Figure-14

Results represent the mean number of circulating human RA PB lymphocytes (x 10^9/litre) from whole blood samples (n= 8), from patients receiving 500mg pulse steroid therapy over 24 hours.

Table 16.1

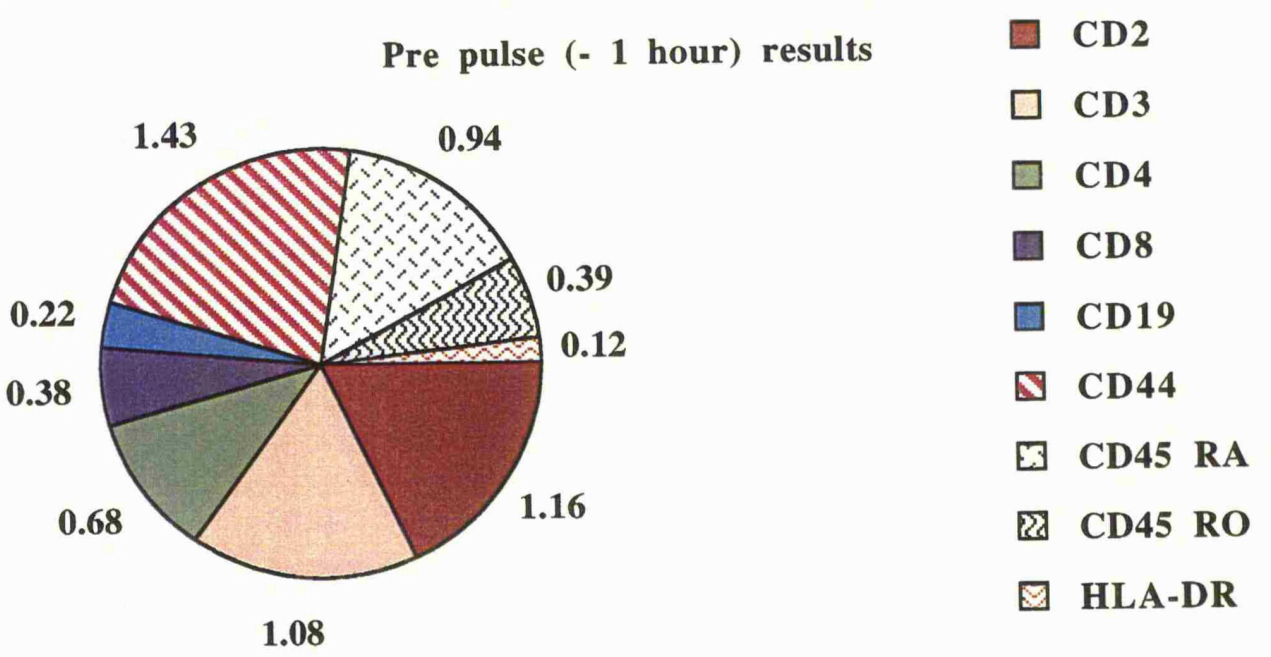
Phenotypic analysis of mononuclear cells (MCs) obtained by density centrifugation from human RA peripheral blood (PB). Samples were obtained from 8 patients undergoing intravenous pulse (500 mg) methylprednisolone therapy, over a 24 hour period. Analysis of circulating MCs was done by flow cytometry. Results are expressed as percentage positively stained MCs for each monoclonal concerned. Mean results are given as (μ), with standard error of mean (+/- SEM).

1) Pre pulse (- 1 hour) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD2	=	75	93	75	83	72	85	82	88	82(+/- 3)
CD3	=	64	58	73	85	64	85	79	67	72(+/- 4)
CD4	=	47	30	58	64	62	54	24	31	46(+/- 6)
CD8	=	23	39	11	17	7	29	47	38	26(+/- 5)
CD19	=	21	2	18	9	27	12	14	3	13(+/- 3)
HLA-DR	=	23	3	24	16	26	13	18	23	18(+/- 3)
CD44	=	99	99	96	99	97	99	99	99	98(+/- 1)
CD45 RA	=	54	62	49	67	81	77	77	48	64(+/- 5)
CD45 RO	=	34	24	43	26	14	18	18	57	29(+/- 5)

2) Post pulse (+ 24 hours) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD2	=	61	84	53	71	85	88	78	90	76(+/- 5)
CD3	=	49	36	48	64	73	83	72	61	61(+/- 6)
CD4	=	36	14	36	51	63	36	20	26	35(+/- 6)
CD8	=	17	41	18	19	14	46	47	38	30(+/- 5)
CD19	=	39	2	40	30	8	7	16	2	18(+/- 6)
HLA-DR	=	38	11	43	29	11	16	17	22	23(+/- 4)
CD44	=	99	98	98	99	95	97	99	99	98(+/- 1)
CD45RA	=	66	68	60	69	71	77	75	45	66(+/- 4)
CD45RO	=	30	25	32	23	22	18	20	53	28(+/- 4)



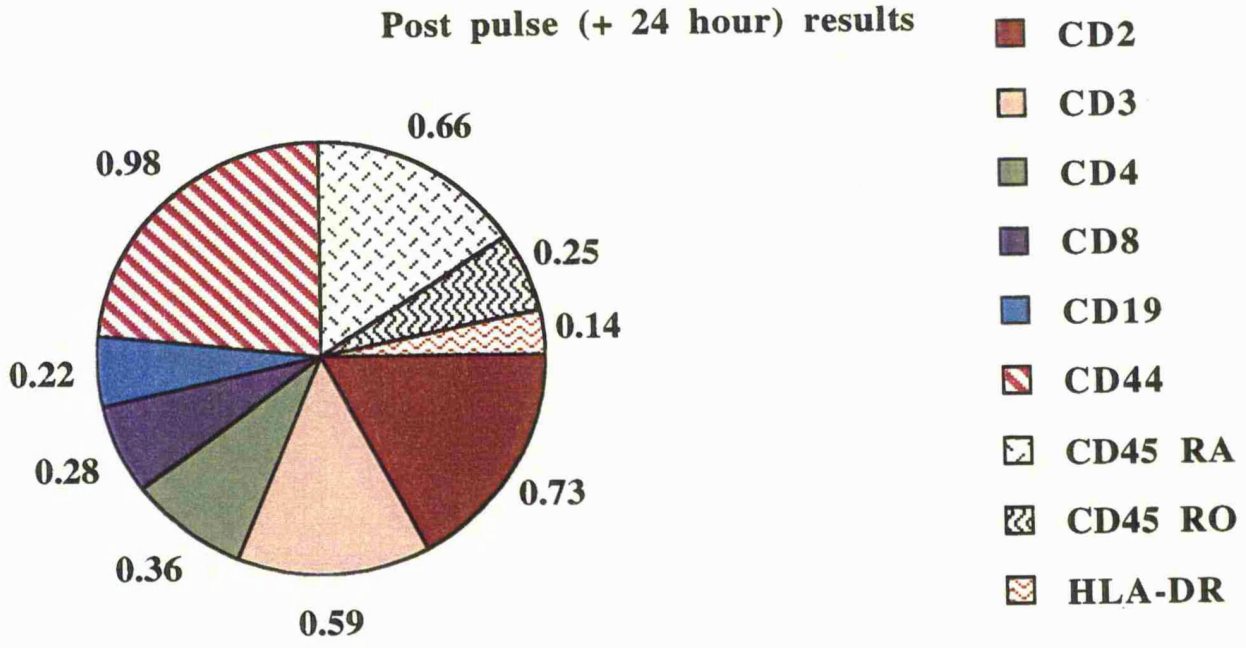


Figure-14.1

Results represent the mean number (x 10^9/litre) of circulating human RA PB lymphocytes from seperated blood samples (n= 8), after 500mg iv pulse steroid therapy over 24 hours.

Table 16.2

Phenotypic analysis of human control and RA peripheral blood (PB) mononuclear cells (MCs) adherent to gut lamina propria endothelial cells. Data from 8 patients undergoing intravenous pulse (500 mg) methylprednisolone therapy, over 24 hour period, also from 4 healthy volunteers. Analysis of adherent MCs was done by Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP). Results are expressed as percentage positively stained adherent MCs, for each monoclonal concerned. Mean results are given as (μ), standard error of mean (+/- SEM).

1) Pre pulse (-1 hour) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD3	=	86	69	76	53	77	73	65	69	71(+/- 4)
CD4	=	77	61	65	38	64	53	51	59	59(+/- 4)
CD8	=	25	52	58	13	48	31	42	29	37(+/- 5)
CD19	=	11	46	26	6	30	21	16	18	22(+/- 4)
HLA-DR	=	41	71	58	39	59	52	46	41	51(+/- 4)

2) Post pulse (+ 24 hour) results

		Patient No.								
Monoclonal		1	2	3	4	5	6	7	8	μ
CD3	=	77	75	55	63	61	73	70	65	67(+/- 3)
CD4	=	67	66	48	38	48	61	56	52	55(+/- 4)
CD8	=	21	52	21	30	31	23	29	38	31(+/- 4)
CD19	=	30	34	30	9	21	13	22	31	24(+/- 3)
HLA-DR	=	74	71	62	60	66	73	50	69	66(+/- 3)

3) Control results (Monoclonal used)			1	2	3	4	μ
	CD3	=	69	70	78	71	72(+/- 2)
	CD4	=	58	61	54	66	60(+/- 3)
	CD8	=	31	43	34	20	32(+/- 5)
	CD19	=	18	24	15	19	19(+/- 3)

Table 17

Analysis of the levels of circulating soluble VCAM-1 in plasma over a 24 hour period, from 8 RA patients undergoing intravenous pulse (500 mg) methylprednisolone therapy. Results are expressed as ng/ml of circulating VCAM-1. Mean results are given as (μ), with standard error of mean is given in parentheses (+/- SEM).

Patient No.	Pre pulse (- 1 hr)	Post pulse (+ 24 hrs)
1 =	650	525
2 =	700	690
3 =	1200	1275
4 =	1100	975
5 =	640	750
6 =	725	650
7 =	800	790
8 =	920	850
μ =	842 (+/- 50)	813 (+/- 81)

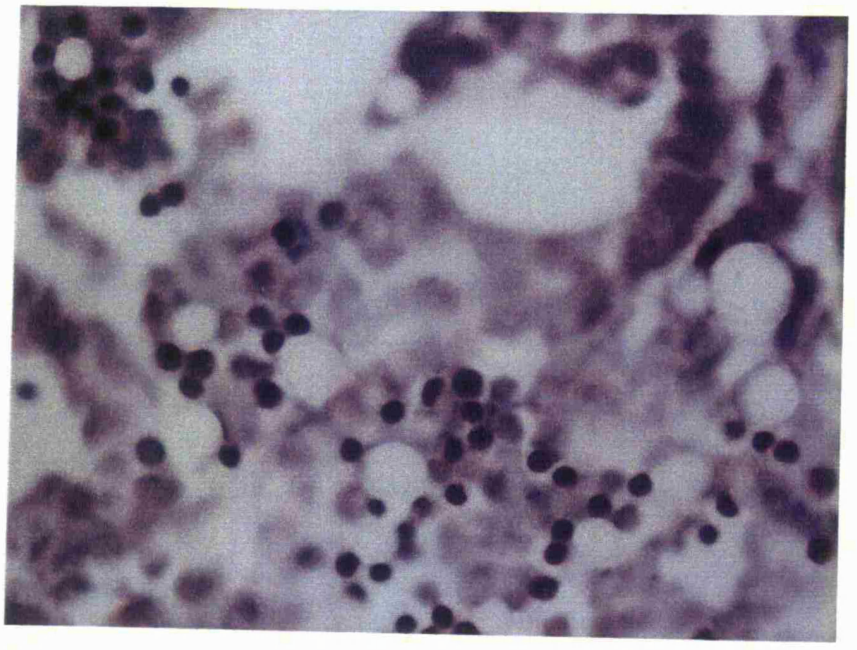
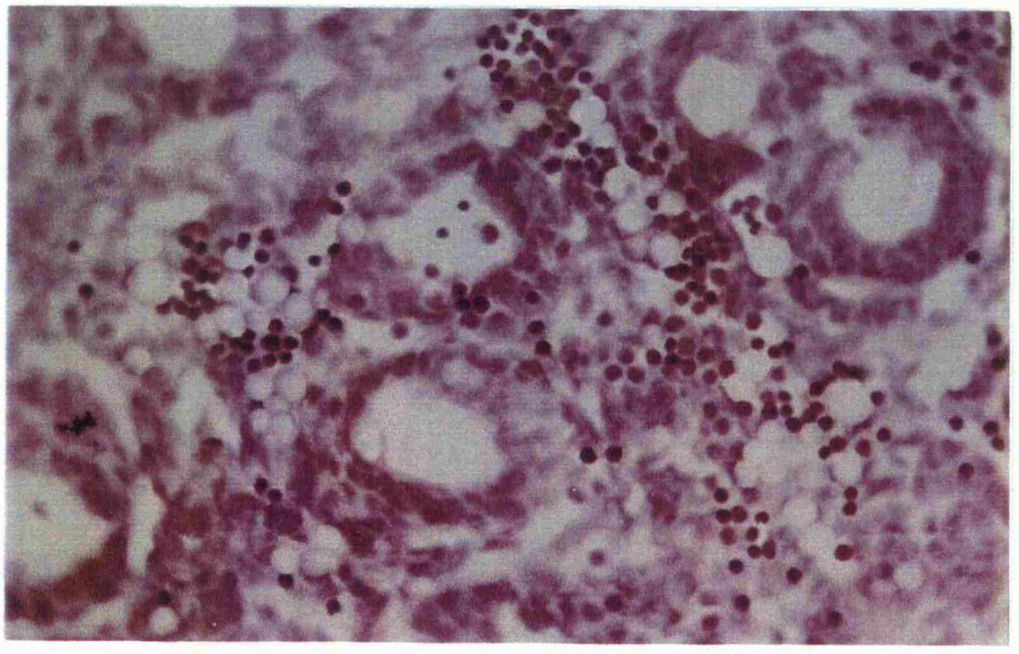


Photo-1 & 2. Adherence of RA SF lymphocytes and latex particles to endothelium-containing porcine gut lamina propria. Haematoxylin and eosin staining, x 400 magnification (above) and x 250 magnification (below).



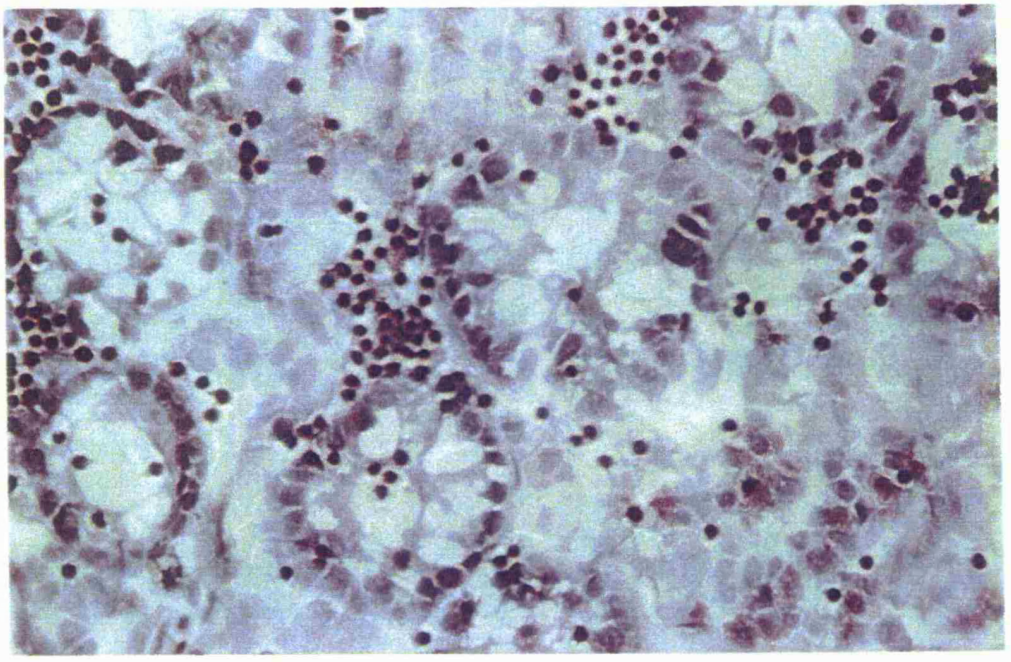


Photo-3. Adherence of RA SF lymphocytes to endothelium containing human gut lamina propria. Haematoxylin and eosin staining, x 250 magnification.

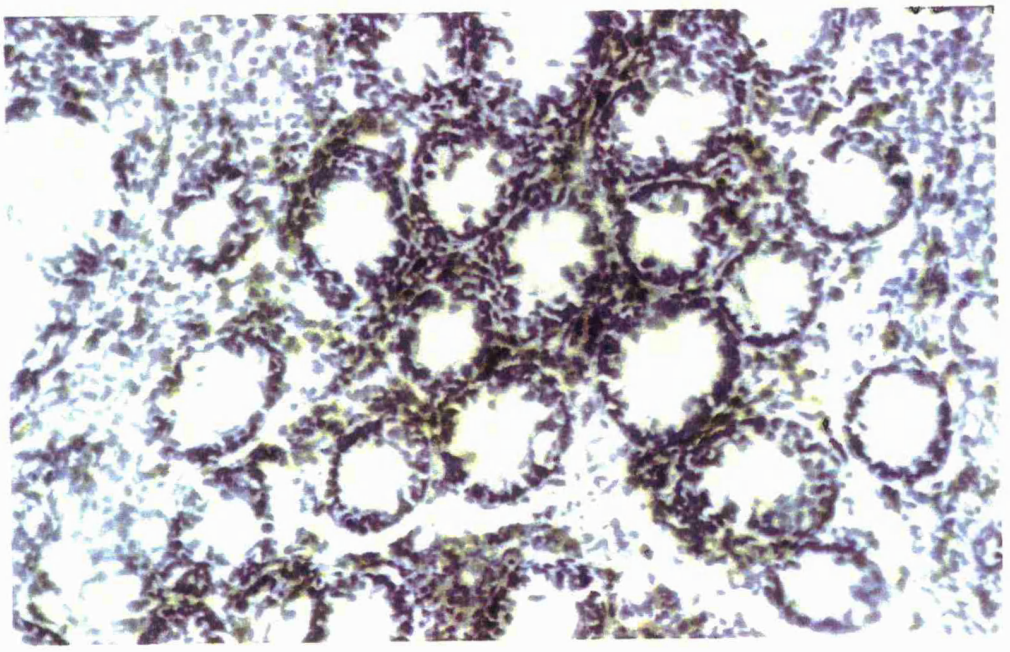


Photo-4. Indirect two-step immunoperoxidase staining of endothelium containing porcine gut lamina propria, using monoclonal antibody, Meca-367 (positive staining appears dark brown). Counterstained with haematoxylin, x 250 magnification.

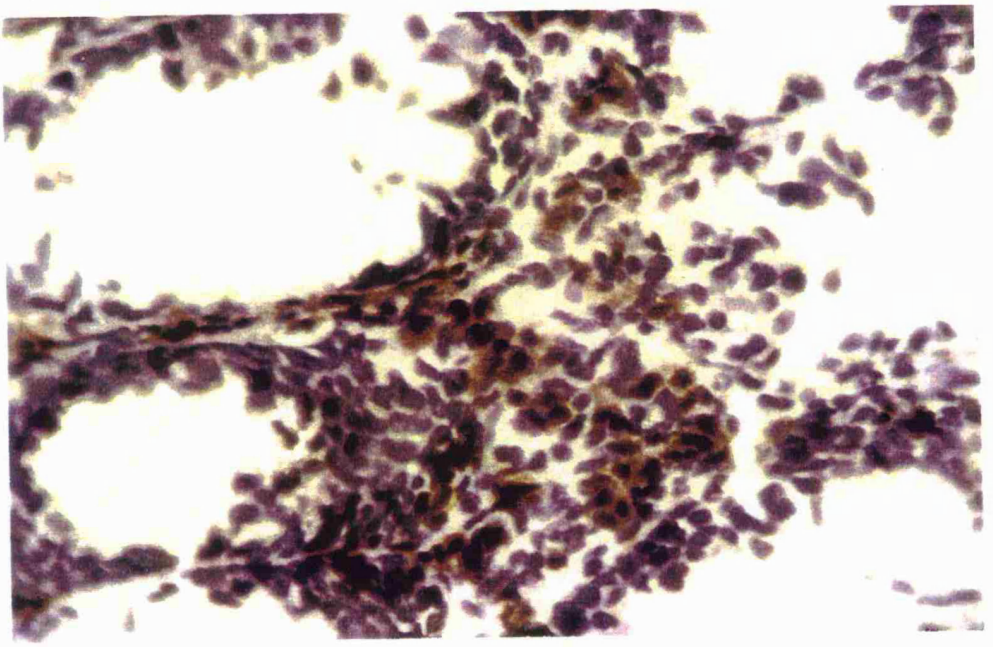


Photo-5. Indirect two-step immunoperoxidase staining of endothelium containing porcine gut lamina propria, using monoclonal antibody, Meca-367 (positive staining appears dark brown). Counterstained with haematoxylin, x 400 magnification.

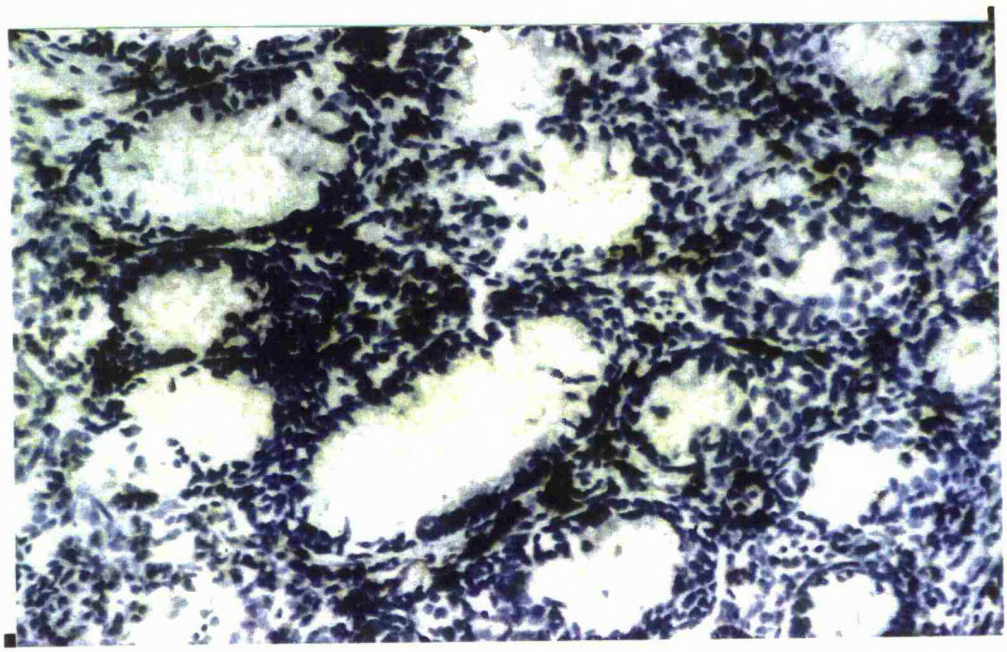
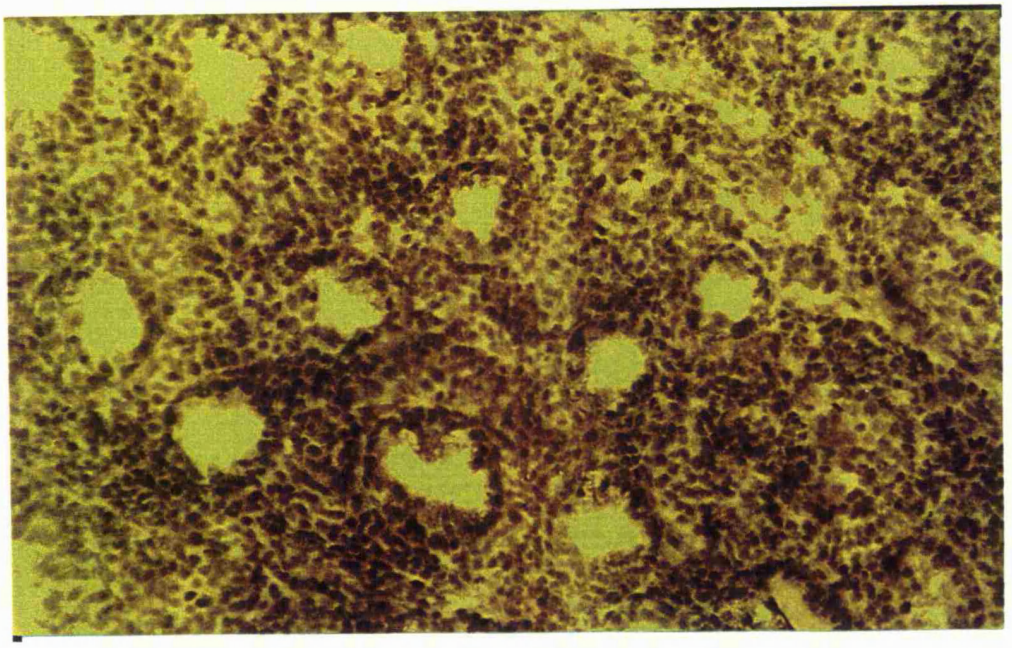


Photo-5.1. Indirect two-step immunoperoxidase staining of endothelium-containing porcine gut lamina propria, using monoclonal antibody, Meca-367 (positive staining appears dark brown). Counterstained with haematoxylin, x 300 magnification.



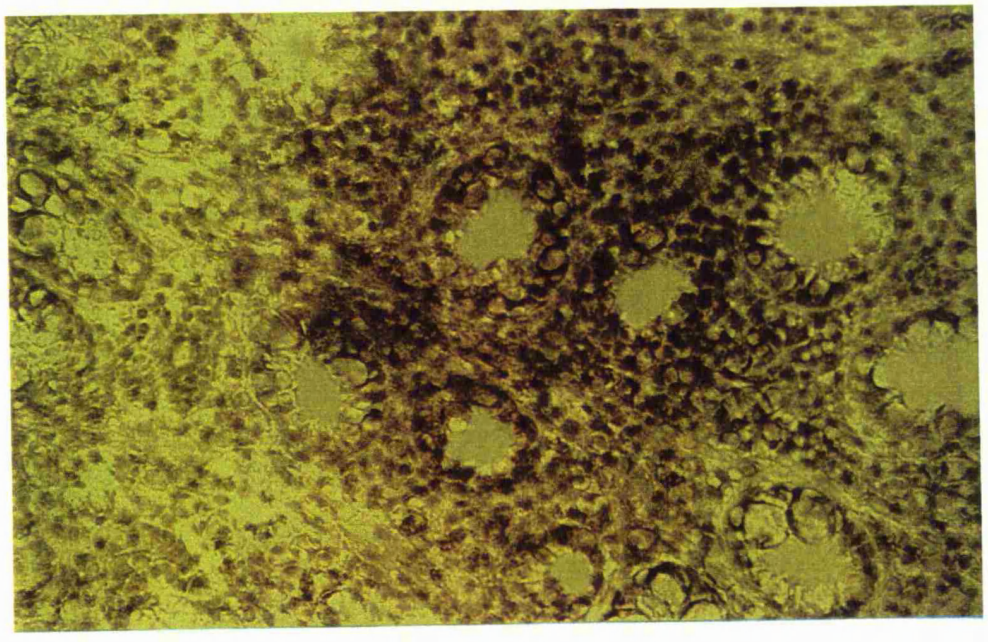


Photo-6 & 7. Indirect two-step immunoperoxidase staining of endothelium-containing porcine gut lamina propria, using anti-factor VIII monoclonal antibody (positive staining appears dark red/brown colour). Both photos at x 250 magnification.

CHAPTER 4

Discussion

A series of adhesion experiments were performed in order to validate and further optimise certain aspects of the in vitro adherence assay, which was used in most of the experiments presented within this thesis. Therefore it was of importance to develop a reproducible assay system reflecting certain features of cell adhesion (as ascertained by other researchers using the same system) and possibly also reflecting some aspects of cell adhesion in vivo.

To this end, experiments were performed examining the effects of temperature, cations, phosphorylated monosaccharides, metabolic activity, protease, mucus and anatomic locations of cells used, on cell adhesion in the adherence assay used. The discussion of these experiments will be dealt with first, before the question of adhesion within the context of different populations of mononuclear cells is addressed.

4.1 Discussion of general adhesion experiments and their implications on the in vitro adherence assay

The effects of temperature, EGTA, manganese sulphate, sodium azide, mannose 6-phosphate and galactose 6-phosphate on human control peripheral blood (PB) mononuclear cells (MCs) adherence to gut lamina propria (GLP) endothelial cells was studied.

The effects of temperature

The in vitro adherence assay was initially performed at 7^{0}C (Stamper & Woodruff 1976, Butcher et al., 1980). Others have

reproduced the same conditions at 4^{0}C with no apparent differences in lymphocyte adhesion (Willfuhr et al., 1990). Indeed the general tendency has been to perform the assay at 4^{0}C rather than at 7^{0}C. Our results showed that increasing the assay temperature does have a considerable effect upon human PB MC adhesion as compared to the 4^{0}C results. We observed maximal adhesion at 4^{0}C (the standard assay temperature), with reduced adhesion at 20^{0}C (adhesion down by 24%) and slightly less adhesion at 37^{0}C (adhesion down by 14%). These results are interesting since it was initially demonstrated that the adhesion of rat thoracic duct lymphocytes to rat peripheral lymph node HEV was maximal at 7^{0}C, significantly reduced by 70% at 24^{0}C and virtually eliminated at 37^{0}C (Stamper & Woodruff 1977). Although the results presented in our study do not show the same level of inhibition at 20^{0}C or 37^{0}C, it should be noted that the adherence of a different cell population to a different organ tissue was examined. The adherence properties of a human PB MC population to gut lamina propria rather than to peripheral lymph node HEV, may be expected to be different with regards to the adhesion molecules involved. If it is assumed that reductions in adherence at higher temperatures are due to the dissociation of adherent cells from endothelium or the inability to adhere successfully in the first place then one would assume that the required molecules for maximal adhesion at 4^{0}C or 7^{0}C are somehow altered or switched off at higher temperatures. Indeed the converse effect at higher temperatures has been reported. $\alpha 4\beta 7$ mediated adherence of a murine T cell lymphoma line to murine VCAM-1 occurs at 21^{0}C and 37^{0}C, but not at 4^{0}C (Andrew et al., 1994). Similarly, L-selectin mediated adhesion of peripheral blood T lymphocytes to peripheral lymph node high endothelial cells is increased substantially at 37^{0}C (as compared to 4^{0}C), as is LFA-1 / ICAM-1 mediated interaction (Ager, personal communication 1994). It should be

noted though that these experiments were not done by using a frozen section in vitro adherence assay, but instead by experiments using purified VCAM-1 and ICAM-1 as endothelial addressins coated on multiwell tissue culture plates. Although these findings are in contrast to those of Stamper & Woodruff (1977), simply because they showed increased levels of adherence at higher temperatures as compared to lower ones, they successfully highlight the apparent functional differences in some adhesion molecules reflected by increased or reduced adhesion.

Regarding our own results, though no substantial analysis considering the effects of temperature on specific adhesion molecules can be made from one such experiment, it is clear that at least within the in vitro adherence assay used, differences in temperature correspond to differences in PB MC adherence, suggesting that the assay system is influenced by physical factors.

The effects of EGTA & manganese sulphate

The calcium (Ca^{2+}) chelator EGTA (50mM) inhibited the adherence of PB MCs to GLP endothelial cells by 75% suggesting the involvement of Ca^{2+} in the adherence of these cells. Manganese sulphate (Mn^{2+}) on the other hand increased PB MC adhesion to GLP endothelial cells by 39% at concentrations of 2mM and 52% at 4mM, while showing no substantial effect at concentrations of 0.5mM or 1mM, also suggesting a role for Mn^{2+} at higher concentrations in MC adherence.

The role of divalent cations in lymphocyte adherence to endothelium has been known for some time. It was first demonstrated in

the rat, when rat thoracic duct lymphocyte adherence to rat peripheral lymph node HEV was inhibited with the calcium and magnesium chelators EGTA and EDTA respectively (Woodruff et al., 1977). It was observed that the inhibitory effects of EGTA were prevented by the addition of Ca^{2+}, but no reversal of inhibition occured with the addition of magnesium (Mg^{2+}). These findings were later reproduced in the mouse, with the added observation that besides Ca^{2+}, Mg^{2+} is also required (but to a lesser extent) in Peyer's patch lymphocyte adherence to gut endothelium (Yednock et al., 1988). More recently, individual adhesion molecules have been studied for their cation requirements. Integrin adherence for example, is largely dependent on the presence of divalent cations, consistent with the presence of multiple cation binding sites in the integrin extracellular domain, one such cation being Mn^{2+} which has been shown to increase the functional activity of LFA-1, VLA-3, VLA-4, VLA-5, and VLA-6 (Shimizu & Mobley 1993). However Mg^{2+} has also been found to upregulate LFA-1 mediated human PB T cell adhesion to purified ICAM-1, but not VLA-4 mediated adhesion to fibronectin and VCAM-1 (Shimizu & Mobley 1993). Likewise Ca^{2+} by itself is sufficient to support VLA-4 mediated PB T cell adhesion to VCAM-1, but not to fibronectin or LFA-1 mediated adhesion to ICAM-1. Such experiments demonstrate the various different cation requirements of several adhesion molecules. It is clear that these requirements differ for each receptor / ligand interaction and although one cation may be enough to up-regulate an adhesive interaction, an additional cation may increase adhesion still further. It has been shown for example that VLA-4 mediated PB T cell adhesion to purified VCAM-1 and $\alpha4\beta7$ mediated murine T cell lymphoma line adhesion to mouse mesenteric lymph node derived MAdCAM-1 is up-regulated by increased concentrations of Mn^{2+} in the presence of Ca^{2+}, with the $\alpha4\beta7$ / MAdCAM-1 adhesive interaction

increasing in proportion to the increase in Mn^{2+} levels (Berlin et al., 1993). On the other hand, it has also been shown that the B lymphoma cell line RPMI 8866 can adhere to MAdCAM-1 quite sufficiently without further activation from Mn^{2+} (in fact increased concentrations of this cation had no enhancing effect), while the T lymphoma cell line HUT 78 is unable to adhere to MAdCAM-1 without the presence of Mn^{2+}, although VLA-4 mediated adhesion to fibronectin is independent of Mn^{2+} presence (Erle et al., 1994). Hence not only do cation requirements differ for individual adhesion molecules for different populations of cells, but also within the same cell line.

Within the light of such knowledge, it would seem reasonable to conclude that the adherence of human PB MCs to GLP endothelial cells (in our adherence assay) could involve adhesion molecules of the integrin family, based upon Ca^{2+} requirement and the observed increase in adhesion following added Mn^{2+}. It would seem that Ca^{2+} is not the only cation of importance in such interactions though, since 25% of PB MCs still managed to adhere to gut endothelium despite EGTA chelation. Of course it is possible that higher concentrations of EGTA might have inhibited adherence further still, but it has been shown that EGTA concentrations as small as 1mM managed to inhibit thoracic duct lymphocyte adherence to lymph node HEV (with higher EGTA concentrations not significantly adding to its inhibitory effect), using essentially the same in vitro adherence assay (Woodruff et al., 1977). The adhesion enhancing effects of Mn^{2+} would also seem to justify the involvement of another cation besides Ca^{2+}, and the possible involvement of integrin adhesion molecules. Concentrations of 2mM Mn^{2+} increased $\alpha4\beta7$ mediated adhesion of mouse mesenteric lymph node lymphocytes to purified MAdCAM-1 coated slides (Berlin et al., 1993) lending support to

our observed increases in PB MC adhesion to gut endothelium at similar Mn^{2+} concentrations, albeit in a different adhesion assay.

Although such assumptions are difficult to make, based upon one set of experiments, it is nevertheless interesting to observe adhesive interactions influenced by cations in a manner similar to those reported by others for integrin adhesion molecules. The demonstrated involvement of such cations is indicative of the capability of the in vitro adherence assay's capability to reflect certain aspects of in vivo cell adhesion as well.

The effects of sodium azide and phosphorlyated monosaccharides

Sodium azide is a metabolic inhibitor of oxidative phosphorylation, previously shown to inhibit rat thoracic duct lymphocyte adherence to lymph node endothelium by over 85% (at a concentration of 10mM) in the in vitro adherence assay (Woodruff et al., 1977). We have also shown that sodium azide at a concentration of 20mM inhibits the adherence of PB MCs to GLP endothelial cells by 98%, using a largely similar in vitro adherence assay. Cell mortality did not account for the observed inhibition, since all MCs were tested for viability as measured by trypan blue exclusion before each assay, and found to be over 90% viable. This result would suggest that MCs need to be metabolically intact in order to adhere to gut endothelium in the adherence assay used.

The phosphorylated monosaccharides galactose 6-phosphate and mannose 6-phosphate (at a concentration of 20mM) were also studied. Mannose 6-phosphate inhibited MC adherence by 65%, whereas galactose

6-phosphate showed no inhibitory effect. Inhibition by mannose 6-phosphate and its structural analog fructose 1-phosphate were initially demonstrated in rat lymphocyte binding to peripheral lymph node HEV, where both molecules produced a 80-90% inhibition of adherence at a concentration of 10mM, while another monosaccharide galactose 6-phosphate (at the same concentration) showed no such inhibitory effect (Stoolman et al., 1984). Similar results were also demonstrated in the mouse, mannose 6-phosphate inhibiting lymphocyte adherence to lymph node HEV (Yednock et al., 1987). It has been suggested that mannose 6-phosphate acts as an inhibitor of cell-endothelium interactions, while galactose 6-phosphate inhibits cell-epithelium interactions, based on adherence of thoracic duct MCs to lymph node endothelium and intestinal and lacrimal gland epithelium, using an in vitro adherence assay (O'Sullivan & Montgomery 1990). Mannose 6-phosphate inhibited MC adhesion to lymph node endothelium, while having no effect on adhesion to intestinal or lacrimal gland epithelium, whereas galactose 6-phosphate inhibited adhesion to intestinal and lacrimal gland epithelium, while having no effect on adhesion to peripheral lymph node endothelium. Although there have been speculative suggestions as to the exact function of these molecules, it would appear that the inhibitory effects of mannose 6-phosphate may be due to blocking of ligand binding regions of adhesion molecules, either by competion with the adhesion molecule's natural ligand, or by interactions with sugar moieties present on such molecules, although there is no satisfactory experimental evidence to support these assumptions as yet. Perhaps adhesion molecules of the Ig superfamily with their abundance of protein bound sugar side chains, and the selectin family with their carbohydrate ligand binding lectin domains may possibly be affected by such mannose 6-phosphate interactions.

Mannose 6-phosphate led to a substantial inhibition in our adherence assay. However up to 35% of PB MCs did manage to adhere despite its presence, suggesting that whilst this molecule does indeed prevent the adherence of the majority of MCs, nevertheless a sizeable proportion is not affected. It is suggested that these cells adhere instead to a third tissue component such as fibronectin, which is present within Peyer's patch tissue, and which is also known to support the adhesion of $\alpha 4\beta 7$, CD44 and VLA-4. Alternatively, mannose 6-phosphate may preferentially interfere with the adhesive interactions of one set of adhesion molecules (which in the assay used mediates the majority of adhesive interactions) without affecting a second set of adhesion molecules mediating the adherence of a smaller proportion of MCs. Or the second set of adhesion molecules could be taking part as accessory molecules able to support adhesion on their own, but not to a great extent.

In summary therefore, the consensus of opinion is that PB MCs do not adhere to epithelium. The questions regarding the inhibitory effects of mannose 6-phosphate on PB MCs adherence to gut endothelium cannot be answered clearly. It is of value however to note that the in vitro adherence assay does support adhesive interactions that can be modulated as such and that these interactions require functional activity on the part of MCs.

The other aspects investigated with regards to the adherence assay, were to do with the nature of the tissue used in the assay, rather than those affecting the cells.

Effects of cell source, proteased tissue and mucus on adhesion

It has been suggested that Peyer's patch (PP) tissue from different regions of small intestine may yield MCs with different adhesion characteristics (Pabst 1992). This would also imply that the adhesive properties of the tissue itself could also be different. To address this question, PP MCs from ileal, jejunal and duodenal PPs were used to study their adherence to jejunal PP tissue (which was the standard PP tissue used in all of the in vitro adherence assays). No significant difference in adhesion was observed in PP MCs from any of the three different regions. Additionally, when adherence of the same PP MC populations were examined on PP tissue subjected to proteolysis the level of MC adhesion was reduced on average by 60% for all three populations. These results would suggest that PP MCs from different regions do not show appreciable differences in adhesion, and that these levels of adhesion are substantially reduced when the counter-receptors present on gut endothelium are proteolytically altered or destroyed.

The low concentration of protease used may account for the 40% or so of PP MCs that still managed to adhere to gut endothelial cells. It would be reasonable to expect even greater levels of reduction in adherence if higher concentrations of protease or longer incubation periods were used. This was not done however since it was of importance to demonstrate the sensitivity of the gut tissue used against proteolysis, rather than trying to abolish adhesion totally. Although differences in ileal and jejunal PP histology have been reported in 1.5 month old pigs (differing follicle shapes and smaller inter-follicular areas in ileal PPs as compared to jejunal PPs), no such differences were seen in older animals of 12 months or more (Rothkotter & Pabst 1989). Additionally,

differences were also reported to occur in the composition of lymphocytes in ileal as compared to jejunal PPs in young pigs, with more B lymphocytes in the former and more CD4+ and CD8+ T lymphocyte populations in the latter (Rothkotter & Pabst 1989). These differences were not evident in older pigs. Since tissue from pigs at least 12 months old was used in our experiments, one would not expect differences between PPs from differing regions to be substantial enough to affect the adhesive properties of the cells concerned. Indeed at least in terms of adhesion to gut endothelial cells in the assay system used, this can be said to be true.

It is known that mucus is present within gut tissue, therefore the possible effects of such mucus on PP MC adhesion to gut endothelium was tested for within the in vitro adherence assay system. Although a fairly simple experiment, it had been reported previously that at concentrations of 1-2mM, dithiothreitol (DTT) could successfully remove residual mucus present on porcine PP tissue segments prior to the in vitro adherence assay (O'Sullivan, personal communication 1992). Tissue samples treated with 2mM of DTT in our assay system, showed no appreciable difference in MC adhesion, when compared to non-DTT treated tissue samples. Residual mucus would not, therefore seem to effect MC adhesion.

The in vitro adherence assay used, would seem to reflect certain aspects of cell adhesion. Factors such as temperature and the presence of certain cations, and phosphorylated monosaccharides previously demonstrated by others to affect adhesive interactions, would also appear to affect such interactions in our assay. Additionally, the requirement of metabolic activity and the necessity that both cell and tissue adhesion

molecules are intact and functional, in order to support adhesive interactions lends further credibility to the assay system used.

One final point has to be made about the composition of the cells used in the experiments discussed so far, and throughout the rest of the discussion. As described earlier (see section 2.5.1) all cells were obtained as mononuclear cells separated by density gradient centrifugation. Therefore the MC populations did not only contain B and T lymphocytes, but also a small proportion of monocytes and natural killer (NK) cells. The main problem of using such mixed cell populations is that it is impossible to say which subset is adhering to the tissue used and in what proportions, unless the MC population used, is further separated into individual subsets or the phenotypic analysis of adherent cells is done. Since it was our intention to use B and T lymphocytes together in our adherence assays rather than separating them, we chose the latter option. Therefore the phenotypic analysis of healthy human volunteer control PB MCs adherent to GLP endothelial cells was examined to see what proportion of adherent cells were B or T lymphocytes or neither (see section 2.8.2.2). It was seen that on average more than 90% of all positively stained adherent cells were of the pan T (72% CD3+) or pan B (19% CD19+) lymphocyte subsets. Therefore nearly 10% of adherent MCs did not express the pan B or T lymphocyte marker. Monocytes and NK cells do not express these markers, hence it is possible that the unidentified 10% were made up of these cells (however monocyte or NK cell surface markers were not tested, hence it is difficult to be accurate on this matter). Also on average, 60% of adherent cells stained positive for the CD4 subset and 32% for the CD8 subset of T lymphocytes (phenotypic analysis was also done for adherent rheumatoid PB MCs, which can be seen together with these results in table 16.2).

Although a small proportion of adherent cells do not express B or T lymphocyte cell surface markers, at least in terms of human MC adherence to porcine tissue the majority of adherent cells consist of B and T lymphocytes.

4.2 Discussion of the adherence characteristics of porcine mononuclear cells

One of the first attempts at using an vitro adherence assay in determining organ specific migratory pathways was done by studying the adherence of mouse peripheral lymph node (LN) lymphocytes, mesenteric LN lymphocytes and PP lymphocytes, to PP and peripheral LN HEV (Butcher et al., 1980). This early work showed that each cell population adhered best to its own tissue HEV (with the exception of mesenteric LN lymphocytes which showed similar levels of adhesion to both PP and peripheral LN HEVs) with peripheral LN lymphocytes binding twice as well as PP lymphocytes to peripheral lymph node HEV, and PP lymphocytes binding 1.5 times as well as LN lymphocytes to PP HEV. These in vitro results were comfirmed by in vivo studies, where the relative localisation of ^{51}Chromium labelled PP, mesenteric and peripheral LN lymphocytes to HEV bearing organs was determined 30 minutes after intravenous injection, with essentially the same levels of selective lymphocyte adherence as in the in vitro study (Butcher et al., 1980). We likewise have demonstrated the adherence of porcine PP, LN PB MCs to porcine GLP endothelial cells and to peripheral LN HEV in a similar fashion. The experiments were primarily done to determine the adhesion characteristics of such different populations of MCs in the pig, where information on the selective adherence of cells to different organ

tissue endothelium is minimal (the majority of information concerning recirculatory migration being derived from studies using mice, rats and to a lesser extent sheep as experimental animal models). The second added benefit of such studies was to demonstrate the experimental validity of the in vitro adherence assay used, with respect to previously published work and the current knowledge regarding recirculation and cell migration, and thirdly, to use results obtained from porcine MC adhesion experiments as a guiding model for future adhesion studies with different cell populations.

The results show that both PP and LN MCs demonstrated the greatest levels of adherence to their own tissue endothelium, with PP MCs adhering to gut endothelium more than 3 times as well as to LN endothelium, and similarly LN MCs adhering to LN endothelium 2 times as well as to gut endothelium. Additionally, important differences were detected in the adherence characteristics of PP, LN and PB MCs when compared against each other. PP MCs adhered to gut endothelium more than 2.5 times as well as LN and PB MCs did, while LN MCs adhered to LN endothelium nearly 2.5 times as well as PP MCs did. PB MCs exhibited similar levels of adherence to gut endothelium as LN lymphocytes did, with no significant differences.

These results are in keeping with previously published work insofar as the relative adherence levels of PP and LN MCs are concerned (Butcher et al., 1980). These results would therefore suggest that a higher proportion of PP MCs have the required adhesion characteristics for binding to gut endothelium than to LN endothelium, and likewise a higher proportion of LN MCs have the necessary adhesion characteristics for binding to LN endothelium than to gut endothelium. Suggesting specific

mucosal tissue homing adhesion characteristics. These would largely depend upon whether or not the cells in question were naive or memory cells, and also the adhesion molecules expressed on their cell surfaces. Recirculation patterns of naive and memory cells are distinct, insofar as their capabilities to migrate to specific organs (see section 1.4 for details). Selective migration is due to the expression of specific homing receptors on cell surfaces and their successful interaction with complimentary endothelial addressins on target organ tissues. Naive cells on the other hand express adhesion molecules enabling them to adhere to different lymphoid organ endothelia non-selectively.

Although the proportions of naive and memory cells within the populations of porcine PP, LN and PB MCs is not known, one may be able to deduce from the results, that the PP and LN populations had significantly greater proportions of memory cells, expressing adhesion molecules specific for own tissue endothelium (e.g. LN MCs adhering to LN endothelium), due to the significantly higher levels of adherence than to other tissue endothelium. Also a smaller proportion of naive cells exist, capable of adhering to both tissue endothelium (e.g. LN MCs adhering to gut endothelium and PP MCs adhering to LN endothelium) by means of expressing the necessary adhesion molecules to do so. While it is harder to make such an assumption for PB MCs, which exhibit adherence to gut endothelium just as well as LN MCs and hence may possess equal numbers of naive cells or indeed that many memory cells with specific binding affinity for gut endothelium. It is impossible to come to a well supported analysis of the results, without exactly knowing the numbers of naive and memory cells in the MC populations as well as the numbers of adherent naive and memory cells following the adherence assay. It was not possible to undertake such an study since at the time of the experiments being

performed (and still today) the identification of naive and memory cells by phenotype available for human lymphocytes (CD45RA and CD45RO respectively) is not available for porcine cells, although a naive cell marker for mouse, rat and sheep lymphocytes has been identified recently, with the memory cell phenotype being identified by default as non-naive cells which do not express the naive cell marker (Westermann & Pabst 1996). Other than this the only conclusion can be made is that at least two different sets of adherence properties exist (mucosal and peripheral adhesion characteristics) amongst two different populations of porcine cells with a third population sharing adhesion characteristics with possibly both of the other populations.

Of course such conclusions are largely based on the assumption that naive and memory cells are indeed distinct in their migratory patterns, with specific adhesion molecules mediating such diverse migration. But recently there have been suggestions to dispute this accepted notion, at least when it comes to in vitro studies. The researchers found that mesenteric LN derived gut lymphocytes bound 3 times better to endothelium of PPs than to the endothelium of peripheral LNs in the rat, using the in vitro adherence assay (Westermann et al., 1992), in keeping with our own results. However the levels of gut lymphocyte adherence to PP and LN endothelium was nearly equal when studied by fluorescence labelling in vivo, suggesting that experimental approach may have a considerable effect on the results of migration experiments when comparing different populations. More surprisingly perhaps, it has been reported that memory T cells isolated from thoracic duct lymphocytes are found in the wall of HEVs of peripheral (cervical) LNs and PPs 30 minutes after intravenous injection into rats, and that memory T cells are found in large numbers in the efferent lymph after such injection

(Westermann & Pabst 1996), showing a pattern that is comparable with that of naive T cell circulation patterns, since it had previously been demonstrated that more than 90% of sheep T cells obtained from the efferent lymph had a naive phenotype, whilst more than 90% of T cells obtained from the afferent lymph exhibited a memory phenotype (Mackay et al., 1990). The expression of different adhesion molecules mediating naive and memory cell migration has also been questioned, since most adhesive interactions have been studied in vitro and in vivo studies have occasionally casted doubt on certain aspects of in vitro experiments. One such example is the ability of naive cells to use L-selectin mediated adherence to peripheral LN endothelium when using in vitro adherence assays, with no L-selectin involvement in adherence to PP endothelium (Gallatin et al., 1983) with more recent in vivo studies demonstrating L-selectin involvement in adherence to LN and PP endothelium (Hamann et al., 1994, see L-selectin heading in section 1.7.1).

The suggestions that naive and memory cell recirculation may not be as different as currently accepted is a contentious issue, which until quite recently had been generally ignored. The questions regarding results obtained from in vivo as compared to in vitro adherence assays is quite the opposite however, with the general acceptance that in vitro adherence assays can only reflect certain aspects of the whole picture (in terms of adhesive interactions between cells and endothelium) and that although results from in vivo adherence assays (being performed under flow conditions more recently) are reproduced to a certain extent in vitro, such conclusions should be taken as informative rather than as concrete evidence.

The results presented so far are as such informative, as to the general capabilities of the in vitro adherence assay used and by inference the migratory patterns of different porcine MC populations.

4.3 Discussion of the adherence characteristics of rheumatoid and control mononuclear cells

The adherence of paired RA synovial fluid (SF), and PB MCs and synovial membrane (SM) MCs, along with the adherence of human control PB MCs to GLP endothelial cells was studied. Additionally the adherence of RA SF and PB MCs adherence to peripheral LN endothelium was also examined and compared to GLP endothelial cell adherence. The overall aim was to demonstrate (if present) any differences in adhesion to gut or LN endothelium between any of the MC populations studied, and also to compare results to those obtained earlier with porcine MCs, with the intention of suggesting possible migratory characteristics within the confines of the in vitro adherence assay used.

The results would suggest that differences in adhesion between the populations studied does indeed exist. With RA SF MCs adhering to GLP endothelial cells nearly 1.8 times as well as RA PB MCs and 1.6 times as well as control PB MCs and interestingly nearly 4.5 times as well as SM MCs. With no significant differences in adhesion to GLP endothelial cells between RA PB MCs and control PB MCs, although both MC populations adhered nearly 2.5 and 2.7 times as well as SM MCs did respectively. Recent research in humans, using the in vitro adherence assay has demonstrated the adherence of gut lamina propria lymphocytes to gut endothelium and to rheumatoid synovial membrane endothelium in

largely equal numbers, with minimal adherence to peripheral LN endothelium, leading the authors to conclude that normal mucosal lymphocytes can interact with endothelium in mucosa and synovium, but not in peripheral LN (Salmi et al., 1995). Similar results were obtained using mouse peripheral LN and PP tissue endothelium with human lamina propria lymphocytes exhibiting the same levels of adherence as they did with human LN and gut endothelium, suggesting that lymphocyte-endothelium interactions in corresponding human and mouse tissue is comparable and that cross-species reactivity does occur to a certain degree (Salmi et al., 1995). These results would lend some support to our own findings indirectly, since the ability of gut lamina propria lymphocytes to adhere to mucosal and synovial endothelium in equivalent numbers would suggest that they share similar adhesion characteristics, different from those required for peripheral LN adhesion. Therefore one might assume that synovial lymphocytes would exhibit a similar pattern of adhesion to those of gut lamina propria lymphocytes. As our results indicate RA SF MCs, exhibited significantly greater levels of adhesion to gut endothelium than to peripheral LN endothelium comparable to the levels of porcine PP MC adherence to gut and LN endothelium (see section 4.2). Although RA PB MCs also exhibited greater levels of adherence to gut than to peripheral LN endothelium, it was not as prominent as that demonstrated by SF MCs. The level of adherence of RA PB MCs to gut endothelium was far smaller than that of RA SF MCs. While both MC populations proved capable of adhering to LN endothelium, this was less so for SF MCs, which exhibited a greater mucosal tissue endothelium binding capability. Others have shown that human T cell lines derived from gut lamina propria, peripheral blood and rheumatoid synovial lymphocytes adhered to mucosal and rheumatoid synovial endothelium in significantly higher numbers than to peripheral

LN endothelium. Gut lamina propria and synovial lymphocytes adhered significantly better than peripheral blood lymphocytes to mucosal and synovial endothelium while adhering equally well to each others tissue endothelium (Salmi et al., 1992). Additionally these experiments were also performed by using the in vitro adherence assay and reproduced with corresponding levels of adherence using mouse rather than human tissue (Salmi et al., 1992). Such work lends further support to our results insofar as gut and synovial lymphocytes (and in our case MCs) seem to exhibit similar patterns of adhesion. RA SF MCs used in our assays exhibited adhesion properties more in keeping with MCs that home to mucosa, than those that home to peripheral lymph nodes. In other words, it has been shown that an appreciable proportion of the MCs found within the joints share adhesion characteristics with MCs homing to the gut.

The point that such experiments were performed by using an in vitro adherence assay essentially the same as ours, with similar results is of value at least in terms of the further validity of the assay system used. The observations of comparable results when using human cells and mouse tissue is also of importance, when the question of cross-species cell-endothelium interactions is discussed (this point shall be taken up later with more detail in the next section). The assumptions made about RA SF MCs do however need consideration when the results concerning RA SM MCs is addressed (this point shall be addressed in section 4.3.2). As mentioned previously RA SF, PB and control PB MCs all exhibited significantly greater levels of adherence to gut endothelium then RA SM MCs did.

4.3.1 The use of monoclonal antibodies and the question of cross-species reactivity

A series of monoclonal antibody (Mab) experiments was performed using RA SF and RA PB MCs. The adherence of these MCs to GLP endothelial cells was studied in the presence of Mabs directed against specific adhesion molecules present on cell or endothelial surface. The rat anti-mouse Mab Mel-14 is directed against the L-selectin adhesion molecule (see section 1.71 for more details) and experiments using the in vitro adherence assay have demonstrated that Mel-14 inhibits the adherence of mouse lymphocytes to peripheral lymph node HEV but not to PP HEV (Gallatin et al., 1983) although more recent in vivo work has also demonstrated a role for Mel-14 in inhibiting naive lymphocyte adherence to PP HEVs, but having no effect on the adherence of memory/activated blast cells to this lymphoid organ (Hamann et al., 1994). These findings are in keeping with the role L-selectin has to play as a peripheral LN homing receptor (most apparent when using in vitro adherence assays) and as an early stage contact adhesion molecule for recirculating naive lymphocytes to LN and PP lymphoid organs (apparent in adhesion assays under conditions of flow within the physiological range of venular shear stresses in vivo). When Mel-14 was used in our assay, it had no inhibitory effect whatsoever on either SF or PB MCs adherence to gut endothelium as compared to controls. The rat anti-mouse Mab Meca-367 on the otherhand, which is directed against the mucosal vascular addressin MAdCAM-1 (which is a counter-receptor for the gut homing adhesion molecule $\alpha 4\beta 7$, see section 1.7.2.3), inhibited the adherence of SF and PB MCs to gut endothelium significantly by 69% and 74% respectively as compared to controls ($P= 0.03$ for both). These results are in agreement with the findings of others insofar as the ability of this Mab

to block the adhesion of cells to gut endothelium. It has been shown for example, that Meca-367 inhibits mouse gut lymphocyte adherence to mouse Peyer's patch HEV by over 90%, while adherence to peripheral lymph node is unaffected when using the in vitro adherence assay (Streeter et al., 1988). Intravenous injection of Meca-367 inhibits lymphocyte localisation within Peyer's patches by over 95% but does not affect localisation within peripheral lymph nodes (Streeter et al., 1988). More recently, the characterisation of the primary structure and adhesion characteristics of MAdCAM-1 and its interaction with $\alpha 4\beta 7$, as a specific receptor/counter-receptor pair in mucosal homing to the gut, has also validated the role played by Meca-367. Anti-$\alpha 4\beta 7$ antibodies parallel the inhibitory effects of the anti-MAdCAM-1 Mab Meca-367 (Briskin et al., 1993). It would be reasonable to suggest therefore, that the inhibition of adherence observed in RA SF and PB MCs, is in part due to the blocking of the MAdCAM-1 vascular addressin by Meca-367. The third Mab used was the mouse anti-human Hermes-3 (directed against the human CD44 adhesion molecule, see section 1.7.4). It also inhibited the adherence of SF and PB MCs to gut endothelium. This monoclonal has been demonstrated to inhibit the adherence of human lymphocytes to HEV in either mouse PP or human appendix, but not to mouse or human peripheral lymph node HEV when using the in vitro adherence assay (Jalkanen et al., 1987). It is known to specifically block CD44 mediated adherence of lymphocytes to mucosal HEV (Goldstein et al., 1989). CD44 expression is widespread, with expression known to be significantly greater in RA SF than PB lymphocytes, although whether CD44 positive cells accumulate within the synovial fluid or whether the expression of CD44 is upregulated within the synovial fluid is not clear (Kelleher et al., 1995). However, this increase in expression may explain the higher levels of

Hermes-3 induced inhibition observed in SF lymphocytes (79%) as compared to PB lymphocytes (57%).

As far as the results are concerned, it is evident that the adherence of a large proportion of RA SF and PB MCs to porcine gut endothelium is inhibited by Hermes-3 and Meca-367. Since the Mab Mel-14 exhibited no inhibitory effect, it would appear that L-selectin was not playing an important role in the adherence of these cells. This would make sense, since to date L-selectin has only been shown to mediate adherence to peripheral LN endothelium, and not to gut endothelium, when used in the in vitro adherence assay. This is an important point to make, since in vivo work with Mel-14 has demonstrated the ubiquitous role of this adhesion molecule, with regards to naive cell recirculation. Perhaps a more vital issue with regards to the current results is one of cross-species reactivity. Mel-14 is a rat anti-mouse Mab. This casts doubt over the capability of this Mab to recognise L-selectin on human MCs. Though falling far short of absolute proof, Mel-14 has been shown to inhibit the adherence of mouse and human PB lymphocytes to lymph node endothelium to the same degree, but not to mucosal or synovial endothelium when using the in vitro adherence assay (Jalkanen et al., 1987). Experimental procedures involving Mel-14 were the same as described for our own experiments (see section 2.7.5 for details). Additionally Mel-14 was shown to immunoprecipitate a 90kD band from lysates of surface iodinated human PB lymphocytes, and this Mel-14 antigen was precleared by absorption with another Mab called Hermes-1, which itself defines a 85-95kD human lymphocyte surface glycoprotein involved in binding to peripheral lymph node HEV (Jalkanen et al., 1986). Such findings have led the authors to suggest that the Mel-14 epitope must be present on human as well as on mouse lymphocyte surfaces. The reproducibility by others of similar

levels of Mel-14 induced inhibition of adhesion using mouse and human PB cells is encouraging for our own results and would suggest that the observations may indeed be genuine.

Likewise this same problem arises for the anti-MAdCAM-1 Mab Meca-367 which like Mel-14 is a rat anti-mouse Mab. We have shown that this Mab inhibits the adherence of SF and PB MCs to gut endothelium. Meca-367 would mediate such inhibition by binding to the MAdCAM-1 vascular addressin on gut endothelium, preventing the adherence of such cells to MAdCAM-1. The use of Meca-367 in the identification of gut endothelium and in lymphocyte adherence inhibition studies is well documented. It has been established that Meca-367 recognises HEV within gut associated lymphoid tissues in the mouse and that this Mab positively stains HEV within the Peyer's patches and mesenteric lymph nodes (Streeter et al., 1988) and also endothelium within the lamina propria of the small intestine (Andrew et al., 1994), while failing to stain HEV within peripheral lymph nodes, such as the axillary, brachial, popliteal and inguinal lymph nodes (Streeter et al., 1988). To date however, no evidence to suggest that Meca-367 can positively stain or bind to *porcine* MAdCAM-1 has been forthcoming. We have shown positive staining with Meca-367 within the porcine gut lamina propria in areas corresponding to MC adherence (Kadioglu & Sheldon 1996, and see photos 4 and 5). Such staining was similar to positive endothelial staining (within the same tissue organ and within similar vicinities) using a Mab directed against porcine Factor VIII, (which acts as an endothelial cell marker in the pig, see photos 6 and 7). However, we also failed to show any positive staining with the rat anti-mouse Mab Meca-325, which has been reported to react with HEVs in mouse peripheral lymph node and Peyer's patch tissue (Duijvestijn et al., 1987) and we have also failed to do so with an mouse

anti-human Mab directed against CD31 (PECAM-1) expressed on endothelial cell surfaces. Such positive endothelial staining with porcine Factor-VIII (despite the failures with Meca-325 and anti-CD31 Mabs) lends support to the Meca-367 results, since taken together with the MC adhesion results, this would suggest that Meca-367 recognises the murine equivalent of MAdCAM-1 in the pig. Although great care was taken in such experiments (such as using several negative controls and unrelated control antibodies) they may still require additional support. Unfortunately the vast majority of research on adhesion molecules is done with mouse, rat or human lymphocytes, generally using mouse tissue, or endothelial cells derived from human tissue. Needless to say published work using porcine tissue in adherence studies is minimal and the availability of Mabs directed against specific porcine adhesion molecules virtually non-existent during the period when these experiments were being performed. Hence any supportive evidence is rather indirect. For example in vitro adherence studies have shown human $\alpha 4\beta 7$ expressing lymphocytes adhere to murine MAdCAM-1 as well as mouse $\alpha 4\beta 7$ expressing lymphocytes (Erle et al., 1994). Hence human lymphocytes can adhere to mouse gut endothelium by recognising murine MAdCAM-1, suggesting that this addressin is conserved to a certain extent in humans and mice. Human MAdCAM-1 has recently been identified and cloned (Shyjan et al., 1996) and it has been shown that human MAdCAM-1 selectively binds both murine and human lymphocyte cell lines expressing $\alpha 4\beta 7$ and that Meca-367 inhibits the adherence of $\alpha 4\beta 7$ expressing mouse lymphocytes to human MAdCAM-1 as well (Shyjan et al., 1996). Although human MAdCAM-1 would seem to have retained sequences important in selective adherence to $\alpha 4\beta 7$ expressing cells, mucin like sequences between mouse and human MAdCAM-1 exhibit substantial divergence. The functional aspects of this difference is not yet known.

The exact role of Meca-367 in binding to porcine MAdCAM-1 and hence inhibiting MC adherence is difficult to assess, since neither porcine MAdCAM-1 has been identified as yet, nor is there much evidence to suggest that cross-species reactivity may occur with this Mab on porcine tissue. But our own adhesion inhibition results along with tissue staining experiments supported by similar endothelial staining with different Mabs would suggest that some sort of reactivity does occur. Direct analysis of porcine MAdCAM-1 with murine and human MAdCAM-1 is awaited.

Recently cross-species reactivity has been shown for a mouse anti-human E-selectin Mab. This Mab has been shown to cross-react with porcine E-selectin in endothelium and porcine leukocyte adherence to human E-selectin was also shown to be inhibited by this antibody. It has also been shown to inhibit porcine leukocyte adhesion to porcine E-selectin, as well as human leukocyte adherence to porcine E-selectin (Tsang et al., 1995). The authors suggest that the immunological similarities between the pig and human adhesion molecule E-selectin in terms of structure and function in particular, plus conserved sequence homologies (e.g. conserved amino acid residues important for adhesion to human E-selectin being conserved in the porcine sequence for E-selectin) are also suggestive of such a cross-reactivity (Graves et al., 1994, Tsang et al., 1995). It is conceivable that such similarities may also exist for other porcine adhesion molecules leading to cross-reactivity with equivalent human adhesion molecules.

We have also shown a pattern of cross-species reactivity, at least in terms of MC adherence to human and porcine gut tissue. The adherence of paired RA SF and PB MCs as well as human control PB MCs to *human* as well as *porcine* gut lamina propria, was compared. The results showed

no appreciable differences in adhesion to either human or porcine tissue, with differences in adhesion between each population being maintained. That is to say while RA PB and control PB MCs adhered to human gut lamina propria in similar levels (maintaining the same levels of adherence as to porcine tissue), RA SF lymphocytes adhered on average nearly 1.8 times as well as RA PB or control PB lymphocytes, mirroring the differences observed when using porcine tissue. The point to make is that differences observed in adhesion to *porcine* GLP endothelial cells was maintained even when *human* gut lamina propria tissue was used. Although these findings do not pretend to propose that porcine and human gut tissue are interchangable, they nevertheless suggest that the same levels of selective MC adhesion as quantified by the in vitro adherence assay using porcine tissue, can also be mirrored when human tissue of comparable organs are used, additionally suggesting that the adhesion molecule(s) required for such selective adherence may be to a certain extent conserved in human gut tissue.

4.3.2 The synovial membrane discrepancy

Rheumatoid arthritis SM MCs exhibited significantly lower levels of adherence to GLP endothelial cells than any other MC population studied, and especially less than RA SF MCs. This inconsistency is intriguing since one would expect SM MCs to exhibit similar adhesion charcteristics as SF MCs, because such cells enter the synovium via endothelium, and subsequently transmigrate into the synovial fluid, presumably using the same set of adhesion molecules. Indeed, similar levels of VLA-4 expression on RA SM and SF lymphocytes has been demonstrated (van Dinther-Janssen et al., 1991) with CD44 expression

being reported on RA SM MCs (Johnson et al., 1993) but to a lesser extent than to its expression on RA SF lymphocytes (Kelleher et al., 1995). In order to rule out the possibility of experimental procedure affecting the adherence levels of SM MCs by possibly disrupting cell surface adhesion molecules, two different extraction methods were used for SM MCs (porcine PP MCs were also extracted by the same methods from PP tissue to see if the results were comparable). SM MCs were extracted from SM tissue via an enzymic extraction method (see section 2.5.2) and a alternative mechanical extraction method (see section 2.5.3). It would seem unlikely that either method used would lead to the low levels of SM MC adherence, since MCs obtained from both extraction methods showed no appreciable differences in adherence to gut endothelium (in fact when tested for PP MCs, enzymic extraction method obtained MCs showed on average a 5% increase in adhesion, which was not statistically significant). Therefore the reason for low SM lymphocyte adherence, may lie in the differing levels of adhesion molecule expression rather than experimental procedure. If there are significant differences in the expression of adhesion molecules on the surface of SM MCs as compared to SF MCs this may be reflected in their capacity to adhere to gut endothelial cells within the adherence assay used. Such differences in the adhesion of SM MCs may have to do with the functional state of the MC when extracted from tissue.

The adhesion molecules which function in adherence to endothelium and subsequent transmigration are generally different. The transmigration phase of lymphocyte adherence is not so well understood although adhesion molecules pairings such as LFA-1/ICAM-1, VLA-4/fibronectin, or CD44/fibronectin are thought to be involved (Hogg 1993, Hogg & Berlin 1995). Of these, the VLA-4 or CD44 /fibronectin

pairings would seem more likely to be involved in transmigration across the synovial membrane due to the higher levels of VLA-4 and CD44 expressing SM lymphocytes and the abundance of fibronectin within the synovial compartment (Rodriguez et al., 1992). Were this the case, it could be suggested that tissue extracted SM lymphocytes may express different adhesion molecules on their cell surfaces due to their migratory state within the tissue, than SF lymphocytes. We have demonstrated the possible role of $\alpha 4\beta 7$ mediated adherence of SF MCs to gut endothelial cells by blocking its counter-receptor MAdCAM-1 via Meca-367. Since the $\alpha 4\beta 7$/MAdCAM-1 pair have not yet been demonstrated in the transmigration stage, this would suggest that SM MCs may indeed be using other adhesion molecules to cross the endothelial basal membrane. Subsequently the differences of adhesion observed in our assay could depend upon the migratory state of the SM MC at the time of tissue extraction. In other words the SM MCs extracted may be expressing the adhesion molecule(s) required for transmigration, rather than strong adhesion (see section 1.7.5 for the different stages of adhesion). Since the in vitro adherence assay is not capable of reflecting all stages of adhesion, and since the assay would ideally mediate the adherence of MCs in the secondary, "strong adhesion" stage of adherence (due to the transient nature of expression of adhesion molecules involved in the first and third stages of adherence, which are best assayed in conditions of flow in vivo), it is possible that SM MCs extracted from SM express adhesion molecules not detected by the in vitro adherence assay.

The question regarding the low adherence of SM MCs to gut endothelium remains enigmatic. Further work needs to be done to solve this discrepancy and the most informative way forward would be to

identify the exact adhesion molecules involved in adhesion to gut endothelium in SM and SF MCs.

4.3.3 Adhesion in other inflammatory arthritic diseases

The adherence of SF and PB MCs to GLP endothelial cells from patients with other arthritic conditions such as reactive arthritis, Reiter's syndrome, osteoarthritis and Paget's disease was studied in order to compare the findings with those obtained in RA. The results in reactive arthritis (ReA) were similar to rheumatoid arthritis. MCs from the synovial fluid of patients with reactive arthritis adhered to gut endothelial cells in significantly greater (P= 0.03) numbers than did their PB MCs. The mean adherence levels for both cell populations were closely similar to those obtained from the equivalent cell populations in RA. SF MCs manifested on average twice the adherence of PB MCs. It is known that certain arthritic diseases such as Reiter's syndrome and ReA are associated with gut infections. Antigenic material from causative organisms such as *Salmonella*, *Yersinia* and *Shigella* species have been identified within inflamed joints (Granfors et al., 1989). More recently *Chlamydia trachomatis* DNA has been shown using the polymerase chain reaction (PCR) to be present within the joints of patients with ReA, suggesting a link between synovial inflammation and abnormalities in the gut (Sieper & Kingsley 1996). Such a link may also be reflected in the MCs that populate the synovial joint. It would appear from the results obtained in our experiments that an appreciable proportion of ReA SF MCs share adhesion characteristics similar to those of gut homing MCs, as do RA SF MCs. This may be indicative of a similar pathogenic process in terms of the onset of disease and the subsequent migration and infiltration

of MCs into the joint. The fact that the opposite was observed in osteoarthritis and Paget's disease would suggest a difference in the adhesion characteristics of these MCs, setting them apart from ReA and RA MCs.

4.3.4 Overview of RA results

The main purpose of studying the adhesion characteristics of RA MCs to GLP endothelial cells and to peripheral LN endothelium was to compare the levels of adherence to those of human control PB MCs and porcine PP, PB and LN MCs. Overall, the picture to emerge was that RA SF MCs exhibited adhesion properties similar to those of normal gut homing PP MCs and that RA PB MCs exhibited adhesion properties similar to those of normal LN homing MCs, but with adherence to gut endothelial cells as well (though to a much smaller extent than SF MCs). Such adhesive properties were also demonstrated with human gut. Adhesion molecules such as MAdCAM-1 (and by inference $\alpha 4\beta 7$) and CD44 have been demonstrated to play a significant part in the adherence of MCs to gut endothelial cells, while L-selectin has not (within the confines of the in vitro adherence assay used and questions regarding cross-species reactivity permitting). The unexpected differences in RA SM and SF MC adherence has also been highlighted, and suggested to be due to differences in adhesion molecule expression rather than experimental procedure. Finally, similarities between the adhesive properties of ReA and RA SF and PB MCs have also been suggested.

Overall, it is felt that whilst falling short of certainty there is a modicum of experimental evidence to suggest the existence of a

connection between SF MCs and gut homing MC populations, in terms of their adhesive properties only. The data presented does attempt speculation as to how this may come about or why and these are questions which need to be addressed within the framework of future studies.

4.4 Discussion of the effects of methylprednisolone treatment on mononuclear cell adherence, in vitro and ex-vivo

Corticosteroids have been used to suppress synovial inflammation in rheumatoid arthritis since the late forties, with superior effectiveness to non-steroidal anti-inflammatory drugs (NSAIDs) and possibly with fewer disadvantages than immunosuppressants (Kirwan 1994). Although a significant amount of work has been published regarding the effects of the most commonly used corticosteroids on the levels of MC and polymorphonuclear cell circulation in peripheral blood, and on circulating cytokine levels with suggested effects of such corticosteroids on DNA-RNA function, there is little on the effects of corticosteroid action on adhesion molecules and the adhesion characteristics of circulating MC populations. It was therefore our intention to see if the glucocorticosteroid methylprednisolone (MP) used in pulse therapy to induce remission in RA patients (see section 1.8.5 for more details) would have an effect on the adherence characteristics of circulating PB MCs obtained from such patients. The effect of MP was first examined on porcine PB MCs in vitro, as an initial experimental stage to see if adhesion was indeed affected. It should be stressed that all the following experiments where the effect of MP on porcine and human MC adhesion was studied, were always performed coded and read double blind to avoid any observer bias.

In vitro effects of methylprednisolone on porcine mononuclear cell adhesion

The effects of methylprednisolone (MP) on the adherence of porcine PB MCs to GLP endothelial cells was studied over a 24 hour period in vitro. Inhibition of adherence was seen to be initially related to the length of MP treatment, with maximal inhibition (34%) achieved after 4 hours of incubation as compared to controls. However after 24 hours MC adherence levels returned to those comparable of control samples adherence levels. It would seem that in vitro at least the duration of MP inhibition was short lived, yet although inhibition was not statistically significant, there was an appreciable difference by 2-4 hours. It has been known for some time that on average a 70% decline in circulating MCs occurs following a single dose (of equivalent concentration to that used in our assay) of glucocorticosteroid in humans (Fauci & Dale 1974). These workers reported that the decrease in circulating MC levels occurs within 4-6 hours of glucocorticoid administration and reverts to normal levels by 24 hours and has been suggested to be due to the redistribution of circulating MCs to other lymphoid compartments, in particular the bone marrow, and not due to cell lysis or death (Fauci et al., 1976). Although the time scale of inhibition of adherence of PB MCs observed in vitro seemed to follow that of the fluctuating levels of circulating MCs over a 24 hour period in vivo, it is apparent that the observed levels of adherence inhibition in vitro are not due to the redistribution of MCs or other such events that could occur in vivo. It would seem more likely that the observations made in vitro were due to differences in adhesion molecule expression following PS therapy (it should be noted that MC populations were tested for cell viability at all time points studied and all were found to be more than 90% viable).

Indeed there is some evidence to suggest that glucocorticoids such as dexamethasone can inhibit the expression of E-selectin (in a dose dependent manner, at nanomolar concentrations) on human umbilical vein endothelial cells, and thereby inhibit the adhesion of polymorphonuclear cells to this adhesion molecule (Cronstein et al., 1992). The authors suggested that such inhibitory action occured at the transcriptional level, with dexamethasone inhibiting the levels of mRNA message required for E-selectin expression and subsequent display on the surface of endothelial cells. Also, it has recently been demonstrated that MP treatment at 30mg/day orally in RA patients, can downregulate the expression of LFA-1, as ascertained by flow cytometric analysis (Pitzalis 1995). So there is some evidence to suggest that corticosteroids can interfere with the expression of adhesion molecules leading to inhibition of binding to endothelium. The next logical step was to investigate the effects of MP treatment in RA patients, in order to see whether or not the same levels of inhibition were also present, and to try to understand the sequence of events that could be taking place in terms of the expression of adhesion molecules.

Effects of methylprednisolone therapy on mononuclear cell adhesion ex-vivo and on circulating subsets in vivo

The adherence of human RA PB MCs to GLP endothelial cells was studied over a 48 hour period. In all cases studied the adherence of PB MCs was inhibited on average by over 58% 24 hours after pulse steroid (PS) therapy with MP as compared to the pre-pulse adherence levels. This reduction in adherence was not evident by 1 hour following PS therapy,

but was maximal by 24 hours. Prior to PS therapy, no significant change in MC adherence levels was observed over a 24 hour run-in period. The adherence levels of MCs (24 and 1 hour prior to PS therapy) along with the adherence levels of MCs 1 hour after PS therapy were significantly different than those obtained 24 hours after PS therapy (P= 0.03 for both and P= 0.001 respectively). These results would suggest, that similar to the in vitro studies previously, MP leads to a significant decrease in PB MC adherence to gut endothelial cells, this time the degree of inhibition being greater and the length of effect possibly longer. What was not possible in vitro, is now possible, hence the levels of inhibition observed by 24 hours could be due to lymphocytopenia and the redistribution of circulating subsets of MCs which would have normally presented equivalent levels of adherence to gut endothelial cells as the pre-pulse samples did. To answer this question and to see if indeed any particular subset exhibited preferential adherence to gut tissue, immunophenotyping of circulating MC subsets 1 hour prior to and 24 hours after PS therapy was carried out in conjunction with the adherence assay experiments described above.

Of the nine subsets studied, only the CD3, CD4 and CD45 RA positive populations in whole blood, showed any significant differences in circulating levels 24 hours after PS therapy (when maximal inhibition of MC adherence occured) when compared to pre-pulse levels. No significant changes were observed in MCs separated from whole blood samples, both when analysed as percentage change or as absolute numbers. In samples taken as whole blood the percentage level of the CD3 positive subset decreased significantly as compared to pre-pulse levels and when absolute numbers were analysed only CD4 and CD45 RA positive cells showed a significant decrease as compared to pre-pulse levels. These

findings are partially in keeping with the findings of others where it has been reported that following intravenous infusions of 200mg dexamethasone over a three day period in patients with active RA, there was a transient decrease in the number of circulating peripheral blood CD3, CD4, and CD8 positive cells with an increase in the number of HLA-DR positive cells (ascertained by flow cytometric analysis) 24 hours after steroid infusion as compared to pre-steroid levels with most other circulating subsets unaltered (van den Brink et al., 1994). We have also shown a similar decrease in the levels of circulating CD3 and CD4 positive cells 24 hours after MP PS therapy as compared to the pre-steroid levels, however we failed to show any significant differences in the CD8 positive or for that matter the HLA-DR positive subsets. Although we also found a significant decrease in the CD45RA positive subset following PS therapy, the remaining subsets were unaltered. This would be in accordance with the work of others, who also found the CD4 positive subset from peripheral blood samples to be decreased following pulse methylprednisolone therapy (given intravenously 1000mg, hence double the dose that our patients were receiving) in RA patients with active disease (Smith et al., 1987). No other subsets were reported to be affected, however the CD3 positive and CD45RA/RO positive subsets were not immunophenotyped. Additionally, when adherent cell subsets were examined in our assay, only the HLA-DR positive subset showed a significant increase following PS therapy as compared to the pre-pulse levels ($P= 0.01$).

As with the adherence results, PS therapy also exhibited a significant effect on the circulation of certain MC subsets 24 hours following treatment. This effect was not however translated to those subsets of MCs which were actually adherent to gut endothelium, since no

significant differences were found in MC subset adherence before or after PS therapy (with the exception of the HLA-DR subset). In other words more or less the same percentage of subsets adhered to GLP endothelial cells regardless of PS therapy. Since these subsets adhered in similar quantities to gut endothelium irrespective of PS therapy and yet the average 58% inhibition of adherence was still observed, this would imply that the relatively small changes observed in certain circulating subsets in PB could not be held responsible for such a large decrease in adherence. Additionally, the levels of adherent MC subsets were similar to those from human control PB MCs. Hence it would appear that PB MC subsets that adhere to GLP endothelial cells from healthy volunteers or from RA patients are not substantially different.

The decreases observed in certain circulating subsets are interesting yet in themselves are not enough to explain the large inhibition of adherence observed. Therefore it seems more likely that differences in the expression of adhesion molecules following PS therapy may account for the high levels of adhesion inhibition observed 24 hours after PS therapy. The circulating levels of the CD44 positive subset were however unaltered following PS therapy. This suggests that CD44 function was not affected by corticosteroid action. It should not be overlooked however that over 40% of PB MCs did manage to adhere to gut endothelial cells 24 hours after PS therapy. Perhaps the reduction in adherence was due to downregulation of a methylprednisolone sensitive gut endothelium specific adhesion molecule, while another adhesion molecule (such as CD44) which may have more of an accessory role in adhesion to gut endothelium, remained insensitive to PS therapy thereby retaining its ability to assist in the adherence of a smaller proprotion of MCs. Since no other adhesion molecules were tested (and Mabs suitable for flow

cytometric analysis of $\alpha 4\beta 7$ were unavailable at the time of study) it is impossible to speculate about the possible fluctuations of such molecules on MC surfaces following PS therapy. What is not known however is whether MP is specific or general in its inhibitory action.

Glucocorticoids such as dexamethasone have been shown to inhibit RNA transcription of the IL-2 gene and increase the degradation of IL-2 mRNA (Boumpas et al., 1991). So it is conceivable that such steroids could effect the expression of adhesion molecules by altering the levels of cytokines present within PB, or indeed act directly upon particular adhesion molecules by inhibiting their nuclear transcription. Such studies of the effects of corticosteroid therapy on adhesion molecule expression is a very recent development and therefore many questions still remain unanswered. What can be said in view of the results obtained however, is that pulse methylprednisolone therapy does lead to a significant reduction in MC adherence, causes minor fluctuations in a small number of circulating subsets, but with no substantial effect on the actual subsets which adhere to GLP endothelial cells, suggesting a possible inhibitory action on the expression of one or more adhesion molecules required for adhesion to gut endothelium. It would seem that methylprednisolone is a potent inhibitor of adhesion related mechanisms, as demonstrated both in vitro and in vivo.

Effect of methylprednisolone therapy on circulating VCAM-1 levels

Increased levels of circulating soluble (shed) adhesion molecules have been reported recently in a number of conditions, including RA

(Blann et al., 1995). Of the small number of patients studied, the overall levels of soluble VCAM-1 in RA PB were found to be higher (by nearly 50%) than previously reported levels of soluble VCAM-1 in PB from normal donors. However when soluble VCAM-1 levels were compared in RA PB samples before and after PS therapy, no significant difference was found, with a decrease of only 3.4% 24 hours after PS therapy. Thus methylprednisolone treatment appeared to have little effect on the circulating levels of soluble VCAM-1 and probably not on cell surface shed VCAM-1 either. Although reports have demonstrated raised levels of soluble VCAM-1 in SF as compared to plasma levels in RA patients (Mason et al., 1993) the importance of these findings with regards to the pathogenesis of RA is not clear, despite the obvious benefit as a good indicator of inflammatory disease. What is especially interesting is how and why such adhesion molecules are shed from cell surfaces. In the case of the presented findings, the significant decrease in MC adherence to gut endothelium would not seem be attributed to any fluctuations in circulating soluble VCAM-1 levels.

4.5 The importance of studying the adhesion characteristics of RA mononuclear cells and possible further avenues of research

By studying the adhesion characteristics of RA lymphocytes to lymph node and gut endothelium, and by taking the migratory patterns of adhesion of porcine lymphocytes as a guiding model, it has been sought to elucidate to some extent the complicated and often overlapping models of lymphocyte trafficking. The effects of various elements on lymphocyte

adhesion have also been studied, with a view to highlight the properties and nature of certain adhesion molecules which may have a role to play in the pathogenesis of RA, and finally the possible control of such molecules by therapeutic agents.

One of the most important questions to be asked in future work should be which adhesion molecule RA SF lymphocytes use when adhering to gut endothelium in vitro. The results obtained here provide valuable clues, however the exact nature of this molecule must be elucidated, in order to be of value in the future treatment of RA. Is the ligand for this adhesion molecule also present within the rheumatoid synovial membrane and is that the reason why a certain population of lymphocytes migrate to RA joints? Is the disease itself the cause for this alteration in lymphocyte migration? And what is the exact mechanism of action of corticosteroids (which are commonly used as therapeutic agents in the treatment of RA) on adhesion molecule expression? It is vital to know whether such agents can specifically alter adhesion molecule function in terms of developing future treatment regimens which are much more specific and less toxic then current ones.

The present work has demonstrated a possible relationship between gut homing lymphocytes and those that migrate to the rheumatoid joint. The probable participation of mucosal adhesion molecules in this process has been highlighted along with questions concerning the interaction of such molecules with their ligands. The interesting role of methylprednisolone in lymphocyte adherence has been brought forward and examined within the context of circulating lymphocytes and adhesion. Finally suggestions have been made concerning the origin of rheumatoid

synovial lymphocytes, with the intention of presenting the important yet often overlooked connection between the gut and rheumatoid arthritis.

CHAPTER 5

References

Abbas AK, Lichtman AH, Pober JS. (1994) *Cellular and Molecular Immunology*. 2 nd. edition, WB Saunders Company.

Ager A. (1994) Lymphocyte recirculation and homing: roles of adhesion molecules and chemoattractants. *Trends in Cell Biology*, 4: 326-333.

Ahmed R, Gray D. (1996) Immunological memory and protective immunity: understanding their relation. *Science*, 272: 54-60.

Airas L, Salmi M, Jalkanen S. (1993) Lymphocyte-vascular cell adhesion protein-2 is a novel 70-kDa molecule involved in lymphocyte adhesion to vascular endothelium. *Journal of Immunology*, 151: 4228-4238.

Albelda SM, Muller WA, Buck CA, Newman JJ. (1991) Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *Journal of Cell Biology*, 114: 1059-1068.

Anderson AO, Anderson ND. (1976) Lymphocyte emigration from high endothelial venules in rat lymph nodes. *Immunology*, 31: 731-748.

Andrew DP, Berlin C, Honda S, Yoshino T, Hamann A, Holzmann B, Kilshaw PJ, Butcher EC. (1994) Distinct but overlapping epitopes are involved in $\alpha 4\beta 7$-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *Journal of Immunology*, 153: 3847-3861.

Arnett FC; Edworthy SM, Bloch DA. (1988) The American Rheumatoid Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis & Rheumatism*, 31: 315-406.

Berg E, Robinson M, Warnock R, Butcher EC. (1991) The human peripheral lymph node vascular addressin is a ligand for LECCAM-1, the peripheral lymph node homing receptor. *Journal of Cell Biology*, 114: 343-349.

Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC. (1993) L-selectin mediated lymphocyte rolling on MAdCAM-1. *Nature*, 366: 695-698.

Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A, Butcher EC. (1993) $\alpha 4\beta 7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell*, 74: 185-195.

Berlin C, Bargarze RF, Campbell JJ, von Andrian UH, Szabo MC, Hassien SR, Nelson RD, Berg EL, Erlandsen SL, Butcher EC. (1995) $\alpha 4$ integrins mediate lymphocyte attachment and rolling under physiological flow. *Cell*, 80: 413-422.

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA. (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. *Proceedings of the National Academy of Sciences, USA*, 84: 9238-9242.

Bevilacqua MP, Spengeling S, Gimbrone MA Jr, Seed B. (1989) Endothelial leukocytes adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*, 243: 1160-1165.

Bevilacqua M, Butcher E, Furie B, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G. (1991) Selectins: a family of adhesion receptors. *Cell*, 67: 233-234.

Blann AD, Herrick A, Jayson MIV. (1995) Altered levels of soluble adhesion molecules in rheumatoid arthritis, vasculitis and systemic sclerosis. *British Journal of Rheumatology*, 34: 814-819.

Boumpas DT, Paliogianni F, Anastassiou ED, Balow JE. (1991) Glucocorticosteroid action on the immune system: molecular and cellular aspects. *Clinical and Experimental Rheumatology*, 9: 413-423.

Boyle JA, Buchanan WW. (1971) In: *Clinical Rheumatology*, Davis FA; editor, Lippincott, 71-72.

Brandtzaeg P. (1996) The human intestinal immune system: basic cellular and humoral mechanisms. *Clinical Rheumatology*, 10: 1-25.

van den Brink HR, van Wijk MJG, Geertzen RGM, Bijlsma JWJ. (1994) Influence of corticosteroid pulse therapy on the serum levels of soluble interleukin 2 receptor, interleukin 6 and interleukin 8 in patients with rheumatoid arthritis. *Journal of Rheumatology*, 21: 430-434.

British National Formulary. (1995) British Medical Association & Royal Pharmaceutical Society of Great Britain, 29: 286-291.

Burnstein KL, Cidlowski JA. (1989) Regulation of gene expression by glucocorticoids. *Annual Review of Physiology*, 51: 683-699.

Butcher EC, Scollay RG, Weissman IL. (1979) Lymphocyte adherence to high endothelial venules: characterization of a modified in vitro assay, and examination of the binding of syngeneic and alogeneic lymphocyte populations. *Journal of Immunology*, 123: 1996-2003.

Butcher EC, Scollay RG, Weissman IL. (1980) Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *European Journal of Immunology*, 10: 556-561.

Butcher EC. (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell*, 67: 1033-1036.

Butcher EC, Picker LJ. (1996) Lymphocyte homing and homeostasis. *Science*, 272: 60-66.

Cahill RNP, Poskitt DC, Frost H, Trnka Z. (1977) Two distinct pools of recirculating T lymphocytes: migratory characteristics of nodal and intestinal T lymphocytes. *Journal of Experimental Medicine*, 145: 420-428.

Carlos T, Kovach N, Schwartz B, Rosa M, Newman B, Wayner E, Benjamin C, Osborn L, Lobb R, Harlan J. (1991) Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular adhesion molecule-1. *Blood*, 77: 2266-2271.

Cepek KL, Shaw SK, Parker CM, Russel GJ, Morrow JS, Rimm DL, Brenner MB (1994) Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. *Nature*, 372: 190-193.

Cerf-Bensussan N, Jarry A, Brousse N, Lisowska-Grospierre B, Guy-Grand D, Griscelli C. (1987) A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. *European Journal of Immunology*, 17: 1279-1285.

Chao C-C, Sandor M, Dailey MO. (1994) Expression and regulation of adhesion molecules by γδ T cells from lymphoid tissues and intestinal epithelium. *European Journal of Immunology*, 24: 3180-3187.

Chin YH, Rasmussen RA, Woodruff JJ, Easton TG. (1986) A monoclonal anti-HEBF PP antibody with specificity for lymphocyte surface molecules mediating adhesion to Peyer's patch high endothelium of the rat. *Journal of Immunology*, 136: 2556-2561.

Chiu HH, Crowe DT, Renz ME, Presta LG, Jones S, Weissman IL, Fong S. (1995) Similar but nonidentical amino acid residues on vascular cell adhesion molecule-1 are involved in the interaction with α4β1 and

α4β7 under different activity states. *Journal of Immunology*, 155: 5257-5267.

Conn DL, McOuffie FC, Dyck PJ. (1972) Immunopathologic study of sural nerves in RA. *Arthritis & Rheumatism*, 15: 135-137.

Cronstein BN, Kimmel SC, Levin RI, Martiniuk F. (1992) A mechanism for the anti-inflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. *Proceedings of the National Academy of Sciences, USA*. 89: 9991-9995.

Cronstein BN, Weissmann G. (1993) The adhesion molecules of inflammation. *Arthritis and Rheumatism*, 36: 147-157.

van Dinther-Janssen ACHM, Horst E, Koopman G, Newmann W, Scheper RJ, Meijer CJLM, Pals ST. (1991) The VLA-4/ VCAM-1 pathway is involved in lymphocyte adhesion to endothelium in rheumatoid synovium. *Journal of Immunology*, 147: 4207-4210.

de Fourgerolles AR, Springer TA. (1992) Intercellular adhesion molecule-3, a third adhesion counter receptor for lymphocyte function-associated molecule-1 resting lymphocytes. *Journal of Experimental Medicine*, 175: 185-190.

de Fourgerolles AR, Quin X, springer TA. (1994) Characterisation of the function of intercellular adhesion molecules (ICAM)-3 and

comparison with ICAM-1 and ICAM-2 in immune responses. *Journal of Experimental Medicine*, 179: 619-629.

Drayson MT, Ford WL. (1984) Afferent lymph and lymph borne cells: their influence on lymph node function. *Immunobiology*, 168: 362-379.

Drickamer K. (1989) *Ciba Foundation Symposium*, 145: 45-61.

Dunon D, Ruiz P, Imhof BA. (1993) Pro-T cell homing to the thymus. *Current Topics in Microbiology and Immunology*, 184: 139-150.

Ebringer A, Baines M, Ptaszynska T. (1985) Spondyloarthritis, uveitis, HLA-B27 and klebsiella. *Immunological Reviews*, 86: 101-116

Ebringer A, Cox NL, Abduljadayel I, Ghuloom M, Khalafpour S, Ptasztnska T, Shodjaimoradi F, Wilson C. (1988) Klebsiella antibodies in ankylosing spondylitis and proteus antibodies in rheumatoid arthritis. *British Journal of Rheumatology*, 27: 72-85.

Ebringer A, Cunningham P, Ahmadi K, Wrigglesworth J, Hosseini R, Wilson C. (1992) Sequence similarity between HLA-DR1 and DR4 subtype associated with RA and proteus / serratia membrane haemolysins. *Annals of Rheumatic Diseases*, 51: 1245-1246.

Elkon KB, Delacroix DL, Gharavi AE, Vaerman JP, Hughes GRV. (1982) Immunoglobulin A and polymeric IgA rheumatoid factors in systemic sicca syndrome: partial characterisation. *Journal of Immunology*, 129: 576-581.

Erlandsen SL, Hassien SR, Nelson RD. (1993) Detection and spatial distribution of the β2 integrin (Mac-1) and L-selectin (LECAM-1) adherence receptors on human neutrophils by high resolution field emission SEM. *Journal of Histochemical Cytochemistry*, 41: 327-333.

Erle DJ, Briskin MJ, Butcher EC, Garcia-Pardo A, Lazarovits AI, Tidswell M. (1994) Expression and function of the MAdCAM-1 receptor, integrin α4β7, on human leukocytes. *Journal of Immunology*, 153: 517-528.

Fairburn K, Kunaver M, Wilkinson LS, Cambridge G, Haskard D, Edwards JCW. (1993) Intercellular adhesion molecules in normal synovium. *British Journal of Rheumatology*, 32: 302-306.

Fan PT, Yu DTY, Clements PJ, Fowlston S, Eisman J, Bluestone R. (1978) Effect of corticosteroids on the human immune response: comparison of one and three daily 1 g intravenous pulses of methylprednisolone. *Journal of Laboratory Clinical Medicine*, 91: 625-634.

Fauci AS, Dale DC. (1974) The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. *Journal of Clinical Investigations*, 53: 240-246.

Fauci AS, Dale DC, Balow JE. (1976) Glucocorticosteroid therapy: mechanism of action and clinical considerations. *Annuals of Internal Medicine*, 84: 304-315.

Fearon DT, Locksley RM. (1996) The instructive role of innate immunity in the acquired immune response. *Science*, 272: 50-54.

Feldmann M, Brennan FM, Maini RN. (1996) Rheumatoid arthritis. *Cell*, 85: 307-310.

Firestein GS. (1994) Rheumatoid synovitis and pannus. In: *Rheumatology*, Klippel JH, Dieppe PA; editors, Mosby, 12: 12.1-12.30.

Fitzgerald O, Bresnihan B. (1995) Synovial membrane cellularity and vascularity. *Annals of Rheumatic Diseases*, 54: 511-515.

Folkman J, Klagsbrun M. (1987) Angiogenic factors. *Science*, 235: 44-47.

Ford WL. (1975) Lymphocyte migration. *Progress in Allergy*, 19: 1

Freemont AJ, Jones CJP. (1983) Light microscopic, histochemical and ultrastructural studies of human lymph node paracortical venules. *Journal of Anatomy*, 136: 349-362.

Freemont AJ. (1988) Functional and biosynthetic changes in endothelial cells of vessels in chronically inflamed tissues: evidence for endothelial control of lymphocyte entry into diseased tissues. *Journal of Pathology*, 155: 225-230.

Fries JW, Williams AJ, Atkins RC, Newman W, Lipscomb MF, Collins T. (1993) Expression of VCAM-1 and E-selectin in an in vivo

model of endothelial activation. *American Journal of Pathology*, 143: 725-737.

Gallatin WM, Weissman IL, Butcher EC. (1983) A cell surface molecule involved in organ-specific homing of lymphocytes. *Nature*, 304: 30-34.

Gaston JSH. (1995) The involvement of the gut in the pathogenesis of inflammatory synovitis. *British Journal of Rheumatology*, 34: 801-804.

Geoffroy JS, Rosen SD. (1989) Demonstration that a lectin-like receptor ($gp90^{mel}$) directly mediates adhesion of lymphocytes to high endothelial venules of lymph nodes. *Journal of Cell Biology*, 109: 2463-2469.

Girard J-P, Springer TA. (1995) High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunology Today*, 16: 449-457.

Goebeler M, Meinardus-Hager G, Roth J, Goerdt S, Sorg C. (1993) Nickel chloride and cobalt chloride, two common contact sentisizers, directly induce expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) by endothelial cells. *Journal of Investigative Dermatology*, 100: 759-765.

Goldstein LA, Zhou DFH, Picker LJ, Minty CN, Bargatze RF, Ding JF, Butcher EC. (1989) A human lymphocyte homing receptor, the

hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell*, 56: 1063-1072.

Gordon DA, Hastings DE. (1994) Rheumatoid arthritis: Clinical features: early, progressive and late disease. In: *Rheumatology*, Klippel JH, Dieppe PA: editors, Mosby.

Gowans JL. (1957) The effects of continous reinfusion of lymph and lymphocytes from the thoracic duct of unanaesthetized rats. *British Journal of Experimental Pathology*, 38: 67-78.

Gowans JL. (1959) The recirculation of lymphocytes from blood to lymph in the rat. *Journal of Physiology*, 146: 54-69.

Gowans JL, Knight EJ. (1964) The route of re-circulation of lymphocytes in the rat. *Proceedings of the Royal Society London*, 159: 257-282.

Gransfors K, Jalkanen S, von Essen R, Lahesmaa-Rantala R, Isomaki O, Pekkola-Heino K, Merilahti-Palo R, Saario R, Isomaki H, Toivanen A. (1989) Yersinia antigens in synovial fluid cells from patients with reactive arthritis. *New England Journal of Medicine*, 320: 216-221.

Graves BJ, Crowther RL, Chandran C, Rumberger JM, Li S, Huang KS, Presky DH, Familletti PC, Wolitzky BA, Burns DK. (1994) Insight into E-selectin / ligand interaction from the crystal structure and mutagenesis of the lec / EGF domains. *Nature*, 367: 532-539.

Gray D. (1992) The dynamics of immunological memory. *Seminars in Immunology*, 4: 29-34.

Greenwood J, Calder VL. (1993) Lymphocyte migration through cultured endothelial cell monolayers derived from the blood-retinal barrier. *Immunology*, 80: 401-406.

Griebel PJ, Ferrari G. (1995) *International Immunology*, 7: 369-379.

Griebel PJ, Hein WR. (1996) Expanding the role of Peyer's patches in B-cell ontogeny. *Immunology Today*, 17: 30-39.

Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder TF. (1990) GM-CSF and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes and their precursors. *Journal of Immunology*, 145: 576-584.

Griscelli C, Vassalli P, McCluskey RT. (1969) The distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection. *Journal of Experimental Medicine*, 130: 1427-1442.

Grober JS, Fox DA, Bowen B, Stoolman LM. (1991) LECCAM (selectin) mediated monocyte-endothelial adhesion in chronic rheumatoid arthritis. *Arthritis & Rheumatism*, supplement 9, 34: S59.

Gunthert U. (1993) CD44: a multitude of isoforms with diverse functions. *Current Topics in Microbiology and Immunology*, 184: 47-63.

Guy-Grand D, Griscelli C, Vassalli P. (1974) The gut-associated lymphoid system: nature and properties of the large dividing cells. *European Journal of Immunology*, 4: 435-443.

Halberg P. (1994) Rheumatoid arthritis: history. In: *Rheumatology*, Klippel JH, Dieppe PA; editors, Mosby, 3: 2.1-2.4

Hamann A, Jablonski-Westrich D, Duijvestijn A, Butcher EC, Baisch H, Harder R, Thiele H-G. (1988) Evidence for an accessory role of LFA-1 in lymphocyte-high endothelium interaction during homing. *Journal of Immunology*, 140: 693-699.

Hamann A, Jablonski-Westrich D, Jonas P, Thiele H-G. (1991) Homing receptors reexamined: mouse LECAM-1 (MEL-14 antigen) is involved in lymphocyte migration into gut-associated lymphoid tissue. *European Journal of Immunology*, 21: 2925-2929.

Hamann A, Rebstock S. (1993) Migration of activated lymphocytes. Adhesion in leukocyte homing and differentiation. *Current Topics in Microbiology and Immunology*, 184: 109-124.

Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. (1994) Role of α4-integrins in lymphocyte homing to mucosal tissues in vivo. *Journal of Immunology*, 152: 3282-3293.

Harris ED. (1990) Rheumatoid arthritis: pathophysiology and implications for therapy. *New England Journal of Medicine*, 322: 1277-1289.

Haynes BF, Telen MJ, Hale LP, Denning SM. (1989) CD44- a molecule involved in leukocyte adherence and T-cell activation. *Immunology Today*, 10: 423-428.

Haynes BF, Liao H-X, Patton KL. (1991) The transmembrane hyaluronate receptor (CD44): multiple functions, multiple forms. *Cancer Cells*, 3: 347-350.

Hench PS, Kendall EC, Slocumb CH, Polley HF. (1950) Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever, and certain other conditions: study in clinical physiology. *Archives in Internal Medicine*, 85: 545-666.

Hendricks HR, Eestermans IL. (1983) Disappearance and reappearance of high endothelial venules and immigrating lymphocytes in lymph nodes deprived of afferent lymphatic vessels: a possible regulatory role of macrophages in lymphocyte migration. *European Journal of Immunology*, 13: 663-669.

Hogg N. (1993) A model of leukocyte adhesion to vascular endothelium. *Current Topics in Microbiology and Immunology*, 184: 79-86.

Hogg N, Berlin C. (1995) Structure and function of adhesion receptors in leukocyte trafficking. *Immunology Today*, 16: 327-330.

Holzmann B, McIntyre BW, Weissman IL. (1989) Identification of a murine Peyer's patch specific lymphocyte homing receptor as an

integrin molecule with an alpha chain homologous to human VLA-4 alpha. *Cell*, 56: 37-46.

Howard JC, Hunt SV, Gowans JL. (1972) Identification of marrow-derived and thymus derived small lymphocytes in the lymphoid tissue and thoracic duct lymph of normal rats. *Journal of Experimental Medicine*, 135: 200-219.

Hynes RO. (1992) Integrins: versatility, modulation, and signalling in cell adhesion. *Cell*, 69: 11-25.

Imhof BA, Dunon D. (1995) Leukocyte migration and adhesion. *Advances in Immunology*, 58: 345-416.

Isaacs JD, Watts RA, Hazleman BL. (1992) Humanised monoclonal antibody therapy for rheumatoid arthritis. *Lancet*, 340: 748-752.

Isaacs JD, Manna VK, Rapson N, Bulpitt KJ, Hazleman BL, Matteson EL, St.Clair EW, Schnitzer TJ, Johnston JM. (1996) CAMPATH-1H in rheumatoid arthritis-an intravenous dose ranging study. *British Journal of Rheumatology*, 35: 231-240.

Isdale A, Wright V. (1989) Seronegative arthritis and the bowel. In: *Bailliere's Clinical Rheumatology*. The gut and rheumatoid disease. Rooney PJ; editor, Saunders, 285-301.

Issekutz TB. (1993) The contributions of integrins to leukocyte infiltration in inflamed tissues. *Current Topics in Microbiology and Immunology*, 184: 177-185.

Issekutz TB, Issekutz AC. (1991) T lymphocyte migration to arthritic joints and dermal inflammation in the rat: differing migration patterns and the involvement of VLA-4. *Clinical Immunology & Immunopathology*, 61: 436-447.

Jalkanen ST, Bargatze RF, Herron LR, Butcher EC. (1986) A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *European Journal of Immunology*, 16: 1195-1202.

Jalkanen S, Bargatze RF, del los Toyos J, Butcher EC. (1987) Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95 kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal or synovial endothelial cells. *Journal of Cell Biology*, 105: 983-990.

Janeway CA, Travers P. (1996) Immunobiology, the immune system in health and disease. Second edition, Current Biology Ltd.

Jeurissen SHM, Duijvestijn AM, Sontag Y, Kraal G. (1987) Lymphocyte migration into the lamina propria of the gut is mediated by specialized HEV-like blood vessels. *Immunology*, 62: 273-277.

Johnson BA, Haines GK, Harlow LA, Koch AE. (1993) Adhesion molecule expression in human synovial tissue. *Arthritis & Rheumatism*, 36: 137-146.

Johnson GI, Cook RG, McEver RP. (1989) Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. *Cell*, 56: 1033-1044.

Kadioglu A, Sheldon P. (1996) Adhesion of rheumatoid lymphocytes to mucosal endothelium: the gut revisited. *British Journal of Rheumatology*, 35: 218-225.

Kagnoff MF. (1981) Immunology of the digestive system. *Physiology of the Gastrointestinal Tract*, 1337-1352, Raven Press.

Kelleher D, Murphy A, Hall N, Omary MB, Kearns G, Long A, Casey EB. (1995) Expression of CD44 on rheumatoid synovial fluid lymphocytes. *Annals of Rheumatic Diseases*, 54: 566-570.

Kilger G, Needham LA, Nielsen P, Clements J, Vestweber D, Holzmann B. (1995) Differential regulation of α4 integrin-dependent binding to domains 1 and 4 of vascular cell adhesion molecule-1. *Journal of Biological Chemistry*, 270: 5979-5984.

Kirwan JR. (1994) Systemic corticosteroids in rheumatology. In: *Rheumatology*, Klippel JH, Dieppe PA; editors, Mosby, 8: 11.1-11.6.

Koopman WJ, Miller RK, Crago SS, Mestecky J, Schrohenloher RE. (1983) IgA rheumatoid factor: evidence for independent expression at local sites of tissue inflammation. *Annals of the New York Academy of Sciences*, 409: 258-272.

Kraal G, Schomagel K, Streeter PR, Holzmann B, Butcher EC. (1995) Expression of the mucosal vascular addressin, MAdCAM-1, on sinus-lining cells in the spleen. *American Journal of Pathology*, 147: 763-771.

Kurosaka M, Ziff M. (1983). Immunoelectron microscopic study of the distribution of T cells subsets in rheumatoid synovium. *Journal of Experimental Medicine*, 158: 1191-1210.

Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B. (1990) PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell*, 63: 467-474.

Lasky LA, Singer MS, Yednock TA, Dowbenko D, Fennie C, Rodriguez H, Nguyen T, Stachel S, Rosen SD. (1989) Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell*, 56: 1045-1055.

Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD. (1992) An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell*, 69: 927-938.

Lawrence MB, Springer TA. (1991) Leukocytes role on a selectin at physiological flow rates: distinction from and prerequiste for adhesion through integrins. *Cell*, 65: 859-873.

Lawrence RC. (1994) Rheumatoid arthritis: classification and epidemiology. In: *Rheumatology*, Klippel JH, Dieppe PA; editors, Mosby, 3: 3.3-3.4.

Lazarovits AI, Karsh J. (1993) Differential expression in rheumatoid synovium and synovial fluid of $\alpha 4\beta 7$ integrin. *Journal of Immunology*, 151: 6482-6489.

MacDermott RP, Nash GS, Bertovich MJ, Mohrman RF, Kodner IJ, Delacroix DL, Vaerman J-P. (1986) Altered patterns of secretion on monomeric IgA and IgA subclass-1 by intestinal mononuclear cells in inflammatory bowel disease. *Gastroenterology*, 91: 379-385.

MacDonald TT, Horton MA, Choy MY, Richman PI. (1990) Increased expression of laminin / collagen receptor (VLA-1) on epithelium of inflamed human intestine. *Journal of Clinical Pathology*, 43: 313-315.

Mackay CR, Marston WL, Dudler L. (1990) Naive and memory T cells show distinct pathways of lymphocyte recirculation. *Journal of Experimental Medicine*, 171: 801-817.

Mackay CR, Imhof BA. (1993) Cell adhesion in the immune system. *Immunology Today*, 14: 99-102.

Maini RN, Eliott MJ, Brennan Fm, Williams RO, Chu CQ, Paleolog E, Charles PJ, Taylor PC, Feldmann M. (1995) Monoclonal anti-TNFα antibody as a probe of pathogenesis and therapy of rheumatoid disease. *Immunological Reviews*, 144: 195-223.

March RE, Kirwan JR, Davies PG, Winrow VR, Holborrow EJ. (1987) Sensitive ELISA measurement of IgA, IgG and IgM antiglobulins in rheumatic and other diseases. *Journal of Rheumatology*, 16: 445-449.

Marchesi VT, Gowans JL. (1964) The migration of lymphocytes through the endothelium of venules in lymph nodes. *Proceedings of the Royal Society London*, 159: 283-290.

Mason JC, Kapahi P, Haskard DO. (1993) Detection of increased levels of circulating intercellular adhesion molecule 1 in some patients with rheumatoid arthritis but not in patients with systemic lupus erythematosus. *Arthritis & Rheumatism*, 36: 519-526.

Masuyama J, Berman JS, Cruikshank WW, Morimoto C, Center DM. (1992) Evidence for recent as well as long term activation of T cells migrating through endothelial cell monolayers in vitro. *Journal of Immunology*, 148: 1367-1374.

Matteson EL, Cohen MD, Conn DL. (1994) Rheumatoid arthritis: Clinical features-sytemic involvement. In: *Rheumatology*, Klippel JH, Dieppe PA: editors, Mosby.

Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. (1993) Leukocyte rolling and extravasation are severly compromised in P-selectin deficient mice. *Cell*, 74: 541-554.

McDermott MR, Bienenstock J. (1979) Evidence for a common mucosal immunologic system. *Journal of Immunology*, 122: 1892-1892-1898.

McEver RP. (1991) GMP-140: a receptor for neutrophils and monocytes on activated platelets and endothelium. *Journal of Cellular Biochemistry*, 45: 156-161.

Mebius RE, Dowbenko D, Williams A, Fennie C, Lasky LA, Watson SR. (1993) Expression of GlyCAM-1, an endothelial ligand for L-selectin, is affected by afferent lymphatic flow. *Journal of Immunology*, 151: 6769-6776.

Mertens AV, de Clerck LS, Moens MM, Bridts CH, Stevens WJ. (1994) Lymphocyte activation status, expression of adhesion molecules and adhesion to human endothelium in rheumatoid arthritis-relationship to disease activity. *Res. Immunology*, 145: 101-108.

Moore KL, Stults NL, Diaz S, Smith DF, Cummings RC, Varki A, McEver RP. (1992) Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *Journal of Cell Biology*, 118: 445-456.

Neutra MR, Pringault E, Kraehenbuhl J-P. (1996) Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology*, 14: 275-300.

O'Brien WM, Bagby GF. (1985) Rare adverse reactions to non-steroidal anti-inflammatory drugs. *Journal of Rheumatology*, 12: 13-20, 347-353, 562-567, 785-790.

Ohmori K, Takada A, Yoneda T, Buma Y, Hirashima K, Tsynoka K, Hasegawa A, Kannagi R. (1993) Differentiation-dependent expression of sialyl stage-specific embryonic antigen-1 and I-antigens on human lymphoid cells and its implications for carbohydrate mediated adhesion to vascular endothelium. *Blood*, 81: 101-111.

Ollier WER, MacGregor A. (1995) Genetic epidemiology of rheumatoid disease. *British Medical Bulletin*, 51: 267-285.

O'Neill JK, Butter C, Baker D, Gschmeissner SE, Kraal G, Butcher EC, Turk JL. (1991) Expression of vascular addressins and ICAM-1 by endothelial cells in the spinal cord during chronic relapsing experimental allergic encephalomyelitis in the biozzi AB/H mouse. *Immunology*, 75: 520-525.

Oppenheimer-Marks N, Davis LS, Bogue DT, Ramberg J, Lipsky PE (1991) Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. *Journal of Immunology*, 147: 2913-2921.

O'Sullivan NL, Montgomery PC. (1990) Characterisation of the lymphocyte-glandular mucosal tissue interaction using an in vitro adherence assay. *Advances in Mucosal Immunology, "Proceedings from the Fifth International Conference on Mucosal Immunology"*, 222-224.

Palecanda A, Walcheck B, Bishop DK, Jutila MA. (1992) Rapid activation-independent shedding of leukocyte L-selectin induced by cross-

linking of the surface antigen. *European Journal of Immunology*, 22: 1279-1286.

Panayi GS, Lanchbury JS, Kingsley GH. (1992) The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis & Rheumatism*, 35: 729-734.

Paulus HE, Machleder HI, Levine S, Yu DTY, MacDonald NS. (1977) Lymphocyte involvement in rheumatoid arthritis. Studies during thoracic duct drainage. *Arthritis & Rheumatism*, 20:1249-1262.

Paulus HE, Machleder HI, Clements PJ. (1979) Prolonged thoracic duct drainage in rheumatoid arthritis and systemic lupus erythematosus. *Western Journal of Medicine*, 130: 309-324.

Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A. (1993) Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *Journal of Cell Biology*, 122: 257-264.

Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC. (1991) The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell*, 66: 921-933.

Pigott R, Power C. (1993) The adhesion molecule facts book. *Academic Press*, Harcourt Brace & company; publishers.

Pitzalis C. (1995) Regulation of cellular adhesion in rheumatoid arthritis by corticosteroids. *Annals of Rheumatic Diseases*, Proceedings

from Third International Symposium on the Immunology of the Rheumatic Diseases, 54: 757-778.

Reynolds JD. (1988) *Migration and homing of lymphoid cells*, 113-136.

Rice GE, Munro JM, Bevilacqua MP. (1990) Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18 independent adhesion mechanism. *Journal of Experimental Medicine*, 171: 1369-1374.

Rodriguez RM, Pitzalis C, Kingsley GH, Henderson E, Humphries MJ. T lymphocyte adhesion to fibronectin (FN): a possible mechanism for T cell accumulation in the rheumatoid joint. *Clinical Experimental Immunology*, 89: 439-445.

Rothkotter HJ, Pabst R. (1989) Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs. *Immunology*, 67: 103-108.

Roux ME, McWilliams M, Phillips-Quagliata JM, Weisz-Carrington P, Lamm ME. (1977) *Journal of Experimental Medicine*, 146: 1311-1317.

Sako D, Chang X-J, Barone KM, Vachino G, White HM, Shaw G, Veldman GM, Bean KM, Ahern TJ, Furie B, Cumming DA, Larsen GR. (1993) Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell*, 75: 1179-1186.

Salmi M, Granfors K, Leirisalo-Repo M, Hamalainen M, MacDermott R, Leino R, Havia T, Jalkanen S. (1992) Selective endothelial binding of Interleukin-2 dependent human T cell lines derived from different tissues. *Proceedings of the National Academy of Sciences*, 89: 11436-11440.

Salmi M, Kalimo K, Jalkanen S. (1993) Induction and function of vascular adhesion protein-1 at sites of inflammation. *Journal of Experimental Medicine*, 178: 2255-2260.

Salmi M, Andrew DP, Butcher EC, Jalkanen S. (1995) Dual binding capacity of mucosal immunoblasts to mucosal and synovial endothelium in humans: dissection of the molecular mechanisms. *Journal of Experimental Medicine*, 181: 137-149.

Salmi M, Jalkanen S. (1995) Different forms of human vascular adhesion protein-1 (VAP-1) in blood vessels in vivo and in cultured endothelial cells: implications for lymphocyte-endothelial cell adhesion models. *European Journal of Immunology*, 25: 2803-2812.

Sampaio SO, Li X, Takeuchi M, Mei C, Franke U, Butcher EC. (1995) Organization, regulatory sequences, and alternatively spliced transcripts of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) gene. *Journal of Immunology*, 155: 2477-2486.

Schoefl GI. (1972) The migration of lymphocytes across the vascular endothelium in lymphoid tissue. *The Journal of Experimental Medicine*, 136: 568-585.

Scollay R, Hopkins J, Hall J. (1976) Possible role of surface Ig in non-random recirculation of small lymphocytes. *Nature*, 260: 528-529.

Schrieber L. (1994) The endothelium in rheumatoid arthritis. In: *Rheumatology*, Klippel JH, Dieppe PA; editors, Mosby, 10: 10.1-10.6.

Scott DGI, Bacon PA, Tribe CR. (1981) Rheumatoid vasculitis: a clinical and laboratory study of 50 cases. *Medicine*, 60: 288-293.

Segal AW, Isenberg DA, Hajirousou V, Tolfree S, Clark J, Snaith ML. (1986) Preliminary evidence for gut involvement in the pathogenesis of rheumatoid arthritis? *British Journal of Rheumatology*, 25: 162-166.

Sewell KL, Trentham DE. (1993) Pathogenesis of rheumatoid arthritis. *The Lancet*, 341: 283-290.

Sheldon P. (1988) Rheumatoid arthritis and gut related lymphocytes: the iteropathy concept. *Annals of Rheumatic Diseases*, 47: 697-700.

Shimizu Y, Shaw S, Graber N, Gopal TV, Horgan KJ, Van Seventer GA, Newmann W. (1991) Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. *Nature*, 349: 799-802.

Shizimu Y, Newman W, Tanaka Y, Shaw S. (1992) Lymphocyte interactions with endothelial cells. *Immunology Today*, 13: 106-112.

Shimizu Y, Mobley JL. (1993) Distinct divalent cation requirements for integrin-mediated CD4 T lymphocyte adhesion to ICAM-1, fibronectin, VCAM-1, and invasin. *Journal of Immunology*, 151: 4106-4115.

Short CL. (1974) The antiquity of rheumatoid arthritis. *Arthritis & Rheumatism*, 17: 193-205.

Shyjan AM, Bertagnolli M, Kenney CJ, Briskin MJ. (1996) MAdCAM-1 demonstrates structural and functional similarities to the a4b7-integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin like sequences. *Journal of Immunology*, 156: 2851-2857.

Sieper J, Kingsley G. (1996) Recent advances in the pathogenesis of reactive arthritis. *Immunology Today*, 17: 160-163.

Sikorski EE, Hallman R, Berg EL, Butcher EC. (1993) The Peyer's patch high endothelial receptor for lymphocytes, the mucosal vascular addressin, is induced on a murine endothelial cell line by tumour necrosis factor-α and IL-1. *Journal of Immunology*, 151: 5239-5250.

Smith CH, Barker JN, Morris RW, MacDonald DM, Lee TH. (1993) Neuropeptides induce rapid expression of endothelial cell adhesion molecules and elicit granulocytic infiltration in human skin. *Journal of Immunology*, 151: 3274-3782.

Smith CW. (1993) Transendothelial migration. *Current Topics in Microbiology and Immunology*, 184: 201-212.

Smith MD, Ahern MJ, Brooks PM, Roberts-Johnson PJ. (1988) The clinical and immunological effects of pulse methylprednisolone therapy in rheumatoid arthritis. II. Effects on immune and inflammatory indices in peripheral blood. *Journal of Rheumatology*, 15: 233-237.

Smith MD, Ahern MJ, Brooks PM, Roberts-Johnson PJ. (1988) The clinical and immunological effects of pulse methylprednisolone therapy in rheumatoid arthritis. III. Effects on immune and inflammatory indices in synovial fluid. *Journal of Rheumatology*, 15: 238-241.

Smith MD, Ahern MJ, Roberts-Thomson PJ. (1990) Pulse methylprednisolone therapy in rheumatoid arthritis: unproven therapy, unjustified therapy, or effective adjunctive treatment? *Annals of Rheumatic Diseases*, 49: 265-267.

Snorrason E. (1952) Landre-Beauvais and his goutte asthenique primitive. *Acta Medical Scandinavian*, 142: 115-118.

Sonnenberg A. (1993) Integrins and their ligands. *Current Topics in Microbiology and Immunology*, 184: 7-35.

Spertini O, Kansas GS, Munro JM, Griffin JD, Tedder TF. (1991) Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. *Nature*, 349: 691-694.

Springer TA. (1990) Adhesion receptors of the immune system. *Nature*, 346: 425-434.

Springer TA, Lasky LA. (1991) Sticky sugars for selectins. *Nature*, 349: 196-197.

Springer TA. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*, 76: 301-314.

Stamper HB Jr, Woodruff JJ. (1976) Lymphocyte homing into, lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *Journal of Experimental Medicine*, 144: 828-833.

Steegmaier M, Levinovitz A, Isenmann S, Borges E, Lenter M, Kocher HP, Klevser B, Vestweber D. (1995) The E-selectin ligand ESL-1 is a variant of a receptor for fibroblast growth factor. *Nature*, 373: 615-620.

Stoolman LM, Tenforde TS, Rosen SD. (1984) Phosphomannosyl receptors may participate in the adhesive interaction between lymphocytes and high endothelial venules. *Journal of Cell Biology*, 99: 1535-1540.

Streeter PR, Berg EL, Rouse BTN, Bargatze RF, Butcher EC. (1988) A tissue specific endothelial cell molecule involved in lymphocyte homing. *Nature*, 331: 41-46.

Sugayama Y, Tiruppathi C, Janakidevi K, Andersen TT, Fenton II JW, Malik AB. (1992) Thrombin induced expression of endothelial P-selectin and intercellular adhesion molecule-1: amechanism for stabilising neutrophil adhesion. *Journal of Cell Biology*, 119: 935-944.

Symmons DPM. (1995) What is rheumatoid arthritis? *British Medical Bulletin*, 51:243-248.

Szekanecz Z, Humpries MJ, Ager A. (1992) Lymphocyte adhesion to high endothelium is mediated by two β1 integrin receptors for fibronectin, α4β1 and α5β1. *Journal of Cell Science*, 101: 885-894.

Targan SR. (1992) The lamina propria: a dynamic complex mucosal compartment. *Annals of the New York Academy of Sciences*, 664: 61-68

Tedder TF, Penta AC, Levine HB, Freedman AS. (1990) Expression of the human leukocyte adhesion molecule, LAM1. *Journal of Immunology*, 144: 532-540.

Tessier P, Audette M, Cattaruzzi P, McColl SR. (1993) Up-regulation by tumor necrosis factor a of intercellular adhesion molecule 1 expression and function in synovial fibroblasts and its inhibition by glucocorticoids. *Arthritis & Rheumatism*, 36: 1528-1539.

Trollmo C, Sollerman, Carlsten H, Tarkowski A. (1994) The gut as an inductive site for synoial and extraarticular immune responses in rheumatoid arthritis. *Annals of Rheumatic Diseases*, 53: 377-382.

Tsang YTM, Stephens PE, Licence ST, Haskard DO, Binns RM, Robinson MK. (1995) Porcine E-selectin: cloning and functional characterisation. *Immunology*, 85: 140-145.

Vestweber D. (1993) The selectins and their ligands. *Currents Topics in Microbiology and Immunology*, 184: 65-75.

Veys EM, Mielants H, deVos M, Cuvelier C. (1996). Spondylarthropathies: from gut to target organs. *Clinical Rheumatology*, 10:123-146.

Walchek B, Watts G, Jutila MA. (1993) Bovine γ/δ T cells bind E-selecting via a novel glycoprotein receptor: characterisation of a lymphocyte / E-selectin interaction in an animal model. *Journal of Experimental Medicine*, 178: 853-863.

Webb C. (1992) Sulfasalazine and mitogen-induced lymphocyte proliferation. *PhD thesis*, Department of Microbiology, University of Leicester.

Weller A, Isenmann S, Vestweber D. (1992) Cloning of the mouse endothelial selectins: expression of both E and P-selectin is inducible by tumour necrosis factor-α. *Journal of Biochemical Chemistry*, 267: 15176-15183.

Wellicome SM, Thornhill MH, Pitzalis C, Thomas DS, Lanchbury JSS, Panayi GS, Haskard DO. (1990) A monoclonal antibody that detects a novel antigen on endothelial cells that is induced by tumour necrosis factor, IL-1, or lipopolysaccharide. *Journal of Immunology*, 144: 2558-2565.

Wellicome SM, Kapahi P, Mason JC, Lebranchu Y, Yarwood H, Haskard DO. (1993) Detection of a circulating form of vascular cell

adhesion molecule-1: raised levels in rheumatoid arthritis and systemic lupus erythematosus. *Clinical Experimental Immunology*, 92: 412-418.

Westedt ML, Daha MR, Baldwin WM, Stijnen T, Cats A. (1986) Serum immune complexes containinig IgA appear to predict erosive arthritis in longitudinal study in rheumatoid arthrits. *Annals of Rheumatic Diseases*, 45: 809-815.

Westermann J, Blaschke V, Zimmermann G, Hirschfeld U, Pabst R. (1992) Random entry of circulating lymphocyte subsets into peripheral lymph nodes and Peyer's patches: no evidence in vivo of a tissue specific migration of B and T lymphocytes at the level of high endothelial venules. *European Journal of Immunology*, 22: 2219-2223.

Westermann J, Pabst R. (1996) How organ specific is the migration of naive and memory T cells? *Immunology Today*, 17: 278-282.

Willfuhr KU, Hirschfeld U, Westermann J, Pabst R. (1990) The in vitro lymphocyte / endothelium binding assay; am improved method employing light microscopy. *Journal of Immunological Methods*, 130: 201-207.

Wilson C, Ebringer A, Ahmadi K, Wrigglesworth J, Tiwana H, Fielder M, Binder A, Cunningham P, Joannou C, Bensal C. (1995) Shared amino acid sequences between MHC class II glycoproteins, type XI collagen and proteus mirablis in RA. *Annals of Rheumatic Diseases*, 54: 216-220.

Woodruff JJ, Katz IM, Lucas LE, Stamper HB. (1977) An in vitro model of lymphocyte homing. *Journal of Immunology*, 119: 1603-1610.

Yednock TA, Butcher EC, Stoolman LM, Rosen SD. (1987) Receptors involved in lymphocyte homing: relationship between a carbohydrate-binding receptor and the MEL-14 antigen. *Journal of Cell Biology*, 104: 725-731.

Yednock TA, Rosen SD. (1989) Lymphocyte homing. *Advances in Immunology*, 44: 313-378.

Young AJ, Hay JB. (1995) Rapid turnover of the recirculating lymphocyte pool in vivo. *International Immunology*, 7: 1607-1615.

Ziff M. (1990) Rheumatoid arthritis: present and future. *Journal of Rheumatology*, 17: 127-133.

Zimmermann GA, Prescott SM, McIntyre TM. (1992) Endothelial cell interactions with granulocytes: tethering and signalling molecules. *Immunology Today*, 13: 93-100.

APPENDIX 1

General reagents

supplemented Iscove's Culture Medium (sICM)

0.5ml	Penicillin / streptomycin
0.5ml	L-Glutamine
	Iscove's Culture Medium to 50ml

Cell Suspension Medium (CSM)

For 25ml.	23.125ml	Iscove's Culture Medium
	1.25ml	Foetal Calf Serum
	0.625ml	Hank's Balanced salt Solution

Dextran Sedimentation Mixture (DSM)

3g	Dextran
3g	D-Glucose
0.9g	Sodium Chloride
5000	units Heparin
	Nanopure water to 100ml

Dextran sedimentation mixture was prepared by dissolving sodium chloride in 100ml nanopure water, followed by dextran and D-glucose. Once dissolved, the required amount of heparin was added and the whole

mixture was filtered, using a Supervac 0.2μm filter and aliquoted into 20ml universals. Stored at -20°C.

Phosphate Buffered saline (PBS)

For 500ml.	470ml	Nanopure water
	25ml	Phosphate Buffered Saline (10 x)

Sterilised by autoclaving at 121°C at 15 pounds per square inch for 120 minutes.

Lymphocyte Freezing Medium (LFM)

For 50ml.	29mls	Iscove's Culture Medium
	15ml	Foetal Calf Serum
	5ml	Dimethylsulphoxide (DMSO)
	0.5ml	L-Glutamine
	0.5ml	Penicillin / Streptomycin

Veronal acetate buffer

1.2g	Sodium acetate (trihydrate)
2.94g	Sodium barbitone
	Nanopure water to 500ml

BDH chemicals

DPX mountant, Iso-pentane, Sodium chloride.

Boehringer Mannheim

Dispase

Dako

APAAP mouse monoclonal, Monoclonal mouse anti-human CD2, CD3, CD4, CD8, CD19, CD44, CD45RA, CD45RO, HLA-DR, Rabbit serum (normal), Rabbit anti-mouse immunoglobulin, Glycergel aqueous mounting medium.

Fisons

D-glucose, Dimethylsulphoxide (DMSO), Glutaraldehyde, Sodium acetate, Sodium barbitone.

Gibco

Hanks balanced salt solution (HBSS), Foetal calf serum (FCS), Penicillin/Streptomycin.

ICN

Dithiothreitol (DTT), Iscove's cell culture medium.

Mercia

Phosphate buffered saline (x 25 stock).

Miles

Tissue TEK OCT tissue embedding compound.

Sigma chemicals

Dextran, Diaminobenzidine tetrahydrochloride (DAB), Eosin Y solution with phloxine, Fast red TR salt, Haematoxylin solution gill no:3, Heparin (100000 units), Histopaque; mononuclear cell separation medium, Levamisole, Naphtol.

Sherwood medical

Q-tips single tipped applicators.

APPENDIX 2

American rheumatism association (ARA) revised criteria for rheumatoid arthritis classification (Arnett et al., 1988).

Criterion	Definition
1) Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.
2) Arthritis of 3 or more	At least 3 joint areas (out of 14 possible joint areas; right or left PIP, MCP, wrist elbow, knee, ankle, MTP joints) simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) as observed by a physician.
3) Arthritis of hand joints	At least one swollen area (as defined above) in a wrist, MCP or PIP joint.
4) Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs or MTPs, without absolute symmetry is acceptable).

5) Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician.
6) Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5% of normal control subjects.
7) Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localise in, or most marked adjacent to, the involved joints (osteoarthritis changes alone do not qualify).

For classification purposes, a patient has RA if at least 4 of these criteria are satisfied (criteria 1-4 must have been present for at least 6 weeks). Abbreviations used; PIP: proximal interphalangeal joint, MCP: metacarpo-phalangeal joint, MTP: metatarso=phalangeal joint.

Appendix : Publications which cover work presented in thesis.

1) Kadioglu A, Sheldon P. (1992). Adhesion of rheumatoid peripheral blood and synovial fluid mononuclear cells to high endothelial venules of gut mucosa. *Annals of Rheumatic Diseases*, 51: 126-128.

2) Kadioglu A, Sheldon P. (1992). Letter to editor, *Annals of Rheumatic Diseases,* 51: 926.

3) Kadioglu A, Sheldon P. (1995). Adherence of porcine Peyer's patch, peripheral blood and lymph node lymphocytes to mucosal endothelial venules. *Advances in Experimental Medicine and Biology* Vol.371, no:A; 81-83.

4) Kadioglu A, Sheldon P. (1995). Adherence of rheumatoid lymphocytes to endothelium of lamina propria and Peyer's patches. *Advances in Experimental Medicine and Biology*, Vol.371, no:A; 171-173.

5) Kadioglu A, Sheldon P. (1996). Adhesion of rheumatoid lymphocytes to mucosal endothelium: the gut revisited. *British Journal of Rheumatology*, 35: 218-225.