NOVEL INHIBITORS OF INFLAMMATION

by

Mark Richard E. Bartlett, B.Sc. (HONS)

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Cell-Cell Interaction Group
Division of Cell Biology
John Curtin School of Medical Research
Australian National University
Canberra 2601 Australia

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STATEMENT

I certify that, except for the experiments represented in section 3.2.1 (paragraph 1) and Figure 2.1, which were done by Dr. H.S. Warren, all experiments described in this thesis represent my own work, were done by me, and have not previously been submitted for a degree at this or any other university

Mark R.E. Bartlett
September 1993
DEDICATION

I would like to dedicate this thesis to

Noriko

and to our children
Daniel, Amanda, Michelle
Stephanie and Sarah
ACKNOWLEDGMENTS

I am grateful to my primary supervisor, Dr Chris Parish, for his unending enthusiasm, his availability to talk and give advice at any time despite many pressures on his time, and his great sense of humour. I am also grateful to Dr Hilary Warren for her guidance, advice and encouragement both before, and during, my studies, and to my advisor, Dr Bill Cowden for always being available, and particularly for his help with computers.

I am also indebted to many of the JCSMR staff including Karen Jacobsen and Susan Maynes from our lab for providing the human and bovine endothelial cells, the photography staff (Stuart Butterworth, Karen Edwards, Marc Fenning and Julie Macklin), the FACs staff (Geoffrey Osborne and Sabine Grueninger), the staff of the histopathology section (Wendy Hughes and Sheila Cook) and the Divisional Technical Manager, Jan Bateman. Without exception, all of these individuals have been a true pleasure to work with.

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Thanks go to all of my fellow students, especially Lyz and Kathie, for willingly sharing their supervisor with me and for helping make the lab a fun place to work.

Finally, and most importantly, eternal gratitude goes to my wife Noriko and our children, for putting up with me, and doing without me, for the past three years.
Summary

In response to inflammatory signals, circulating leukocytes first adhere to the apical surface of the postcapillary venule endothelium and migrate between the endothelial cells (ECs) until they arrive at the subendothelial basement membrane (BM). The subendothelial BM represents a formidable barrier to leukocyte extravasation and, in fact, histological studies have frequently reported that leukocyte migration is impeded at this point, apparently until the matrix is successfully penetrated (Huber, 1989).

While the initial adhesive events which control leukocyte recruitment into inflammatory sites have recently been well characterised at the molecular level, the mechanisms used by leukocytes to traverse the BM remain less clear. However, recent evidence supports the proposal that activated leukocytes secrete hydrolytic enzymes which degrade the BM, facilitating their extravasation. In support of this view, a number of groups have demonstrated that the potent anti-inflammatory activity of certain sulfated polysaccharides (SPS) such as heparin is probably due to inhibition of the BM-degrading endoglycosidase, heparanase. Furthermore, following evidence that heparanase and a number of other BM-degrading enzymes are of lysosomal origin, and that lysosomal enzymes can be expressed on the cell surface via mannose phosphate receptors (MPR), Parish and co-workers put forward the hypothesis that leukocyte extravasation is dependent upon the cell surface expression, and not merely the secretion, of lysosomal enzymes. Willenborg and co-workers subsequently showed that the phosphosugar, mannose 6-phosphate (M6P), as well as castanospermine (CS), an inhibitor of N-linked oligosaccharide processing, are potent inhibitors of experimental autoimmune encephalomyelitis (EAE) and adjuvant induced arthritis. It has been proposed that the anti-inflammatory effects of M6P and CS are due to their ability to inhibit the MPR-lysosomal enzyme interaction which is critical for the intracellular transport and cell-surface expression of lysosomal enzymes. In contrast, the anti-inflammatory activity of heparin is likely to be due to its direct inhibition of heparanase activity. The major aim of this thesis was to test the hypothesis, proposed by Parish et al (1990), that the anti-inflammatory effects of CS and M6P are due to their ability to displace lysosomal enzymes from the leukocyte cell surface, thus preventing degradation of the subendothelial BM and ultimately impeding the ability of leukocytes to transmigrate into tissues.

Chapter 3 describes an initial study conducted to confirm the previous findings that SPS, M6P, and CS are effective inhibitors of inflammation.
Here, the anti-inflammatory activity of these agents was tested in murine models of allograft rejection and elicitation of peritoneal exudates. CS, M6P and the SPS, fucoidan, partially inhibited rejection of permanently accepted thyroid allografts induced by the i.p. injection of donor strain (H-2<sup>d</sup>) spleen cells with a reduction in leukocyte infiltration by 25-36%. Elicitation of peritoneal exudates by thioglycollate was inhibited by CS, M6P and fucoidan with sustained leukopenia being induced by CS. In contrast, CS and fucoidan, but not M6P, inhibited antigen elicited peritoneal exudates. In general, the in vivo data of chapter 3 support the proposal that M6P, SPS and CS inhibit inflammation, showing that they could generally reduce the numbers of leukocytes in inflammatory foci. The results also suggested that CS, M6P and the SPS, fucoidan, exhibit subtle differences in their anti-inflammatory activity.

The study described in chapter 4 compared the ability of the major cellular components of inflammation, namely leukocytes, platelets and EC to degrade a <sup>35</sup>S<sub>0</sub>4-labeled subendothelial extracellular matrix (ECM) and assessed the effect of PMA and various pro-inflammatory cytokines on this degradative activity. The products of degradation of <sup>35</sup>S<sub>0</sub>4-labeled ECM heparan sulfate proteoglycans (HSPGs) were analysed by FPLC gel filtration chromatography and four major mol. wt. species were identified. These products corresponded to intact HSPG (I), free heparan sulfate (HS) chains (II), HS chain fragments (III) and oligo/monosaccharides/free sulfate (IV). Degradation products I and II were indicative of protease activity, product III arose from endoglycosidase (heparanase) activity and product IV suggested exoglycosidase and/or sulfatase activity. In terms of ECM degradation, human umbilical vein ECs (HUVECs) and platelets were the most active, with PMA stimulation further enhancing the degradative activity of these two cell types. However, platelets exhibited predominantly heparanase activity whereas the HUVEC degradation products suggested a range of enzymic activities, namely proteases, heparanases and sulfatases. Interestingly, EC in suspension expressed these three enzymic activities whereas confluent EC monolayers only exhibited sulfatase activity, suggesting that the former situation might represent an angiogenic response. In the case of leukocytes, neutrophils and lymphocytes degraded the ECM to a much greater extent than monocytes. Each cell type differed in the predominant enzymic activities they expressed, i.e., heparanase activity by lymphocytes; protease, and to a lesser extent, heparanase activity by neutrophils; and sulfatase activity by monocytes. PMA stimulation was shown to have differential effects on these enzymic activities. In addition, some pro-inflammatory cytokines were found to be cell type specific in their
effects on ECM degradation. Thus, a combination of IL-1 and TNF enhanced neutrophil and HUVEC degradation of the ECM but inhibited lymphocyte ECM degradation. In contrast, the chemokine IL-8 enhanced ECM degradation by neutrophils, lymphocytes and HUVECs. Of particular interest was the unique sulfatase activity expressed by ECs and monocytes which was induced in HUVECs by TNF + IL-1 and IL-8, and in monocytes by the chemokine MCAF.

Collectively, the results presented in chapter 4 showed that leukocytes differ markedly in the enzymes they express to degrade the BM during extravasation and that PMA and cytokines are cell-type specific in their induction of hydrolytic enzyme activity. These results also suggested that ECs play an important role, not only in the recruitment of leukocytes, but also in the preparation of the vascular BM for extravasation. In particular, the compromised BM integrity resulting from EC sulfatase activity is likely to be important in allowing greater physical interaction between leukocytes and the ECM and increasing the accessibility of BM components to degradation by hydrolytic enzymes.

In chapter 5, heparin, M6P and CS were examined for their ability to inhibit in vitro degradation of $^{35}$SO$_4$-labeled ECMs by leukocytes, ECs and platelets. All three anti-inflammatory compounds analysed in this study inhibited $^{35}$SO$_4$-labeled ECM degradation, but M6P and CS were shown to be cell-type specific in their effects. Heparin inhibited the heparanase activity of all cell types examined while M6P selectively inhibited lymphocyte heparanase, with no effect on the heparanase activity of platelets and the other cells examined. CS selectively inhibited HUVEC heparanase and sulfatase activity, but inhibited only PMA-induced degradative enzyme expression, without affecting the constitutive expression of degradative enzymes by nonstimulated HUVECs. The finding that heparin was able to inhibit heparanase activity in each cell type tested, while the actions of CS and M6P were cell type specific, provided important clues as to the mode of action of these compounds and the cause of the characteristic inflammatory pathology associated with the use of these anti-inflammatory agents. In particular, the data support the view that leukocytes markedly differ in the mechanisms they use to degrade the BM/ECM to enable extravasation, and that some degree of cooperation with ECs is required in this process.

Previous studies have clearly demonstrated that the majority of adhering and extravasating leukocytes in inflammatory responses are found in postcapillary venules, and it is possible that this is due to differences in the EC from the different vascular beds. In order to test this hypothesis, venular
and arterial ECs were examined, in chapter 6, for their expression of adhesion molecules, and their ability to degrade or reorganise the subendothelial BM in response to pro-inflammatory cytokines. The cytokine induced expression of E-selectin, VCAM-1 and ICAM-1 was shown to be similar between early passage venular and arterial EC. However, a number of striking differences between venular and arterial EC were demonstrated. First, arterial ECs expressed significant constitutive levels of VCAM-1 whereas venular ECs expressed low constitutive levels of VCAM-1. Second, TNF and IL-1 were virtually unable to induce adhesion molecule expression on late passage arterial ECs whereas similar passage venular ECs were only slightly reduced in their cytokine responsiveness. Third, analysis of ECM degradation products revealed substantial heparanase activity in venular ECs, an enzymic activity lacking in arterial ECs under a number of activation conditions. Fourth, there were marked differences in the ability of different stimuli to induce ECM degradation by the two types of ECs. Thus, PMA substantially enhanced ECM degradation by venular ECs but had no effect on arterial EC degradation. Furthermore, TNF and IL-1 induced an increase in the release of high mol. wt. proteoglycans from the ECM by venular ECs but had virtually no effect on the release of degradation products by arterial ECs despite these same cytokines inducing adhesion molecule expression on both EC types. Conversely, IFNγ selectively upregulated arterial EC sulfatase expression whilst inhibiting endogenous venular EC sulfatase activity. These data provide evidence for EC heterogeneity between different vascular beds. Further, collectively the adhesion molecule and ECM degradation data support the notion that margination and subsequent extravasation of leukocytes probably depends on a number of contributing processes, and not entirely on increased adhesive interactions mediated by adhesion molecules on endothelium.

In conclusion, the results presented in this thesis show that M6P and CS, compounds proposed to perturb the lysosomal enzyme-MPR interaction, can, as predicted, inhibit degradation of the BM by leukocytes and ECs. The anti-inflammatory activity of CS and M6P, as well as SPS which directly inhibits heparanase activity, was also confirmed. Taken together, these results support the hypothesis that cell-surface expression of hydrolytic enzymes is a critical property of leukocytes which allows them to traverse the BM during inflammation.
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<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>B cell</td>
<td>B lymphocyte</td>
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<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>CD</td>
<td>cluster designation</td>
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<td>CM</td>
<td>conditioned medium</td>
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<td>CMI</td>
<td>cell-mediated immunity</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>ConA</td>
<td>concanavalin A</td>
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<td>CR</td>
<td>complement receptor</td>
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<td>CS</td>
<td>castanospermine</td>
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<td>CsA</td>
<td>cyclosporin A</td>
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<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
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<tr>
<td>DMARD</td>
<td>disease-modifying antirheumatic drug</td>
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<td>DTH</td>
<td>delayed-type hypersensitivity</td>
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<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>ECGF</td>
<td>endothelial cell growth factor</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>ELAM</td>
<td>endothelial leukocyte adhesion molecule</td>
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<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<td>FB</td>
<td>fibrinogen</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FN</td>
<td>fibronectin</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>FUI</td>
<td>fluorescence intensity unit</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
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<td>GMP</td>
<td>granule membrane protein</td>
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<td>HEV</td>
<td>high endothelial venule</td>
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<td>HS</td>
<td>heparan sulfate</td>
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<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
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<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<tr>
<td>LAM</td>
<td>leukocyte adhesion molecule</td>
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<tr>
<td>LFA</td>
<td>lymphocyte function associated antigen</td>
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<td>M6P</td>
<td>mannose 6-phosphate</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<td>MCAF</td>
<td>monocyte chemotactic and activating factor</td>
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<td>MEL</td>
<td>murine erythroleukemia</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
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<tr>
<td>Mol. wt.</td>
<td>molecular weight</td>
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<td>MPR</td>
<td>mannose phosphate receptor</td>
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Abbreviations (cont'd)

MS  multiple sclerosis
NO  nitric oxide
NOD nonobese diabetic
NSAID non-steroidal anti-inflammatory drug
PAF  platelet activating factor
PBMC peripheral blood mononuclear cell
PBS  phosphate buffered saline
PMA Phorbol 12-myristate 13-acetate
PMN polymorphonuclear leukocyte
PPD purified protein derivative
r  recombinant
RA  rheumatoid arthritis
rh  recombinant human
SE  standard error of the mean
SPS sulfated polysaccharide
T cell T lymphocyte
TCR  T cell receptor
TGF transforming growth factor
TH helper T lymphocyte
TNF tumour necrosis factor
tPA tissue plasminogen activator
uPA urokinase plasminogen activator
uPAI urokinase plasminogen activator inhibitor
uPAr urokinase plasminogen activator receptor
VCAM vascular cell adhesion molecule
VLA very late antigen
Publications resulting from this thesis

Papers:

Effects of the anti-inflammatory compounds castanospermine, mannose-6-phosphate and fucoidan on allograft rejection and elicited peritoneal exudates
*Immunol. Cell Biol.* (submitted)

Venular and arterial endothelial cells differ in their expression of adhesion molecules and their ability to degrade the subendothelial basement membrane
Bartlett, M.R. and Parish, C.R.
*Eur. J. Immunol.* (submitted)

Comparative analysis of leukocyte, endothelial cell and platelet degradation of the subendothelial basement membrane: Evidence for cytokine dependence and detection of a novel sulfatase
*J. Immunol.* (In preparation)

Differential effects of the anti-inflammatory compounds heparin, mannose-6-phosphate and castanospermine on degradation of the vascular basement membrane by leukocytes, endothelial cells and platelets
Bartlett, M.R., Cowden, W.B. and Parish, C.R.
*J. Immunol.* (In preparation)

Conference abstracts:

In vitro studies on the degradation of subendothelial extracellular matrix by leukocytes
Bartlett, M.R., and Parish, C.R.
Chapter 1

Literature Review

The inflammatory reaction is the body's most important defence mechanism against injury or invasion by infectious agents and its proper function in some form is crucial to the survival of most multicellular organisms. Inflammation is a localised reaction involving a complex series of events including dilatation of vessels in the microvasculature, increased blood flow, exudation of fluids and leukocyte migration into the inflammatory focus (Gallin et al., 1992). Inflammation serves to eliminate, by means of leukocytes and soluble factors, the injurious agent, with a goal of returning the tissue to normal, or as close to normal as possible.

An integral part of the host's ability to defend itself against foreign antigens is its ability to recognise, and ignore, self components. The tip of this delicate balance of the immune system determines its role in health and disease, hence any deficiency of inflammation in the host is likely to compromise its survival. Conversely, excessive inflammation leads to disease in the host resulting from damage to self components. Such autoimmune diseases are a significant cause of mortality and morbidity in humans. Clearly, an understanding of the mechanisms of inflammation and of the pathogenesis of inflammatory disease is a necessary prerequisite to finding more effective means of therapy.

The purpose of this chapter is to outline current ideas as to the major molecular events of inflammation. Autoimmune disease and current means of therapeutic control will also be discussed. Since the discipline of inflammation has recently experienced an explosion of information at both the basic science and clinical level, a comprehensive review of the subject is beyond the scope of this thesis. Thus, in this review of the literature, only the salient features of inflammation will be described.
1.1 Historical overview

Inflammation was known to the ancients by the appearances it produced in the skin and other surfaces of the body. Its main manifestations were summarised by the Roman encyclopaedist, Celsus (30BC-38AD) as rubor (redness), tumour (swelling), calor (heat) and dolor (pain). However, a real appreciation of the changes in inflamed areas that were responsible for these so-called cardinal signs was not possible until Cohnheim (1839-1884) gave a careful, vivid and exciting description of the changes seen after injury to living transparent tissues. Using the frog's tongue and mesentery, Cohnheim described the circulatory changes which take place in the terminal vascular bed (Cohnheim, 1882). Inflammation was found to be a localised reaction in which there is a rapid and vigorous change at the level of the capillary and post capillary venules. After an initial arteriolar constriction, Cohnheim described a sustained dilatation of all small blood vessels within the injured area. While the volume of blood has been shown to increase up to ten-fold (Ascheim and Zweifach, 1962), the dilatation of blood vessels effectively causes a slowing of the blood flow so that individual blood cells could easily be recognised not only in the capillaries, but also in the veins and arteries.

"...there is a gradual accumulation of large numbers of white cells in the peripheral zone, which come to be comparatively motionless..."

Cohnheim's in vivo observations also resulted in a remarkable description of the margination and sticking of leukocytes and their migration through the vascular wall. Leukocytes were reported to appear in the marginal plasma stream of the venules, and to impinge from time to time on the venular wall.

"At first they stick momentarily to the wall, may roll along it for short distances, and then fall off and pass back into the flowing blood "

In an appearance graphically described by Cohnheim as pavementing, injuries of moderate intensity resulted in progressively more leukocytes passing to the periphery of the blood stream, contacting the venular wall and adhering to it for longer periods, until the luminal surface of many venules within the injured area became covered with a layer of living, adherent leukocytes.

"A striking contrast is presented by the central column of red blood corpuscles, flowing on in an uninterrupted stream of uniform velocity, and the peripheral layer of resting colourless cells, the internal surface of the vein appears paved with a single but unbroken layer of colourless corpuscles..."
Many of the adherent cells were reported to subsequently pass out through the venular wall into extravascular tissues. The leukocyte extravasation was accompanied by an increased loss of fluid from the blood vessels into the extravascular space and the accumulation of such fluid resulted in progressive swelling of the tissues.

In summary, the three major components of the acute inflammatory response are: haemodynamic changes, alterations in the permeability of vessels, and changes in the location and concentration of leukocytes. As a consequence there were alterations in blood flow resulting in the cardinal sign of rubor. Calor also resulted, due to the great increase in local blood flow. During inflammation there are rapid changes in the structure of the vascular wall, with a loss of EC integrity, leakage of fluid and plasma components, and ultimately, emigration of leukocytes from the blood circulation into the extravascular tissue. This escaping of inflammatory exudate and leukocytes from blood vessels, and their accumulation in extravascular tissues, as well as the capture of water by deposited fibrin, are the main factors responsible for tumour, or swelling, of an acutely inflamed area.

While Cohnheim’s studies focused on vascular changes during inflammation, Metchnikoff (1845-1916) emphasised phagocytosis and the elimination of foreign agents by white corpuscles (Metchnikoff, 1893):

"The diapedesis of the white corpuscles, their migration through the vessel wall... is one of the principle means of defence possessed by an animal. As soon as the infective agents have penetrated into the body, a whole army of white corpuscles proceeds towards the menaced spot, there entering into a struggle with the micro-organisms. The leukocytes, having arrived at the spot where the intruders are found, seize them after the manner of the Amoeba and within their bodies subject them to intracellular digestion."

Although phagocytosis alone does not constitute an inflammatory reaction, the removal of injurious agents and injured tissue is one of the principle functions of inflammation. Metchnikoff’s contribution in this area was enhanced by Ehrlich’s further classification of white corpuscles as basophils, eosinophils and neutrophils, referring to the effects of aniline dies which he used in his doctoral thesis (Hirsch and Hirsch, 1980).

The new science of immunology developed in parallel with Cohnheim and Metchnikoff’s work, beginning with the observations of Jenner (1749-1823).
on vaccination, and reaching a peak with the work of Pasteur (1822-1895), von Behring (1854-1917), Ehrlich (1854-1915) and Landsteiner (1868-1943). This new branch of science, while still concerned with the body's defences, accumulated evidence of the humoral aspects of inflammation. These immunologists gathered a body of evidence that body fluids, particularly the blood serum, possessed a protective effect against invasive microorganisms equal, if not greater than, that of cells.

Gradually the various views on inflammation combined to form a general doctrine, allowing for the cooperation of humoral and cellular aspects of inflammation. Rossle (Rossle, 1923) not only recognised that the vascular system facilitated the rapid accumulation of large quantities of phagocytes and antibodies, but also reported quantitative differences in the inflammatory reaction in animals which had previously been in contact with the same antigen. In the first discovery of inflammatory mediators, Lewis presented evidence that the vascular phenomena of the inflammatory reaction are initiated by histamine or other similar "H-substances" (Lewis, 1927) and Menkin isolated thermolabile and thermostable substances which caused an increase in vascular permeability and emigration of leukocytes (Menkin, 1960). The studies of Lewis and Menkin initiated an era in which the mediators of inflammation are still actively being pursued. At the same time extensive studies, initiated by Florey, were carried out on the ultrastructure of the vasculature during exudation and leukocyte emigration (Florey, 1962).

Since the importance of the inflammatory response as a means of host defence began to be recognised over a century ago, there has been an exponential growth in our understanding of the precise biochemical and cellular mechanisms that regulate inflammation. Characterisation of the immunoglobulins, the discovery and availability of cytokines, the characterisation of adhesion molecules and the application of molecular biology to the field has greatly contributed to our understanding of the inflammatory process as well as providing opportunities for the development of new therapeutic approaches for controlling inflammation. Nevertheless, to quote the words of Florey: "while much interest is being taken in the chemical and physical mechanisms (of inflammation)...we are still far from being able to give a complete picture of what happens in the tissues, or to explain how and why the sequence of events that follows injury takes place" (Florey, 1962).
1.2 Types of inflammation

Most of the processes of the immune system occur in an ongoing and covert fashion, successfully eliminating foreign antigens without resulting in clinical symptoms. However when the immune system mounts an inappropriately large or persistent response, clinically detectable inflammation results. An abnormal state of immune reactivity, or hypersensitivity, can result in pathological consequences. Such hypersensitivity reactions can be classified as immediate or delayed, depending upon the speed of the response following exposure to the antigen. A classification of hypersensitivity states has been proposed which further splits hypersensitivity reactions on the basis of their physiopathology and whether humoral and/or cellular components are involved (Altman, 1981). Despite its lack of perfect correspondence with all clinical situations, this sub-division of hypersensitivity into four distinct types aids considerably in our understanding of their pathogenesis. These are summarised in Table 1.1.

1.2.1 **Type I hypersensitivity**

Type I hypersensitivity is also referred to as immediate hypersensitivity or allergic inflammation and actually describes a wide variety of hypersensitivity states. The expression can be predominantly cutaneous or of a systemic nature such as in anaphylactic reactions, of which anaphylactic shock is the most serious (Kay, 1987). The primary event in type I hypersensitivity is the antigen driven cross-linking of IgE antibodies binding to basophil and mast cell Fc receptors. When exposed to the sensitising antigen, the reaction with cell-bound IgE triggers the release of histamine, and the synthesis of leukotrienes C4, D4 and E4. These "Slow Reacting Substances of Anaphylaxis" are potent effectors of vasodilation and bronchial smooth muscle constriction (Goetzl et al., 1984; Kay, 1987). Most frequently, human type I hypersensitivity is responsible for such allergic phenomena as asthma, hay fever and urticaria (Kay, 1987).

1.2.2 **Type II hypersensitivity**

Type II hypersensitivity or antibody mediated inflammation (reviewed by (Cochrane and Koffler, 1973; Kimberly, 1987)) involves, in its most common forms, the recognition of cellular or tissue antigens by complement fixing antibodies leading to the deposition of complement fragments on the surfaces of these cells. In the tissue or ECM, binding of complement fragments to
### Table 1.1

Characteristics of the four types of hypersensitivity reactions

<table>
<thead>
<tr>
<th>Type of inflammation (manifestation)</th>
<th>Immune lag time</th>
<th>Recognition component</th>
<th>Soluble mediator</th>
<th>Inflammatory response</th>
<th>Disease/example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I (Reagenic/allergic or immediate hypersensitivity)</td>
<td>Minutes</td>
<td>IgE</td>
<td>Basophil and mast cell products eg: histamine, arachidonate metabolites</td>
<td>Immediate flare and wheal; smooth muscle constriction</td>
<td>Atopy, anaphylaxis, asthma, rhinitis</td>
</tr>
<tr>
<td>Type II (Cytotoxic antibody)</td>
<td>Variable</td>
<td>IgG, IgM</td>
<td>Complement</td>
<td>Lysis/phagocytosis of circulating antigen; acute inflammation in tissues</td>
<td>Autoimmune haemolytic anemia; thrombocytopenia assoc. with SLE</td>
</tr>
<tr>
<td>Type III (Immune complex)</td>
<td>ca 6 hr</td>
<td>IgG, IgM</td>
<td>Complement</td>
<td>Accumulation of PMN and macrophages</td>
<td>Rheumatoid arthritis, SLE</td>
</tr>
<tr>
<td>Type IV (Delayed hypersensitivity)</td>
<td>12-48 hr</td>
<td>Sensitized T lymphocytes</td>
<td>Cytokines</td>
<td>Mononuclear cell infiltrate</td>
<td>Tuberculosis, sarcoidosis, vasculitis, granulomatosis</td>
</tr>
</tbody>
</table>

Adapted from Gallin (1992) and Virella (1993)
antigen activates the complement cascade. The activation of the complement cascade results in the generation of chemotactic and pro-inflammatory fragments such as C3a, C3b, and C5a. These substances also enhance the expression of adhesion molecules, such as the CD11/CD18 complex on neutrophils (Bevilacqua et al., 1989). The resultant accumulation of inflammatory cells such as neutrophils, which further release PAF (Camussi et al., 1990), or monocytes and macrophages, which possess plasma membrane receptors for the Fc portion of IgG and C3b, ultimately causes tissue damage.

1.2.3 Type III hypersensitivity reactions.
Similarly, when IgM and/or IgG - and specific antigen reach a critical local concentration the deposition of immune complexes leads to complement activation and the release of pro-inflammatory complement fragments (Snyderman, 1985). These complement derived peptides, along with some cytokines, lead to enhanced vascular permeability, mast cell degranulation, and leukocyte accumulation and degranulation and these events can lead to severe loss of vascular integrity and haemorrhagic necrosis (Snyderman, 1985). Associated with immune complex mediated inflammation are arthritis, glomerulitis and serum sickness (Kay, 1987)

1.2.4 Delayed (type IV) hypersensitivity
In contrast to the other three types of hypersensitivity, type IV hypersensitivity is a cell mediated phenomenon. The term "delayed-type hypersensitivity" (DTH) refers to the time elapsed following contact with antigen before the reaction occurs. Whereas allergic reactions occur within minutes and immune complex reactions occur within 5-24 hours, DTH reactions peak at 48-72 hours after deposition of the antigen (Altman, 1981). In DTH reactions, the antigen is processed and presented by antigen presenting cells (eg. macrophages, Langerhans cells) to T cells specific for the processed antigen (see section 1.4.5). In the process of antigen recognition, T cells are induced to release pro-inflammatory cytokines which attract lymphocytes and monocytes into the site, a process which peaks at around 72 hours following the introduction of antigen. The DTH reaction has been shown to be of crucial importance in the resolution of viral infections (Townsend and Bodmar, 1989) and in the destruction of intracellular parasites (Paul, 1992).
1.3 Soluble components of inflammation

The humoral and cellular components of the immune system overlap and synergise with one another in an elegantly orchestrated fashion (reviewed by (Schwartz and Syamal, 1989)). Thus, whatever the source of inflammation, it is not appropriate to ascribe it only to humoral or cellular effector functions. Nevertheless, this section will describe the humoral signals of inflammation, including the circulating cascades of complement, kinins, coagulation and fibrinolysis, the arachidonic acid derivatives and vasoactive amines, as well as the more recently emerged family of cytokines. A detailed description of the individual soluble components of inflammation is beyond the scope of this thesis. However, a number will be considered briefly below.

1.3.1 Immunoglobulins

Immunoglobulins (Ig) or antibodies are a group of glycoproteins present in serum and tissue fluids of all mammals and most vertebrates. It is beyond the scope of this review to discuss Ig structure, but in terms of inflammation, the effector function of the different Ig classes and subclasses plays a critical role and will be considered briefly here. There are five distinct classes of soluble Ig, each with differing biological properties (Jefferis and Pound, 1992). The structural diversity of the Fc region of the different Ig classes and subclasses results in different effector functions once the primary function of antigen binding occurs (Table 1.2).

The primary function of Igs is to bind antigen, although such binding does not always result in neutralisation of the antigen. The significant feature of Ig is its bifunctional nature, allowing it to link the antigen (bound to its Fab region) to its means of elimination (i.e. effector systems) via its Fc region. Depending on the Ig subclass, antigen binding can result in a wide range of biological responses (Jefferis et al., 1990). Since virtually every cell type involved in the immune response binds one or more Ig isotypes through Fc receptors, these biological responses are dependent upon the Fc portion of the molecule (Tables 1.2 and 1.3). The secondary effector functions include complement fixation and cellular elimination mechanisms (Feinstein et al., 1986). The Classical Pathway of Complement, for example, is triggered by the interaction of C1 with either IgG or IgM associated with antigen (Feldbush et al., 1984). The Alternative Pathway of Complement is also activated weakly by IgA-antigen complexes (Feldbush, Hobbs et al., 1984). The other major
Table 1.2

Biological properties of human immunoglobulins

<table>
<thead>
<tr>
<th>Property</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
<th>IgGA1</th>
<th>IgGA2</th>
<th>IgM</th>
<th>IgD</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical p’way</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alternative p’way</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Interaction with FcR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγRI</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>++</td>
<td>?</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcεR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcαR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcμR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reaction with FcR on:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>macrophages</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>neutrophils</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>basophils/mast cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>platelets</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>++</td>
<td>?</td>
<td>++</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mucosal transfer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Adapted from: Jeffers and Pound (1992) and Virella (1993)

a See also Table 2.2
Table 1.3

Cellular expression of human Fc receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Characteristic</th>
<th>Distribution</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>Transmembrane and intracytoplasmic domains; high affinity; binds both monomeric and aggregated IgG</td>
<td>Monocytes/ macrophages</td>
<td>ADCC (monocytes)</td>
</tr>
<tr>
<td>FcγRII (CD32)</td>
<td>Transmembrane and intracytoplasmic domains; low affinity</td>
<td>Monocytes/ macrophages; langerhans cells; platelets; B cells</td>
<td>IC binding; phagocytosis; degranulation; ADCC (monocytes)</td>
</tr>
<tr>
<td>FcγRIII (CD16)</td>
<td>Glycosyl-phosphatidyl inositol anchor in neutrophils; transmembrane segment in NK cells; low affinity</td>
<td>Macrophages; neutrophils; NK and K cells</td>
<td>IC binding and clearance; &quot;priming signal&quot; for phagocytosis and degranulation; ADCC (NK cells)</td>
</tr>
<tr>
<td>FcαR</td>
<td>Transmembrane and intracytoplasmic segments</td>
<td>Neutrophils; monocytes/ macrophages; T &amp; B cells; platelets</td>
<td>Phagocytosis; degranulation</td>
</tr>
<tr>
<td>FcεRI</td>
<td>High affinity</td>
<td>Basophils/ mast cells</td>
<td>Basophils/mast cell degranulation</td>
</tr>
<tr>
<td>FcεRII (CD23)</td>
<td>Low affinity</td>
<td>T &amp; B cells; monocytes/ macrophages; eosinophils; platelets</td>
<td>Mediates parasite killing by eosinophils</td>
</tr>
<tr>
<td>FcµR</td>
<td></td>
<td>T cells</td>
<td>Activates T helper functions (?)</td>
</tr>
</tbody>
</table>

Adapted from Virella (1993)
effector system, the recognition of antibody coated target cells by cells bearing Fc receptors, is associated with phagocytosis (by monocytes, macrophages and granulocytes), antibody-dependent cellular cytotoxicity (ADCC) (by monocytes, macrophages and lymphocytes) and anaphylaxis (by mast cells) (Jefferis and Pound, 1992; Virella and Wang, 1993).

1.3.2 Plasma protein systems
There are four major plasma protein systems which interact with and regulate inflammation. While for the sake of this discussion, these protein systems are divided into the complement, contact, coagulation and fibrinolytic systems, in reality the distinction between the various pathways are not clear-cut, since there are numerous interrelations between them (Fig. 1.1). There are several reviews which discuss these systems in detail (Frank and Fries, 1991; Kozin and Cochrane, 1992). In this section I will briefly discuss the four systems and, in particular, their general role in inflammatory processes.

1.3.2.1 Complement
The complement system is one of the major effector pathways in inflammatory reactions and consists of a group of about 20 serum proteins which interact with one another and with other elements of the immune system, both innate and adaptive (reviewed by (DiScipio, 1987; Frank and Fries, 1991; Goldstein, 1992)). Activation of complement, which can occur via either an antibody mediated (direct) or antibody independent (alternative pathway) (reviewed by (Lachmann and Hughes-Jones, 1984; Müller-Eberhard, 1992)) (Fig. 1.2) is a potent mechanism for the initiation and amplification of the inflammatory response, resulting in the following events:

1) Opsonization, which is accomplished by the fixation of certain complement proteins to either soluble or particulate antigens. Specific receptors on cells then mediate binding and uptake of the opsonized antigen (Table 1.4).

2) Lysis of cells, resulting from complement activation, which is caused by the insertion of a hydrophobic plug into the cell membrane, allowing osmotic disruption of the target (Simone and Henkart, 1980). Cell lysis can result in release of foreign antigen and initiation of non-specific inflammatory responses.

3) Cellular activation and chemotaxis, in which cellular components of inflammation are activated by low molecular weight and readily diffusible cleavage products of complement proteins such as C3a, C4a and C5a (Osler
Fig. 1.1
The plasma enzyme systems and their interactions in inflammation. Adapted from Roitt (1989)
Fig. 1.2
Organization of the complement pathways. The upper segment of the scheme represents the classical pathway, and the lower segment represents the alternative pathway. C3 has a central position in both pathways.

From Müller-Eberhard (1992)
Table 1.4

Complement receptors on human peripheral blood cells

<table>
<thead>
<tr>
<th>Complement receptor</th>
<th>Receptor-positive blood cell</th>
<th>Binding specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1 (CD35)</td>
<td>Erythrocytes - low&lt;sup&gt;α&lt;/sup&gt;</td>
<td>C3b, C4b, iC3b</td>
</tr>
<tr>
<td></td>
<td>Neutrophils - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B cells, eosinophils - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages - low</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T cells (20%) - low</td>
<td></td>
</tr>
<tr>
<td>CR2 (CD21)</td>
<td>B cells - high</td>
<td>C3d region of iC3b</td>
</tr>
<tr>
<td></td>
<td>Follicular dendritic cells - v. high</td>
<td>(C3dg, C3d and C3b)</td>
</tr>
<tr>
<td></td>
<td>Pharyngeal epithelial cells - low</td>
<td></td>
</tr>
<tr>
<td>CR3 (CD11b/CD18)</td>
<td>Neutrophils - high</td>
<td>iC3b (Ca&lt;sup&gt;2+&lt;/sup&gt; required)</td>
</tr>
<tr>
<td></td>
<td>NK cells - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monocytes - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macrophages - low</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils - high</td>
<td></td>
</tr>
<tr>
<td>CR4 (CD11c/CD18)</td>
<td>Neutrophils - low</td>
<td>C3d region of iC3b</td>
</tr>
<tr>
<td></td>
<td>Monocytes - low</td>
<td>(same as CR2),</td>
</tr>
<tr>
<td></td>
<td>Macrophages - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K and NK cells, eosinophils - low</td>
<td></td>
</tr>
<tr>
<td>CR5</td>
<td>Neutrophils - low</td>
<td>C3dg, C3d,</td>
</tr>
<tr>
<td></td>
<td>Platelets - low</td>
<td>C3d region of iC3b</td>
</tr>
<tr>
<td>C5a receptors</td>
<td>Neutrophils - high</td>
<td>C5a</td>
</tr>
<tr>
<td></td>
<td>Monocytes - high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Ross (1989), Virella (1993)

*α* High and low refers to level of expression
Neutrophils and macrophages, for example, express specific receptors for these fragments (anaphylatoxins), the ligation of which results in chemotaxis and activation (Frank and Fries, 1991). The net effects of these activities are histamine and leukotriene-mediated contraction of vascular smooth muscle, increased vascular permeability and emigration of neutrophils and monocytes from the circulation into the inflammatory focus (Hugli, 1984) (Table 1.5). Lymphocytes and antigen presenting cells also express similar receptors which bind complement-opsonized antigens and enhance specific immune responses (Feldbush, Hobbs et al., 1984; Carter and Fearon, 1989).

1.3.2.2 The contact activation system

The contact activation system consists of four main proteins (reviewed by (Cochrane and Griffin, 1982)):

1) The **Hageman factor** (HF) is cleaved by limited proteolysis (probably by plasma kallikrein) resulting in a peptide which activates prekallikrein. HF is also activated upon binding to negatively charged surfaces (Nossel et al., 1968)


3) **High MW kinogen** (HMWK) releases a potent vasoactive peptides (bradykinin) and a histidine rich fragment upon cleavage (Meier et al., 1977).

4) **Coagulation factor XI** plays an important role in intrinsic clotting. Its major function is in cleaving factor IX. Factor XI may also cause proteolysis leading to the activation of HF and plasminogen (Cochrane and Griffin, 1982). The four proteins interact with extrinsic coagulation factors (factor VII) and complement to extend the physiologic effects of the contact system. Contact activation is initiated by a number of substances (Table 1.6) and is also thought to be induced by negatively charged surfaces (Nossel, Rubin et al., 1968; Kozin and Cochrane, 1992), possibly by bringing the four components of the contact system into apposition on the surface, triggering their reciprocal activation (Kozin and Cochrane, 1992). A biologically important site of activation is the extravascular space, due to ECM components such as heparan sulfates which are highly negatively charged. Sulfated polysaccharides including naturally occurring glycosaminoglycans from several sources have been reported to be effective in activating the contact system, releasing kinins (Dos Santos et al., 1970; Meier and Kaplan, 1978). Other studies have shown that some naturally occurring connective tissue
Table 1.5

Biological activities of human anaphylatoxins

<table>
<thead>
<tr>
<th>Anaphylatoxin</th>
<th>Target cell/tissue</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3a, C4a, C5a</td>
<td>Small blood vessels</td>
<td>Increased permeability</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle cells</td>
<td>Contraction</td>
</tr>
<tr>
<td></td>
<td>Mast cells, basophils</td>
<td>Release of histamine</td>
</tr>
<tr>
<td>C5a</td>
<td>Fibroblasts</td>
<td>Directed migration</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>Polarization; directed migration; secretion of lysosomal enzymes; secretion of eicosanoids; increased oxidative metabolism; increased adherence to surfaces; increased expression of C3; Fc receptors increased; expression of integrins</td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages</td>
<td>As for neutrophils plus: generation of IL-1, TNFα and IL-6</td>
</tr>
</tbody>
</table>

Adapted from (Goldstein, 1992)
Table 1.6

Surfaces responsible for contact activation

<table>
<thead>
<tr>
<th>Organic substances</th>
<th>Inorganic substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosodium urate crystals</td>
<td>Silica dioxide</td>
</tr>
<tr>
<td>Bacterial LPS</td>
<td>Glass</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>Kaolin, celite</td>
</tr>
<tr>
<td>Carrageenin</td>
<td>Calcium pyrophosphate</td>
</tr>
<tr>
<td>Vascular basement membrane</td>
<td></td>
</tr>
<tr>
<td>Articular cartilage</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
</tr>
<tr>
<td>Glycosaminoglycans (eg. heparin)</td>
<td></td>
</tr>
<tr>
<td>and glycoproteins</td>
<td></td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td>Sulfatides</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Kozin (1992)
matrix glycosaminoglycans and proteoglycans do not activate the contact system unless they were further sulfated (Hojima et al., 1984). It seems likely that plasma factors must inhibit contact activation by the normally highly negatively charged luminal surface of vascular endothelium, possibly by masking heparan sulfates on cell surfaces. Following the initial burst of HF activity, a series of events occur which serve to amplify the response, generating increased amounts of HF, kallikrein and bradykinin (Cochrane and Griffin, 1979). Further, the contact system activates complement (Ghebrehiwet et al., 1983) and, through bradykinin receptors on a number of cells, activates phospholipases A2 and C resulting in the augmentation of arachidonate mobilisation from cells (Bell et al., 1980). Bradykinin induces the synthesis of PAF and prostacyclin by cultured human EC and also has profound effects on vascular permeability, leukocyte margination, chloride secretion and pain (Regoli and Barabe, 1980). The important role of the contact activation system in the regulation of immunity and inflammation is highlighted by considerable evidence supporting its role in a number of disorders such as arthritis (Sawai et al., 1980) and allergic and anaphylactic reactions (Kier et al., 1990).

1.3.2.3 The coagulation system
In the coagulation, or plasma phase of blood, the change from the fluid to the gelified state is primarily due to the transformation of the soluble protein, fibrinogen, to the insoluble protein, fibrin. The transformation of fibrinogen occurs as a result of the activation and amplification of an enzymic cascade (Fig. 1.3). The cascade is composed of a large number of protein components which are mainly proenzymes transformed to active forms by partial proteolysis (reviewed by (Bennet, 1984)).

The first step of coagulation can occur by either the intrinsic or extrinsic pathway, both of which are of equal importance during normal haemostasis. The intrinsic (or endogenous) route is purely plasmatic and consists of the components of the contact system (described above) as well as two antihaemophilic factors, A and B (VIII and IX) (Bennet, 1984). The extrinsic (or exogenous) route is activated by tissue factors, involving only thromboplastin and proconvertin (factors III and VII). Both routes ultimately lead to the formation of the prothrombinase complex which binds to a surface phospholipid, platelet factor 3 or tissue thromboplastin. Prothrombinase is proteolytic, transforming prothrombin (factor II) to
Fig. 1.3 The intrinsic and extrinsic coagulation pathways. The terminal steps in both pathways are the same. Calcium, factors X and V, and platelet phospholipids combine to form prothrombin activator which then converts prothrombin to thrombin. This interaction, in turn, causes conversion of fibrinogen into fibrin strands that create the insoluble blood clot. Adapted from Machin (1992).
thrombin (factor IIa) resulting in fibrin formation, which constitutes the second step in coagulation (Bennet, 1984).

Thrombin is also involved in the induction of platelet activation, a variety of functional alterations in endothelial cells (EC) and the activation of a number of other essential factors. For example, thrombin induces the production by ECs of platelet activating factor (PAF) which in turn elicits further diverse biological actions, especially by platelets and neutrophils (Prescott et al., 1984). Neutrophil adhesion to thrombin-activated ECs is probably due to the co-ordinate expression of PAF and P-selectin (section 1.5.1) which bind to receptors on the neutrophil causing an activation dependent alteration in neutrophil β2 integrins (Zimmerman et al., 1986; Carveth et al., 1992). Finally, thrombin activity is neutralised by antithrombin III, and heparin, a product of degranulating mast cells, can augment antithrombin III activity (Carrel et al., 1987).

1.3.2.4 The fibrinolytic system
Fibrinolysis (reviewed by (Collen and Lijnen, 1991)) (Fig. 1.1), must occur as a part of the normal dynamic equilibria involved in blood coagulation, where any spontaneously forming fibrin deposits must be dissolved to reopen thrombosed blood vessels. Fibrin is degraded by the broadly acting serine protease, plasmin, which is formed by the activation of plasminogen. There are several different pathways of plasminogen activation, but usually tissue plasminogen activator (t-PA) which is released from vascular EC, and urokinase plasminogen activator (u-PA) are the main activators. As with the coagulation system, fibrinolysis is localised, and counterbalanced by an inhibitory system which exerts its negative control on either the plasminogen activators (eg. plasminogen activator inhibitor or PAI) or plasmin itself (mainly by α2 antiplasmin) (Carrel, Christey et al., 1987; Collen and Lijnen, 1991).

1.3.3 Lipid derived soluble factors
1.3.3.1 Prostaglandins
The oxidation of arachidonic acid in mammalian cells gives rise to a number of biologically active chemicals called eicosanoids, which are intimately involved in inflammation - making important contributions to the signs and symptoms of a number of inflammatory diseases. Prostaglandins were the first members of this family to be identified and characterised (reviewed by (McGiff, 1981)). Prostaglandins and thromboxanes (collectively called
prostanoids) are produced from arachidonic acid via the enzyme, cyclo-oxygenase (Fig. 1.4). Prostanoids are inflammatory mediators and have been described in several types of inflammation. They cause vasodilation, fever and hyperalgesia and act synergistically with other inflammatory mediators to produce oedema and pain. The prostanoids also exert numerous effects on lymphocyte functions (reviewed by (Goodwin and Webb, 1980). Substances such as non-steroidal anti-inflammatory drugs, which inhibit the synthesis of prostaglandins or antagonise their effect, reduce the intensity of inflammatory signs and symptoms (Vane, 1971; Higgs et al., 1980).

1.3.3.2 Leukotrienes
A second major pathway of arachidonic acid metabolism has also been characterised, in which arachidonic acid is converted to pro-inflammatory products known as leukotrienes by a cascade of enzymic activities which are initiated by 5-lipoxygenase (Fig. 1.5) (reviewed by (Lewis and Austen, 1984). Like the prostanoids, leukotrienes have been implicated as potent pro-inflammatory mediators. A number of the leukotrienes are chemotactic for inflammatory cells, cause smooth muscle contraction, vasoconstriction, and increase vascular permeability. Leukotrienes also result in increased EC adhesiveness for leukocyte populations (Chau et al., 1986; Lindstrom et al., 1990). Inhibitors of 5-lipoxygenase have demonstrable anti-inflammatory effects (Batt et al., 1992), an observation which provides strong evidence for the importance of leukotrienes in inflammation.

1.3.3.3 Platelet activating factor
The importance of lipid derived mediators in inflammation has been clearly demonstrated as a result of studies of prostaglandins, thromboxanes and leukotrienes. More recent studies have revealed that platelet activating factor (PAF), a biologically active phospholipid, is a major contributor to the physiopathology of inflammatory reactions (reviewed by (Camussi, Tetta et al., 1990; Prescott et al., 1990). The activity of PAF extends beyond the activation of platelets to such diverse effects as promotion of cellular aggregation, chemotaxis, granule secretion and oxygen bursts in neutrophils (O'Flaherty et al., 1981), eosinophils (Bruynzeel et al., 1986) and macrophages (Hartung, 1983) and induction of smooth muscle contraction in vitro (Stimler and O'Flaherty, 1983). In vivo effects include alteration of vascular permeability, recruitment of inflammatory cells and oedema (Camussi, Tetta et al., 1990). Again, the finding of PAF production in vivo in
Fig. 1.4
The synthesis of prostaglandins and thromboxanes
From Davies and MacIntyre (1992)
Fig. 1.5
5-Lipoxygenase pathway of leukotriene synthesis

From Lam and Austen (1992)
many hypersensitivity reactions suggests its important role in inflammation (Zimmerman et al., 1992).

1.3.3 4 Other lipid-derived mediators of inflammation
In biological systems polyunsaturated fatty acids in membrane phospholipids are also utilised for non-enzymatic lipid peroxidation initiated by free radicals (Slater, 1984). Some end products of lipid peroxidation are the 4-hydroxyalkenals which are capable of producing a wide variety of powerful effects including chemotaxis of neutrophils (Curzio et al., 1982) and potentiation of platelet aggregation (Selley et al., 1988; Selley et al., 1990). Thus, lipid peroxidation products represent important lipid-derived pro-inflammatory mediators.

1.3.4. Cytokines
Cytokines are soluble proteins (often glycoproteins) that are non-immunoglobulin in nature, and are released by living cells of the host. Cytokines act locally and non-enzymatically in extremely low concentrations (pM to nM) to regulate the function of the same or other cells of the host and play a vital role in coordinating immune and inflammatory responses. Most cytokines are pleiotropic, with multiple biological activities, and the coordination of immune and inflammatory responses is the result of a network of differentially activated, unique combinations of cytokines (Fig. 1.6). The source of these cytokines during the earliest phases of the immune response was initially presumed to be antigen activated T cells. However, it has since emerged that cytokines are produced by a variety of other cell types including macrophages, EC, mast cells and smooth muscle cells (reviewed by Arai et al., 1990).

1.3.4.1 The chemokines
Recently, on the basis of amino acid sequence similarities, a number of cytokines have been grouped into a super-family of ten or more pro-inflammatory, chemoattractant cytokines or chemokines. The chemokines have been further divided into the CC and CXC subfamilies on the basis of their organisation of cysteine residues in the primary amino acid sequence. The CC subfamily consists of MIP-1α, MIP-1β, MCAF and RANTES. Members of the CXC subfamily include IL-8, MGSA/Gro, Platelet Factor 4 and β-thromboglobulin.
Fig. 1.6
Role of some T cell- and macrophage-derived cytokines in immune and inflammatory responses
Adapted from Virella (1993)
Interleukin 8 (IL-8, NAP-1, NAF), the prototype member of the CXC family of chemokines, is considered to be a potent pro-inflammatory cytokine due to its chemoattractant and degranulating effect on neutrophils (Djeu et al., 1990; Schroder et al., 1990). IL-8 is produced by monocytes, macrophages, fibroblasts, epithelial cells, chondrocytes (Lotz et al., 1992), mitogen stimulated T cells (Gregory et al., 1988), ECs and keratinocytes stimulated with IL-1 and TNF (Matsushima and Oppenheim, 1989). It is synthesised as a precursor molecule of 99 amino acids, specific proteolytic cleavage resulting in four distinct N-terminal variants of 77, 72, 70 or 69 amino acids (Lindley et al., 1988). Different cells have been shown to preferentially express certain forms, which have varying activities (Hebert et al., 1990). Two high affinity receptors, which exhibit a 77% amino acid homology, have been identified for IL-8. The type I receptor binds only IL-8 (Holmes et al., 1991) while the type II receptor binds IL-8, MGSA/Gro and NAP-2 (Murphy and Tiffany, 1991). These receptors, which belong to a family of receptors containing seven transmembrane domains and are coupled to the guanine nucleotide-binding (G) proteins, are distinct from the other chemoattractant receptors for C5a, fMLP, PAF and leukotriene B4 (Besemer et al., 1989; Grob et al., 1990).

Macrophage Inflammatory Protein-1 (MIP-1) is a key member of the CC subfamily of chemokines. MIP-1α and MIP-1β both contain 69 amino acid residues, and are 60% homologous (Broxmeyer et al., 1991) although MIP-1β possesses a potential N-glycosylation site which MIP-1α lacks. They also differ in their effects on haematopoietic stem cell growth. MIP-1α is produced by activated macrophages and T cells, B cells, mast cells and fibroblasts. MIP-1α inhibits haematopoietic stem cell growth while MIP-1β counteracts this inhibition by MIP-1α (Broxmeyer, Sherry et al., 1991). In vitro, MIP-1α and β bind to, and induce chemotaxis of, T cells and macrophages (Oh et al., 1991) and enhance the activities of GM-CSF and M-CSF (Broxmeyer, Sherry et al., 1991). Both cytokines induce inflammatory infiltrates in skin and CNS as well as pyrogenic effects when injected i.v. into rabbits (Wolpe and Cerami, 1989). Recently, phenotypic analysis revealed that activated CD4+ T cells migrated in response to MIP-1β whereas MIP-1α attracted predominantly activated CD8+ T lymphocytes (Taub et al., 1993). The MIP-1 cytokines also enhanced the ability of T cells to bind to an EC monolayer (Taub, Conlon et al., 1993). These results suggest that MIP-1 cytokines preferentially recruit specific T cell subsets during the evolution of the immune response.
**RANTES**, or Regulated upon Activation, Normal T Expressed and Presumably Secreted, is another member of the CC subfamily of chemokines. It is an 8.4 kD (68 amino acids) non-glycosylated protein expressed by circulating T cells in culture and is unique in that its expression is actually down-regulated by activation (Schall et al., 1988). Unlike IL-8, the RANTES protein fails to act on neutrophils, instead causing selective migration of monocytes and CD4+ T cells expressing markers associated with memory (Schall et al., 1990).

**Monocyte Chemotactic and Activating Factor** (MCAF), another CC chemokine glycoprotein (8.4 kD, 76 amino acids), is produced by human peripheral blood mononuclear cells, fibroblasts and ECs treated with IL-1 or TNFα (Sica et al., 1990). In addition to its monocyte chemoattractant properties, MCAF can activate monocytes, inducing superoxide anion production, calcium flux and lysosomal enzyme release (Rollins et al., 1991).

### 1.3.4.2 Tumour necrosis factor

Tumour necrosis factor (TNFα) (17 kD, 157 amino acids) is mainly a product of stimulated monocytes and macrophages, but it is also produced by lymphocytes, EC and keratinocytes (for review see (Semanzato, 1990)). It is structurally related to lymphotoxin (TNFB) (20 kD, 171 amino acids), and is recognised by the same cell membrane receptor. TNFα is active only in its trimeric form and requires a molar concentration one or two orders of magnitude higher than that of IL-1 to stimulate immunocompetent cells (Ranges et al., 1986). TNFα was originally found to be of interest due to its ability (unlike IL-1) to induce tumour necrosis in tumour-bearing mice (Carswell et al., 1975). However, recent studies have focused on its other widespread biological effects, which are primarily pro-inflammatory (Semanzato, 1990). Like IL-1, TNFα induces a wide variety of pro-inflammatory effects including the induction of other cytokines, PGE2 and collagenase synthesis in a variety of tissues, induction of fever and modulation of vascular endothelium. TNFα causes bone and cartilage resorption, inhibition of lipoprotein lipase, increases in hepatic acute phase proteins and complement components, and a decrease in albumin synthesis, and upregulation of fibroblast proliferation and collagen synthesis (Semanzato, 1990). TNFα also induces the expression of a number of adhesion molecules (see section 1.5.1) which are crucial in the early events of leukocyte migration from the circulation into an inflammatory focus.
(Bevilacqua et al., 1987; Bevilacqua, Stengelin et al., 1989; Carlos et al., 1990).

1.3.4.3 The interleukins

**Interleukin 1** (IL-1) has been isolated in two forms, IL-1α and IL-1β, which represent the products of two distinct genes, but which have similar activities and molecular weights (17 kD, 270 and 269 amino acids respectively). Along with TNFα, IL-1 affects nearly every tissue and organ system, displaying a wide spectrum of biological activities including pyrogenicity, stimulation of the immune response and the release of a number of pro-inflammatory mediators (Dinarello, 1989). As such, these cytokines are the prototype of the pro-inflammatory cytokines. They are early mediators that initiate the tissue inflammatory response, and have been found to induce the expression of a number of genes and the synthesis of several proteins which, in turn, induce acute and chronic inflammatory changes (Gershenwald et al., 1990). For example, in cultured EC alone, IL-1 induces upregulation of adhesion molecule expression (see section 1.5.1), increased production of procoagulant activity, tissue factor, PGE₂, PGI₂, PAF and plasminogen activator inhibitor (PAI) (Rossi et al., 1985; Dejana et al., 1987). In addition, there is an enhancement of thrombin induced von Willebrand’s factor and the synthesis of other cytokines - IL-8, IL-6, TNFα and itself (Dinarello, 1989). Thus, while earlier studies with partially purified IL-1α and β, (along with TNF) were reported to have considerable leukocyte chemotactic activity (Luger et al., 1983; Sauder et al., 1984), later studies with the recombinant cytokines showed that this was due to the diverse secondary effects of IL-1 and TNFα (Yoshimura et al., 1987).

**Interleukin 2** (IL-2) is a 15 kDa protein produced by activated T cells (Smith, 1988). The major function of IL-2 is to stimulate T cell proliferation, enabling their clonal expansion in conjunction with their activation by specific antigen (Dinarello and Mier, 1987). However, the biological activity of IL-2 is not restricted to T cells. IL-2 is also able to stimulate the growth and differentiation of B cells (Ralph et al., 1984), augment the cytotoxic activity of NK cells (Henny et al., 1981) and LAK precursors (Grimm et al., 1983), exert effects on the growth and differentiation of immature thymocytes (Toribio et al., 1989), and induce the production of other lymphokines, including IFN-γ (Farrar et al., 1982). IL-2 can also augment the cytotoxicity of monocytes (Malkovsky et al., 1987). It is a potent stimulus for
inflammation and adaptive immunity, stimulating lymphocyte production of other cytokines (Mier and Gallo, 1980)

**Interleukin 4** (IL-4), formerly known as B cell activating factor, is produced by activated T cells and stimulates growth of activated B cells (Dinarello and Mier, 1987) by interacting with a specific cell-surface receptor (Crawford *et al.*, 1987). However, IL-4 is now known to have a much broader spectrum of activity (reviewed by (Arai, Lee *et al.*, 1990)) including the induction of expression of MHC antigens, promotion of Ig class switching, induction of T cell proliferation and the enhancement of antigen presenting functions of macrophages.

**Interleukin 6** (IL-6), (formerly known as IFNβ2), is produced by a number of lymphoid and non lymphoid cells and has potent antiviral activity (Van Damme *et al.*, 1987). However, IL-6 also exerts important influences on inflammation, especially as it regulates hepatic acute phase protein synthesis (May *et al.*, 1988), induces proliferation of T cells and transformed B cells and induces B cell differentiation and Ig secretion (Arai *et al.*, 1990).

1.3.4.4 **Colony Stimulating Factors**

Human hemopoietins, or colony stimulating factors (CSFs), are chiefly associated with the regulation of the levels of granulocytes and mononuclear phagocytes in the peripheral blood. However the CSFs also have important effects on mature leukocytes (Table 1.7), and play a significant direct role in host defence and inflammation (reviewed by (Golde and Baldwin, 1992; Hamilton, 1993). For example, during cell mediated immunity, the interaction of antigen with the appropriate TCR leads to T cell activation and subsequent elaboration of cytokines including granulocyte/macrophage CSF (GM-CSF) and interleukin 3 (IL-3). GM-CSF is then able to stimulate the local proliferation of monocytes, facilitate chemotaxis towards the inflammatory focus and prime granulocytes and monocytes for heightened cytotoxic activity. This in turn has been reported to lead to the production of TNFα and IL-1 by the activated monocytes, although there is some disagreement as to whether or not this is a direct effect (Hamilton, 1993). Recently, evidence has emerged demonstrating that local GM-CSF action also plays an important role in augmenting antigen presentation (Tao and Levy, 1993).
The effects of GM-CSF on mature leukocytes

**Neutrophils**
- Prolonged *in vitro* survival
- Membrane ruffling and increased adhesion
- Increased expression of CD11b/CD18
- Increased protein synthesis
- Increased chemotaxis
- Decreased random migration
- Priming for enhanced oxidative metabolism
- Increased phagocytosis and intracellular killing
- Enhanced antibody-dependent cellular cytotoxicity
- Priming for increased release of arachidonic acid and LTB4
- Priming for degranulation

**Eosinophils**
- Prolonged *in vitro* survival
- Increased antibody-dependent cellular cytotoxicity against schistosome larvae and tumour cells
- Priming for increased LC4 release

**Mononuclear phagocytes**
- Enhanced cytotoxicity
- Enhanced intracellular killing
- Increased phagocytosis
- Potentiation of antigen processing

Adapted from Golde and Baldwin (1992)
1.3.4.5 **Interferon γ**

Interferon γ (IFNγ) functions primarily as an immunomodulatory cytokine rather than as an antiviral cytokine as do other interferons (Wardle, 1987). For example, IFNγ has marked effects on the cell-surface expression of MHC class I and II molecules (Unanue and Allen, 1987), and is involved in the induction of expression of IL-2 receptors by T cells (Wardle, 1987). Central to the immunomodulatory influence of IFNγ is its ability to potently stimulate several macrophage functions (Unanue and Allen, 1987). IFNγ also upregulates the expression of VCAM-1 and ICAM-1 (Dustin et al., 1986; Thornhill et al., 1991) and enhances the expression of E-selectin (Doukas and Pober, 1990) by ECs (see section 1.5.1). The upregulated expression of these adhesion molecules is important in the early stages of leukocyte migration and entry into inflammatory sites.

1.3.5 **Other soluble mediators of inflammation**

Vasoactive amines such as histamine and serotonin are potent vasoactive substances associated with tissue mast cells and platelets respectively. Most histamine is stored preformed in mast cell and basophil granules, in close association with the anionic side chains of proteoglycans (mainly heparin in human mast cells, and chondroitin sulfate in human basophils) (Atkinson et al., 1992). Histamine, released in response to antigen stimulation and inflammatory mediators, induces vasodilation, increased vascular permeability and pain and is thus capable of augmenting the infiltration of leukocytes into inflammatory foci. In addition, histamine may play an important role in the regulation of immune responses. (Reviewed by (Rocklin, 1990)).

Nitric oxide (NO), which accounts for the biological properties of endothelium derived relaxing factor (EDRF), is a simple and relatively unstable reactive nitrogen intermediate with potent and pleiotropic effects (Moncada et al., 1991). NO is synthesised from L-arginine by constitutive or inducible isoforms of NO synthase found in EC, macrophages, neutrophils and other cells (Moncada, Palmer et al., 1991) and its manifold effects parallel the actions of inflammatory cytokines like IL-1. Recent reports suggest that the synthesis of NO mediates much of the antimicrobial activity of macrophages against a variety of intracellular and extracellular pathogens (Nathan and Hibbs, 1991). Besides its role as a defence molecule, NO is also emerging as an important immunoregulatory molecule, with potent effects on vascular...
permeability and leukocyte and platelet adhesion to endothelium (Moncada, Palmer et al., 1991).

1.4 Cellular components of inflammation

1.4.1 Neutrophils
Neutrophils play an important role in inflammatory reactions by removing foreign particles and damaged cells as well as secreting numerous potent pro-inflammatory and histotoxic agents (i.e., reactive oxygen intermediates, hydrolytic enzymes, chemotactic peptides, cytokines and various lipid-derived mediators) which can, paradoxically, also mediate tissue injury and disease (Malech and Gallin, 1988). This is probably why, traditionally, the neutrophil has been regarded as a primitive end cell which digests its way through tissues in order to ingest and kill bacteria before finally disintegrating (Haslett et al., 1989). Indeed, in the typical acute inflammatory response neutrophil numbers increase rapidly early in the response as they provide a first line of defence against invading foreign material and microorganisms (Huber and Weiss, 1989). But more recent evidence supports the notion that neutrophils are subtle, complex and variable in their functional responses. Like macrophages, neutrophils are capable of expressing a number of different functions and states of reactivity in response to specific factors. This notion is supported by evidence that neutrophils are capable of expressing a variety of functionally important cell-surface molecules (reviewed by (Jack and Fearon, 1988)) such as complement receptors (Table 1.4), Fc receptors (Table 1.3) and receptors for chemotactic agents (such as fMLP) which enable the neutrophil to respond to micro-environmental changes. Similarly, neutrophils are able to synthesise selectively a variety of molecules such as heat shock proteins, Class I MHC molecules and further cell-surface receptors in response to such micro-environmental changes. Neutrophils also express a number of proadhesive molecules on their surfaces, the number and functional state of which can be rapidly altered in response to specific stimuli (Zimmerman et al., 1992). These include all three β2-integrins and L-selectin, which mediate tethering to endothelium during the earliest events of neutrophil transmigration (section 1.5.1).

When primed neutrophils are activated (Fig. 1.7), for example when opsonized antigens bind to neutrophil surfaces, they undergo secretion and/or degranulation (Table 1.8), and initiate the generation of reactive oxygen species (Henson et al., 1992). Neutrophil activation responses are tightly
Fig. 1.7 Neutrophil priming for subsequent enhanced responses to chemotactic peptides has been attributed to a number of agents which have a variety of effects on the neutrophil. GM-CSF, granulocyte/macrophage colony-stimulating factor; FMLP, N-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; MDP, muramyl dipeptide; ROI, reactive oxygen intermediates.

From Haslett et al (1989)
### Table 1.8

Possible secretory products of neutrophils: enzymes, proteins and glycosaminoglycans

<table>
<thead>
<tr>
<th>Azurophil granules</th>
<th>Specific granules</th>
<th>Other granules</th>
<th>Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td></td>
<td>Acid phosphatase</td>
<td>5'-Nucleotidase</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td></td>
<td>Heparitinase</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>β-Glucosaminidase</td>
<td>Alkaline phosphatase</td>
<td>β-Glucosaminidase</td>
<td>Neutral α-glucosidase</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>Histaminase</td>
<td>α-Mannosidase</td>
<td>Deoxyribonuclease ?</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td></td>
<td>Acid proteinase</td>
<td>Ribonuclease ?</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Fucosidase</td>
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<td></td>
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<tr>
<td>Neuraminidase</td>
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<td></td>
<td></td>
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<tr>
<td>Esterase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin A, D, E, F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin G ? (acid)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Collagenolytic cathepsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastase</td>
<td>Collagenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin G (neutral)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histonase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>Lysozyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cationic proteins</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt; binding protein</td>
<td>Laminin receptor</td>
<td>Laminin receptor</td>
</tr>
<tr>
<td>Bacteriological/permeability inducing protein</td>
<td>Laminin receptor</td>
<td>C3bi receptor</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>Defensins</td>
<td>fMet-Leu Phe receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycosaminoglycans</td>
<td>Lactoferrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Chondroitin sulfate, Heparan sulfate)</td>
<td>Cytochrome b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavoproteins</td>
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</tr>
</tbody>
</table>

Adapted from Henson (1992)
regulated and usually result in minimal endothelium and tissue damage (Moser et al., 1989). It has been shown, for example, that transendothelial passage of neutrophils is not accompanied by a neutrophil secretory response (Huber and Weiss, 1989) or significant damage to endothelium or basement membrane (BM) (Huber and Weiss, 1989). Clearly, however, the potential for endothelium injury, excessive ECM degradation and tissue damage exists when defects in the control of any of these processes occurs (Malech and Gallin, 1988).

1.4.2 Mast cells and basophils
Mast cells and basophils are important effector cells in inflammation, particularly in certain forms of IgE-dependent immediate hypersensitivity, by virtue of their expression of high affinity Fc receptors for IgE (FcεRI) (Kinet, 1990) (Table 1.3). Mast cells and basophils also participate in a wide variety of IgE independent activities and play a central role in inflammation due to their ability to release a wide variety of biologically active substances including vasoactive amines, cytokines, lipid derived mediators and proteases (reviewed in (Galli and Lichtenstein, 1988; Galli, 1990)). In fact, recent studies have indicated a role for mast cells in inflammatory joint diseases (Firestein and al, 1988; Allen and al, 1990).

In humans, mast cells are found in the loose connective tissue of all organs, especially around blood vessels, nerves, lymphatics and skin (Atkinson, White et al., 1992). A major function of mast cells is the IgE-mediated release of a number of inflammatory mediators, including histamine, during anaphylactic reactions (Galli, 1990). Mast cells and basophils have also been found to contain significant preformed stores of TNFα available for immediate release upon IgE activation (Gordon et al., 1988; Steffen et al., 1989). Mast cells at sites of delayed type hypersensitivity reactions have been shown to release TNFα, which induces E-selectin expression on EC (Walsh et al., 1991). Such release of TNFα may, in part, account for the striking recruitment of leukocytes that occurs in IgE dependent responses. In fact, studies with mast cell deficient mice have provided evidence that mast cells are essential for virtually all of the early and late phases of tissue swelling and leukocyte infiltration associated with IgE dependent reactions of the skin (Wershill et al., 1991).
1.4.3 Eosinophils

Eosinophils (reviewed by (Hirsch and Hirsch, 1980; Weller, 1991; Gleich et al., 1992)) express Fc receptors for IgG and IgE (Table 1.3), as well as receptors for C1q, C3b/C4b (CR1), iC3b (CR3) and C5a (Table 1.4), and these receptors provide the basis for their effector functions towards targets, particularly parasites (Butterworth et al., 1978). Eosinophil granule proteins are powerful toxins toward multicellular parasites, supporting the role of eosinophils as an effector cell in parasitism, but these granule proteins may also play an important role in inflammation (Gleich and Adolphson, 1986). For example, C5a is chemotactic for eosinophils and stimulates the eosinophil biosynthesis of PAF, a potent mediator of inflammation (Lee et al., 1984). Furthermore, IgG coated beads can stimulate eosinophils to produce leukotrienes (Shaw et al., 1985) under conditions that probably mimic eosinophil degranulation induced by helminths. The production of eosinophils from bone marrow progenitor cells is regulated by GM-CSF and IL-3, which are produced by TH1 and TH2 cells and by IL-5 which is selectively produced by TH2 cells (section 1.4.5). Development of eosinophilia is T cell dependent, and the helminth-induced eosinophilia can be prevented by administration of anti-IL-5 antibodies (Coffman et al., 1989). Interestingly, eosinophils treated in vitro with GM-CSF also express MHC class II molecules (Lucey et al., 1989) and CD4 (Lucey et al., 1989) although the role of this MHC class II expression in inflammation remains uncertain.

There is also much evidence supporting an effector role for eosinophils in mediating tissue damage in hypersensitivity diseases ranging from chronic asthma and atopic dermatitis to cardiac disease (Parillo and Fauci, 1978; Gleich and Adolphson, 1986). Such diseases may be accounted for, in part, by the ability of cytokines, particularly IL-5, to convert the eosinophil to a long-lived cell capable of synthesising large quantities of leukotriene C4 (a potent bronchoconstrictor, section 1.3.2) and toxic granule proteins (Limaye et al., 1990). Like other leukocytes, eosinophils express adhesion molecules important in their recruitment to sites of inflammation. Thus, adhesion of eosinophils to IL-1 stimulated EC can be inhibited by antibodies to E-selectin, VCAM-1 and ICAM-1 (Bochner et al., 1991). Eosinophils (as well as T cells and monocytes, but not neutrophils) also express VLA-4, which binds to VCAM-1, and this may explain the selective accumulation of eosinophils and monocytes in allergic diseases (Weller, 1992). Once in the tissue, eosinophils secrete IL-5, a selective attractant for eosinophils, as well as IL-3 and GM-CSF which synergise to enhance eosinophil survival (Weller, 1992).
Moreover, eosinophils secrete IL-1, IL-8, MIP and TNFα, as well as TGFα and TGFβ which are important in maintaining an inflammatory response in chronic diseases in which eosinophils are involved (Wong et al., 1990; Wong et al., 1991).

1.4.4 Monocytes and macrophages

Usually, 24 hrs or more after the initiation of a typical acute inflammatory response, the prevailing cells in the inflammatory exudate are monocytes and macrophages which are recruited into sites of inflammation by T cells (section 1.4.5) (Paulnock, 1992). Monocytes are produced in the bone marrow and found in the peripheral blood, and are voracious phagocytes when they leave the capillaries and enter the tissues as macrophages (reviewed by (Adams, 1992)). During acute or chronic inflammation the number of monocytes in the circulation has been shown to increase temporarily by about 2-3 fold and the number of cells entering the inflammatory focus is doubled (van Furth et al., 1973). Macrophages that have recently arrived at a site of inflammation have the capacity to respond to a wide range of external stimuli via a battery of cell surface receptors (Spector and Mariano, 1975; van Furth, 1979; Adams and Hamilton, 1992). Receptors expressed include those important for endocytosis such as Fc receptors, complement receptors and receptors recognising carbohydrates and lipoproteins (Adams and Hamilton, 1984; Adams, 1992) (Tables 1.3 and 1.4). Other receptors expressed include those for CSFs, interferons, neuropeptides, adrenergic agents, histamine and adhesion molecules (Adams and Hamilton, 1984; Gordon, Perry et al., 1988; Silverstein et al., 1989; Springer, 1990). Macrophages are also capable of secreting over 100 defined products essential for induction and regulation of inflammation such as elements of complement (at least 10), cytokines such as IL-1, lymphotoxin, TNF-α, IL-6, a wide variety of neutral proteases, protease inhibitors and acid hydrolases, metabolites of arachidonic acid and a number of hormones (reviewed in (Adams and Hamilton, 1984; Unanue, 1984; Gordon, 1986; Helin, 1986; Nathan, 1987; Rich, 1988)).

Monocytes and macrophages are also important in the induction of immune responses due to their ability to endocytose, degrade, and present exogenous antigens in association with class I or II MHC antigens to T cells (Unanue and Allen, 1987). When macrophages act as antigen presenting cells and cause cognate T cell activation, the T cells in turn produce soluble factors, especially IFNγ (Schreiber, 1984), which stimulate macrophages to be much
more efficient in their effector functions. However, while T-cell stimulated macrophage activation is induced in an antigen specific manner, the macrophage is non-specific in its subsequent effector phase.

1.4.5 T Lymphocytes

T lymphocytes, due to their central role in mediating and regulating adaptive immunity, are a key component of many inflammatory responses. The main function of T cells is to initiate cell mediated immunity (CMI) and to control antibody production by B cells, processes which are dependent upon recognition of specific antigen on antigen presenting cells such as macrophages and dendritic cells. Other important functions of T cells include direct lysis of target cells (cytotoxicity) that express the appropriate antigens (Cohen et al., 1985; Young and Cohn, 1986; Trenn et al., 1987; Tschopp and Nabholz, 1990) and the secretion of lymphokines which modulate the activities of various cells including lymphocytes, ECs and phagocytes (reviewed by (Kelso, 1989)).

Central to the effective function of T cells is their capacity to recognise cell bound antigen. To this end, the T cell has evolved a unique antigen recognition system in which it recognises processed, but not native, antigen fragments (Unanue, 1984) which are bound to major histocompatibility complex (MHC) molecules on the cell surface (Germain, 1986). The T cell recognises and binds antigen in association with MHC via the T cell antigen receptor (TCR) (Barclay et al., 1993). Most (about 95%) T cells express TCRs consisting of a heterodimer of two disulfide-linked polypeptides designated the α and β chains. The TCR α and β polypeptides are associated with 5 other chains designated γ, δ, ε, ζ and η which form the CD3 complex - a critical component of the TCR complex (Ashwell and Klausner, 1990). The α and β chains form the antigen binding region of the TCR which interact with peptide-MHC complexes, and as such they possess variable and constant domains much like the antigen combining sites of immunoglobulins (Yin et al., 1990). This allows a high degree of diversity which easily accounts for the vast array of distinct T cell responses available to the immune system. A small subpopulation (about 5%) of peripheral T cells express γδ TCR, whose function is poorly understood.

T cells expressing the αβ TCR can be further subdivided into two distinct populations; the CD4+ and the CD8+ subsets. The major functional difference between CD4+ and CD8+ T cells concerns their recognition of
antigen. CD4+ T cells recognise antigen in association with MHC class II molecules while CD8+ T cells recognise antigens in association with MHC class I molecules. CD4+ T cells can be functionally divided into two further subsets, T_{H1} and T_{H2}, depending upon their distinctive profile of cytokine expression and resultant biological activities (Mosmann and Coffman, 1989). The T_{H1} cells produce mainly IL-2 and IFN\gamma whereas T_{H2} cells secrete IL-4, IL-5 and IL-10. Both sets produce TNF\alpha, IL-3 and GM-CSF. T_{H1} cells are potentially more effective in mediating cellular immunity than T_{H2} cells, which are generally more efficient at inducing antibody production by B cells. These differences in cytokine expression, therefore, have important implications for the protective value of an immune response. For example, murine Leishmania major infections were resolved in mice with an immune response dominated by T_{H1} cells, but progressed in mice with T_{H2} dominated immune responses (Heinzel et al., 1989).

In order for T effector cells to be activated by antigen, they need to encounter MHC-antigen complexes on antigen presenting cells (APC) in inflammatory sites. Since there is a paucity of cells expressing class II MHC in normal tissue, the availability of antigen presenting cells at sites of inflammation must be crucial for the initiation of CD4+ T cell-dependent inflammatory responses. Resident cells that are capable of acting as APC which initiate an inflammatory response include macrophages, Langerhans-dendritic cells and B lymphocytes. In order to present antigen to T cells, APC must be able to take up antigen, process it and present it in association with either class I or class II MHC molecules, as well as express appropriate secondary adhesion molecules and costimulator molecules which activate the cognate T cell (Unanue, 1984; Hodgkin et al., 1990). In this context, the recently characterised CD28/B7 interaction is a key co-stimulatory signal for T cell IL-2 production and proliferation and can determine whether antigen presentation results in T cell anergy or proliferation in T_{H1} cell lines (Parker, 1993).
1.4.6 **B Lymphocytes**

B cells represent about 10% of the circulating lymphoid pool in humans and are defined by their ability to secrete Ig. In contrast to T cells, B cells recognise foreign antigens in an MHC independent manner via cell-surface-bound Ig, and therefore recognise antigenic determinants in their native state. In terms of inflammatory responses, the essential role of B cell derived Ig in several antigen specific inflammatory reactions such as type I, type II and type III hypersensitivity reactions is well characterised (Table 1.1, section 1.2). The process of B cell entry into inflammatory sites and their local production of Ig in these sites is less well understood.

B cells must be activated before they can proliferate and secrete antibody. Certain antigens with repetitive epitopes (eg. LPS, dextrans) are able to activate B cells by cross-linking the membrane Ig as long as certain other soluble growth requirements, such as IL-2, IL-5 and IL-6, are provided (Nakanishi et al., 1984). However most B cell responses to foreign antigen require help mediated by antigen specific T cells. Cognate help by T cells can only occur when B cell surface Ig receptors have bound, internalised, processed, and expressed the antigen on the surface as peptides in association with MHC class II molecules (Vitetta et al., 1989).

Recent studies have shown that although T-B collaboration is initiated by the TCR interaction with peptide-class II MHC complexes on B cells, and while cytokine production by the T cell contributes to the B cell response, activation of the B cell is dependent upon secondary cell adhesion events. For example, membranes prepared from activated T cells can stimulate the activation of resting B cells (Hodgkin, Yamashita et al., 1990). It is now known that CD40 on B cells and an activation dependent CD40 ligand on T cells is essential for B cell activation (Armitage et al., 1992; Castle et al., 1992; Parker, 1993). In contrast, differentiation of B cells into antibody forming cells is cytokine dependent, IL-4 and IL-6 being particularly important (Hodgkin, Yamashita et al., 1990).

1.4.7 **Endothelial cells**

Endothelial cells (EC) do not simply form a passive barrier between the blood circulation and tissues. On the contrary, the strategic location of EC between the circulation and the tissues, ensures that they play an active role in inflammation, with the potential to determine the outcome of any inflammatory response. That EC play such a crucial role during
inflammation was clearly demonstrated by the early observations of Cohnheim (Cohnheim, 1882), who observed that microvessels dilated, became more permeable and increased in adhesiveness for circulating leukocytes. Thus, ECs at sites of inflammation or immune reactions are activated frequently by cytokines such as IL-1, TNFα and IFNγ and undergo dramatic morphological, functional and antigenic changes (Mantovani and Dejano, 1989). EC also produce cytokines that regulate the proliferation and differentiation of lymphocytes and their recruitment at sites of inflammation. For example, after exposure to inflammatory stimuli, EC are important producers of IL-1, IL-6 and CSFs, as well as the potent chemoattractant cytokines (chemokines) MCAF and IL-8 (Harlan, 1985; Mantovani and Dejano, 1989).

During inflammation ECs develop increased adhesiveness for circulating leukocytes due to their induced expression or upregulation of adhesion molecules such as the selectins and integrins (Bevilacqua et al., 1985; Furie and McHugh, 1989; Carlos, Schwartz et al., 1990). These events are discussed in greater detail in section 1.5.1 of this thesis. EC also seem to be able to respond to inflammatory signals, or microenvironments, by becoming angiogenic (Folkman and Brem, 1992). For example macrophages, when activated by cytokines or the appropriate microenvironmental factors such as pH, secrete angiogenic products (Nathan, 1987; Sunderkotter et al., 1991). In fact, in chronic inflammation, neovascularization is one of the principle vascular responses (Folkman and Brem, 1992). This notion seems logical, since following leukocyte extravasation, the endothelium must regenerate to replace damaged or dying EC as well as degraded ECM.

In this thesis, the role of EC in inflammation will be considered with respect to adhesion molecule upregulation and related events leading to leukocyte extravasation. These events will be discussed in more detail in the section entitled "Molecular events of inflammation”

1.4.8 Platelets

Human blood platelets (reviewed by (Weksler, 1992) are anucleate, discoid cells which are highly specialised for haemostasis and wound healing, but also participate in other bodily responses to injury, maintaining numerous inflammatory capacities. During normal conditions of blood flow platelets circulate as inactive repositories of vasoactive and inflammatory mediators sequestered in platelet alpha and dense granules. The platelet plasma membrane is extremely sensitive to many types of inflammatory stimuli
including soluble factors (ADP, thrombin, epinephrine, collagen), contact with foreign surfaces, changes in the vascular surface or even with turbulent blood flow. Activation of the platelet results in rapid (within seconds) gross changes in platelet morphology and metabolism leading to platelet adhesiveness and aggregation, acceleration of coagulation, initiation of synthesis of prostanoids and PAF, and the release of preformed vasoactive substances (Table 1.9). Platelets contain and release adhesion molecules, activate complement, produce prostaglandins and leukotrienes and proteases and enhance vascular permeability (Table 1.10). Most of these activities are mediated by soluble factors expressed by the platelets, but platelet-cell interactions also play an important role. For example, P-selectin, found in the alpha granules of resting platelets, fuses to the plasma membrane after granule secretion where it may mediate platelet interaction with leukocytes (Hamburger and McEver, 1990). Exposure of human ECs to thrombin-activated platelets, which express surface associated IL-1, results in expression of ICAM-1 and E-selectin by the EC, and stimulates endothelial secretion of IL-6 and GM-CSF (Hawrylowicz et al., 1989). Thus, platelets play an important role as they interact extensively with circulating leukocytes on the one hand, and with vascular endothelium on the other hand, to modulate inflammatory activities of each of these cell types.

1.5 Molecular events of inflammation

The processes of inflammation and normal lymphocyte recirculation hinge upon the ability of leukocytes to leave the circulation, and extravasate into the tissues. For example, lymphocyte recirculation through lymph nodes and gut associated lymphoid tissue (GALT) requires lymphocyte adhesion to the specialised cuboidal ECs of high endothelial venules (HEV) followed by migration into lymphoid organs (Gowans and Knight, 1964; Picker, 1992). Similarly, during an inflammatory response, leukocytes also adhere to venular endothelium before migrating between ECs to the underlying tissues (Lasky, 1992; Zimmerman, Prescott et al., 1992; Hogg and Landis, 1993). Research on tumour cell invasion and metastasis, and on the shared capacity of various leukocytes to extravasate, has led over the years to a conceptual roadmap of the processes of invasion (Nicolson, 1982; Terranova et al., 1986; Dustin and Springer, 1991; Smith et al., 1991; Schweighoffer and Shaw, 1992). According to the model that has emerged, recruitment of
Table 1.9

Platelet constituents with inflammatory potential

<table>
<thead>
<tr>
<th>Alpha granule</th>
<th>Dense granule</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>Serotonin</td>
<td>Thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>ADP</td>
<td>12-HETE</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Calcium</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td></td>
<td>Reactive Oxygen species</td>
</tr>
<tr>
<td>Plasminogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine-rich glycoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-2 plasmin inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet factor 4 (antiheparin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFα and TGFβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic FGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-lysin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permeability factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decay-accelerating factor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From Weksler (1992)

Table 1.10

Inflammatory functions of platelets

- Release of adhesion molecules
- Activation/regulation of complement
- Alteration of vascular permeability
- Production of chemotactic factors
- Uptake, release, metabolism of vasoactive mediators
- Release of arachidonic acid metabolites (eicosanoids)
- Release of growth factors
- Acceleration of coagulation and fibrinolysis
- Modulation of leukocyte functions
- Binding to/interaction with microorganisms

From Weksler (1992)
leukocytes from the circulation into the inflammatory focus can be divided into at least three main steps (Fig. 1.8):

1) Adhesion: The first event in leukocyte recruitment involves adhesive interactions between the stimulated endothelium and the circulating leukocytes. These adhesive interactions are mediated by a large variety of adhesion molecules (Springer, 1990; Hynes, 1992; Hogg and Landis, 1993). The selectin family of adhesion molecules is responsible for the initial adhesion event, resulting in transient adhesion, or leukocyte rolling along the venule (Hogg, 1992). These transient interactions lead to activation of the leukocytes and the upregulation of the more avidly binding integrin family of adhesion molecules (Webb et al., 1990; Dustin and Springer, 1991; Schweighoffer and Shaw, 1992).

2) Transmigration: As leukocytes encounter and traverse through the endothelium, and then the sub-endothelial extracellular matrix (ECM), they participate in reversible adhesive interactions with protein and/or carbohydrate components of the ECM. Indeed, a number of lymphocyte adhesion receptors for ECM components have been identified (Dustin and Springer, 1991; Shimizu and Shaw, 1991).

3) BM degradation and repair: According to this model, the elaboration of hydrolytic enzymes is required to clear a channel for movement through the subendothelial BM (Kaiser, 1980; Liotta et al., 1982; Ratner, 1992). At this point, chemotactic gradients and the interaction of ECM components with motility-mediating receptors on leukocytes are probably important in guiding leukocyte migration (Ratner, 1992). Finally, ECs actively repair breaches in the ECM and BM by synthesising their constituent components (Nicolson, 1982; Huber and Weiss, 1989). The following discussion will focus on some of the steps outlined in this model of cellular extravasation.

1.5.1 Adhesion molecules

During inflammation, adhesion molecules serve to enhance cell-cell interactions and transmit signals that direct effector functions. A cascade of adhesion molecule-counter receptor interactions has been described which is important in the binding of leukocytes to the endothelium during leukocyte extravasation (Fig. 1.9, 1.10) (reviewed by Springer, 1990; Hynes, 1992; Zimmerman, Prescott et al., 1992; Hogg and Landis, 1993)). Cell adhesion molecules have been classified into four main groups, or families, on the basis of structural homologies (Table 1.11). These are the selectins, the integrins, the immunoglobulin (Ig) superfamily and the link protein associated family.
Fig. 1.8. Stages in the extravasation of neutrophils (or other leukocytes) through the vascular endothelium
Fig. 1.9 Model for the adhesion mechanisms underlying leukocyte movement from the blood circulation into tissues.

a) Selectins initiate rolling contacts between leukocytes and endothelium allowing time for the leukocytes to become exposed to locally released signaling molecules.

b) Integrins on the activated leukocytes mediate shear-resistant attachment and transendothelial cell migration

Adapted from Springer (1990) and Shimizu et al (1991)
Fig. 1.10 Schematic diagram of molecules mediating adhesion of a) neutrophils and b) lymphocytes to EC. The amino-terminal domains of selectins interact with carbohydrate counter-structures. Leukocyte integrins bind to specific immunoglobulin-like domains of their EC counter-receptors. The surface expression or adhesive function of many molecules is regulated by the state of the cell.

From McEver (1992)
## Table 1.11

Cell adhesion molecules implicated in inflammation

<table>
<thead>
<tr>
<th>Immunoglobulin superfamily</th>
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<tbody>
<tr>
<td>CD2</td>
<td>CD58 (LFA3)</td>
<td>CD31</td>
</tr>
<tr>
<td>CD34 (ICAM-1)</td>
<td>ICAM-2</td>
<td>VCAM-1</td>
</tr>
<tr>
<td>T-Cell receptor (TCR)</td>
<td>CD4 (TCR coreceptor)</td>
<td>CD8 (TCR coreceptor)</td>
</tr>
<tr>
<td></td>
<td>MHC Class I</td>
<td>MHC Class II</td>
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</table>

<table>
<thead>
<tr>
<th>Integrins</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>β1</td>
<td>VLA-1-3 (CD49a, b, c/CD29)</td>
<td>VLA-4 (CD49d/CD29)</td>
</tr>
<tr>
<td></td>
<td>VLA-5 (CD49e/CD29, Fibronectin receptor)</td>
<td>VLA-6 (CD49f/CD29, Laminin receptor)</td>
</tr>
<tr>
<td></td>
<td>CD51/CD29 (αβ1, Vitronectin receptor)</td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>CD11a/CD18 (LFA-1)</td>
<td>CD11b/CD18 (Mac-1 or Mo-1)</td>
</tr>
<tr>
<td></td>
<td>CD11c/CD18 (p150,95)</td>
<td></td>
</tr>
<tr>
<td>β3</td>
<td>CD51/CD61 (Vitronectin receptor)</td>
<td>CD41/CD61 (Platelet gp-IIb/IIIa)</td>
</tr>
<tr>
<td>β4</td>
<td>CD49f/CD-</td>
<td></td>
</tr>
<tr>
<td>β5</td>
<td>CD51/CD-</td>
<td></td>
</tr>
<tr>
<td>β7</td>
<td>LPAM-1, (αβ7)</td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>Selectins</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Selectin (LAM-1, LEC-CAM-1, LHR, MEL-14, Leu 8, Ly-22, gp90MEL)</td>
<td>E-Selectin (ELAM-1, LEC-CAM-2)</td>
<td>P-Selectin (GMP-140, LEC-CAM-3, PADGEM, CD62)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Link protein associated</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>CD44 (Hermes antigen, ECMRIII, HCAM, gp90 Hermes, PGP-1)</td>
<td></td>
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</tr>
</tbody>
</table>

LFA, leukocyte function antigen; ICAM, intercellular cell adhesion molecule; VCAM, vascular cell adhesion molecule; CD, cluster designation; VLA, very late antigen; gp, glycoprotein, MHC, major histocompatibility complex; CAM, cell adhesion molecule; LEC, Lectin endothelial complement domains; LHR, lymphocyte homing receptor; LAM, leukocyte adhesion molecule; ELAM, endothelial leukocyte adhesion molecule; MEL, murine erythroleukemia; GMP, granule membrane protein.
(Adapted from: Arai (1990), Brandley (1990), Long (1992), Springer (1990))
The members of these families and their relevance to cell-cell and cell-ECM interactions during inflammation will be discussed in more detail below.

1.5.1.1 Selectins

The selectin family consists of three membrane glycoproteins which mediate adhesion to ECs via carbohydrate ligands (reviewed by (Springer, 1990; Hogg, 1992; Lasky, 1992; Varki, 1992). During inflammation, selectins mediate the initial adhesive interactions that result in leukocyte rolling along the venule prior to the upregulation of integrins. Each member of the selectin family of adhesion molecules contains a single N-terminal calcium-dependent lectin binding domain, an epidermal growth factor (EGF) receptor domain, and a region of cysteine-rich tandem repeats which are homologous to complement binding proteins (Bevilacqua, Stengelin et al., 1989; McEver, 1992) (Fig. 1.11). The ligands for the selectins are anionic glycoconjugates (Springer, 1991; Imai et al., 1993), (more specifically, sialylated, fucosylated lactosaminoglycans (Brandley et al., 1990; Lowe et al., 1990)) and this sets them apart from adhesion molecules of the Ig and integrin families. The essential features of each type of selectin will be briefly summarised below.

L-Selectin

L-selectin (mol. wt 50 kD) (previously referred to as the MEL-14 antigen or the Leu-8 antigen) was first shown to play an important role in lymphocyte recirculation and homing (Siegelman and Weissman, 1989). L-selectin on lymphocytes was subsequently shown to recognise carbohydrate determinants on the peripheral lymph node addressin expressed by high endothelial venules (HEV) (Imai et al., 1991; Spertini et al., 1991), an interaction which controls the entry of lymphocytes into peripheral lymph nodes. L-selectin is found not only on subsets of lymphocytes, but is also constitutively expressed on the surface of monocytes and neutrophils (Jutila et al., 1989). In fact, L-selectin on neutrophils is responsible for neutrophil rolling along venules prior to extravasation during acute inflammation (Ley et al., 1991; Zimmerman, Prescott et al., 1992). L-selectin also binds to cultured ECs activated by cytokines (Spertini et al., 1991) and has thus been implicated not only in the homing of lymphocytes to peripheral lymph nodes, but also in the adhesion of leukocytes to EC at inflammatory sites.

L-selectin, along with P and E-selectin, can bind to multivalent forms of the tetrasaccharide, sialyl Lewis x (sLe$^x$, Neu5Acα2-3Galβ1-4(Fucα1-
Fig. 1.11 The selectin family showing N-terminal lectin-like domain, EGF-like domain and short consensus repeats roughly to scale.
Adapted from (Springer, 1990, 1991)
3)GlcNAcβR) and its isomer, sialyl Lewis a (sLe\(^a\), Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβR) (Berg et al., 1991; Varki, 1992) but the preferred leukocyte ligand for L-selectin, allowing interactions of higher affinity, appears to require additional features imparted by specific protein determinants. GlyCAM-1, (Lasky, 1992) a 50 kD sulfated glycoprotein heavily substituted with O-linked oligosaccharides, has recently been identified as a specific ligand for L-selectin.

It seems paradoxical that despite the fact that leukocyte extravasation is tightly regulated, individual receptors such as L-selectin can apparently participate in multiple leukocyte-EC interactions that are quite independently regulated. It is obvious that such behaviour cannot be explained simply by the lock and key models of cell-cell recognition, and that more complex controls of cell-cell interaction events must occur in vivo. An explanation has been offered by the report that lineage specific differences in leukocyte migration are controlled by upregulation of the molecule by each cell type following lineage specific stimulation (Spertini, Luscinskas et al., 1991). It also appears certain that multiple receptor-ligand interactions are required for productive cell-cell adhesion to occur (Ley et al., 1991; Ley, Gaehtgens et al., 1991; Spertini, Kansas et al., 1991; Spertini, Luscinskas et al., 1991; Von Adrian et al., 1991).

**P-Selectin**

P-selectin, (Brandley, S.J. et al., 1990; Carlos and Harlan, 1990) is a 140 kD protein found in platelet α granules and EC Weibel-Palade bodies. P-selectin is rapidly mobilised from platelet granules or EC Weibel Palade bodies to the cell surface following stimulation by proteins/enzymes of the coagulation cascade (such as thrombin) or pro-inflammatory agents such as phorbol esters, histamine or oxygen radicals (McEver, 1991; Harlan and Liu, 1992). The evidence so far suggests that, like E-selectin, P-selectin plays an important role in adhesive interactions predominantly between myeloid cells (Bochner, Luscinskas et al., 1991; McEver, 1991), subsets of natural killer cells and memory T lymphocytes (Damle et al., 1992; Moore and Thompson, 1992) and cytokine-activated endothelium (Bevilacqua, Stengtelin et al., 1989). P-selectin has also recently been shown to bind to chronically stimulated, but not resting, CD4\(^+\) T lymphocytes as well as T lymphocytes isolated from the synovial fluid of RA patients (Damle, Klussman et al., 1992).
P-selectin can bind to multivalent forms of the tetrasaccharide, sLex and sLeα (Berg, Robinson et al., 1991; Varki, 1992) but, unlike E-selectin, P-selectin can also bind to sulfatides (3-sulfated galactosyl ceramides) and to sulfated polysaccharides such as fucoidan, heparin and dextran sulfate (Aruffo et al., 1991). This suggests that an anionic carbohydrate ligand is involved, although a clear role for the sulfatide and charged polymer binding regions of P-selectin in leukocyte adhesion is yet to be defined.

E-selectin

E-selectin (mol. wt 115 kD) was first identified using a panel of monoclonal antibodies (mAbs) raised against cytokine-treated EC. These mAbs were screened for their ability to bind selectively to activated EC and to inhibit neutrophil adhesion to the cytokine-activated EC (Bevilacqua, 1987). The nucleotide sequence of E-selectin has revealed its relationship with other cell adhesion molecules, notably murine L-selectin (MEL-14), P-selectin, and complement pathway regulatory proteins (Springer, 1990). E-selectin is minimally expressed on unstimulated EC, but transiently expressed by ECs at their apical surface 2-4 hr after activation by LPS and the cytokines TNFα, lymphotoxin, and IL-1β (Bevilacqua, 1987; Bevilacqua, Stengelin et al., 1989). It is expressed only by EC of veins and venules in pathological settings, mediating the adhesion of myeloid cells (McEver, 1991), eosinophils (Bochner, Luscinskas et al., 1991), subsets of natural killer cells (Moore and Thompson, 1992) and memory T lymphocytes (Shimizu and Shaw, 1991) to cytokine-activated endothelium (Bevilacqua, Stengelin et al., 1989). This is consistent with the role of selectins in recruiting circulating leukocytes as the first step in mediating leukocyte extravasation during acute, chronic and allergic inflammatory responses.

1.5.1.2 Integrins

The integrins (Tables 1.11 and 1,12) include a family of cell membrane heterodimeric glycoproteins consisting of non-covalently linked α and β subunits. They are divided into several groups based on distinct β subunits (e.g. β1 or CD29, β2 or CD18 and β3 or CD61 and others), sharing multiple α subunits (Springer, 1990; Hynes, 1992) (Fig. 1.12). The integrins were first discovered during studies of the molecular nature of cellular interactions with the ECM (Hynes, 1981; Tamkun et al., 1986; Burridge et al., 1988). These studies demonstrated the role of integrins in controlling the ability of the ECM to influence cell growth, differentiation and motility. It is now known that this broadly distributed family of adhesion molecules is expressed on
Figure 1.12

_Schematic Structure of an Integrin._

(Albelda and Buck, 1990).
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leukocyte determinants</th>
<th>Endothelial ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Molecule Structure</td>
<td>Molecule Structure</td>
</tr>
<tr>
<td>Mac-1</td>
<td>CD11b/CD18 $\beta 2$ integrin Neutrophils, monocytes and eosinophils</td>
<td>ICAM-1 Ig superfamily</td>
</tr>
<tr>
<td>LFA-1</td>
<td>CD11a/CD18 $\beta 2$ integrin Neutrophils, monocytes and lymphocytes</td>
<td>ICAM-1 Ig superfamily</td>
</tr>
<tr>
<td>P150,95</td>
<td>CD11c/CD18 $\beta 2$ integrin Neutrophils, monocytes,</td>
<td>Unknown</td>
</tr>
<tr>
<td>VLA-4</td>
<td>$\beta 1$ integrin Lymphocytes, monocytes and eosinophils</td>
<td>VCAM-1 Ig superfamily</td>
</tr>
<tr>
<td>L-selectin</td>
<td>Selectin Neutrophils, eosinophils, monocytes and lymphocytes</td>
<td>glyCAM-1 Anionic polysaccharide with fucose</td>
</tr>
<tr>
<td>Sialyl Lewis X</td>
<td>Sialylated fucosylated lactosaminoglycan Neutrophils, monocytes</td>
<td>E-selectin Selectin</td>
</tr>
<tr>
<td>Sialyl Lewis X</td>
<td>Sialylated fucosylated lactosaminoglycan Neutrophils, monocytes</td>
<td>P-selectin Selectin</td>
</tr>
</tbody>
</table>

Adapted from Kishimoto and Anderson (1992)
virtually every cell type (reviewed by (Springer, 1990; Ruoslahti, 1991; Hynes, 1992)). An important feature of the integrins is that they are unable to bind ligand unless they are activated - either by soluble mediators (hormones, cytokines, etc) or by cellular or ECM components (Hynes, 1992) (Table 1.12). On circulating leukocytes integrins are in an inactive state, and can be activated by phorbol esters and various inflammatory mediators such as C5a and PAF (Hynes, 1992).

During inflammation or lymphocyte recirculation, integrins are largely responsible for the arrest of rolling leukocytes by allowing binding to their counter-receptors on endothelium (discussed below). But these "leukocyte migration" integrins are also responsible for other adhesive interactions of immune cells important during inflammation. LFA-1 participates in T-lymphocyte antigen-dependent adhesion to and killing of some target cells (Kurzinger et al., 1982; Springer et al., 1982; Kohl et al., 1984). Mac-1 is a complement receptor (CR3) (Beller et al., 1982) and thus plays a role in binding and phagocytosis of iC3b-opsonized particles by macrophages and neutrophils (Rothlein and Springer, 1985). Mac-1-dependent adhesion to ECs and matrix proteins has also been shown to elicit massive bursts of reactive oxygen by neutrophils (Shappell et al., 1990). Such a function might not only be important in the elimination of pathogens, but also in contributing to tissue damage during inflammatory pathologies under some circumstances. P150,95 is also a complement receptor (Malholtra et al., 1986) but its functional significance in inflammation is less well defined.

An exhaustive review of the integrins and their many functions is impossible here. Instead, a brief outline of some of the integrins that are involved in leukocyte-EC and leukocyte-ECM interactions will be given below.

1.5.1.2.1 **Integrins involved in leukocyte-EC interactions**

From the earliest observations of inflammation (described in section 1.1 of this thesis) it was clear that, following tissue injury, circulating leukocytes initially formed brief reversible attachments with venular endothelium before adhering tightly and finally migrating into the inflammatory focus. Though demonstrated most clearly with neutrophils (Lawrence and Springer, 1991), it is clear from recent data that selectins support the initial leukocyte rolling events, and that this is a prerequisite for integrin dependent firm adhesion and emigration (Lawrence and Springer, 1991; McEver, 1992; Hogg and Landis, 1993) (Fig. 1.9). It has also become clear that following initial
adhesion by selectins, there may be a number of activation steps, or an activation cascade, invoking integrin activation at inflammatory sites which then mediate strong adhesion to their counter-receptors on inflamed endothelium (Butcher, 1991; Schweighoffer and Shaw, 1992). More than a dozen membrane receptors have been identified so far that cause activation of the β2 integrin, LFA-1 (Pardi and Inverardi, 1992), and recently a lipid intermediate able to modulate integrin affinity was isolated from stimulated neutrophils (Hermanowski-Vosatka et al., 1992). Moreover, evidence now exists for selective activation of integrins on specific cell types. For example, CD31 is expressed at higher levels on naive CD8+ T cells than on CD4+ T cells, and triggering through CD31 could thus activate CD8+ cells preferentially (Tanaka et al., 1992). Further examples of integrin activation and modulation are reviewed extensively elsewhere (Butcher, 1991; Hynes, 1992; Schweighoffer and Shaw, 1992; Zimmerman, Prescott et al., 1992).

The leukocyte, or β2 integrins, which are found to play an important role in cell-cell interactions during inflammation include LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) (Hynes, 1992) (Table 1.12, Fig. 1.9). The β1 integrin, VLA-4 (CD49d/CD29), is also important in such cell-cell interactions, mediating the attachment of monocytes, lymphocytes, eosinophils and basophils to the VCAM-1 counter-receptor on cytokine stimulated EC (Stoolman, 1992). VLA-4 is an unusual integrin in that it has counter-receptors both on EC and on the ECM (Hemler, 1990). LFA-1 is expressed on virtually all immune cells (Miller et al., 1985) with the exception of some macrophages (Kurzinger and Springer, 1982). In contrast, Mac-1 has a more limited cellular distribution than LFA-1, being expressed on myeloid cells including monocytes, macrophages, neutrophils, eosinophils and basophils, as well as large granular lymphocytes and a subset of CD5+ B cells (de la Hera et al., 1988; Hynes, 1992). The distribution of p150,95 is similar to that of Mac-1, but with expression also on activated lymphocytes (Kurzinger, Ho et al., 1982; Springer, Davignon et al., 1982).

The counter-receptors for integrins, produced by endothelium or underlying inflamed tissue, are also upregulated by inflammatory mediators (Table 1.12). Expression of ICAM-1, the ligand for LFA-1, is induced on endothelium by a number of inflammatory mediators, particularly TNFα and IL-1 (Dustin et al., 1988). LFA-1 also binds to ICAM-2, which is constitutively expressed on EC, and possibly to ICAM-3, which is expressed on some haematopoietic cells but not EC (De Fougerolles et al., 1991). Mac-1 and p150,95 function as
complement receptors and are particularly important in adhesion of monocytes and neutrophils to EC and other substrates during activation of the complement and clotting cascades (Kishimoto et al., 1989). Mac-1 also binds to ICAM-1, but not ICAM-2 (Diamond et al., 1990). P150,95 interacts with cytokine stimulated endothelium but its counter-receptor on endothelium is unknown (Stacker and Springer, 1991). As mentioned earlier, VLA-4 binds to VCAM-1 on cytokine-stimulated endothelium (Elices et al., 1990) and mAbs specific for VLA-4 partially inhibit lymphocyte migration into delayed-type hypersensitivity lesions in the skin and joints (Issekutz, 1991). Since VCAM-1 has been found on ECs at sites of atheromas, VLA-4 may also play a role in the recruitment of mononuclear cells in vascular pathologies (Cybulsky and Gimbrone Jr., 1991).

Perhaps the best in vivo example of the importance of integrins in inflammation is in the congenital leukocyte adhesion deficiency (LAD) in which there are mutations in the common β2 subunit. LAD patients experience recurring, life-threatening infections due to the inability of their neutrophils to orient and migrate in response to chemoattractants, and their inability to bind and cross the endothelium at sites of infection (Kishimoto, Larson et al., 1989). The importance of VLA-4 in leukocyte binding and transmigration was also highlighted by recent studies using the animal model of autoimmune encephalomyelitis (EAE) (section 1.6.2) where the entry of myelin basic protein (MBP) specific T cells into the brain was shown to be completely dependent on elevated levels of VLA-4 (Baron et al., 1993).

1.5.1.2.2 Integrins involved in leukocyte-ECM interactions
Integrins appear to be the major receptors by which cells attach to the ECM (Hynes, 1992) and through this function they may play an important role in leukocyte transmigration (Shimizu and Shaw, 1991). For example, members of the β1 integrin family of heterodimeric receptors (also known as the VLA family), which are expressed on many leukocytes, have been reported to mediate the adhesion of leukocytes to ECM components such as collagen, fibronectin and laminin (Albelda and Buck, 1990, Shimizu, 1991; Springer, 1990) (Table 1.11 ). The interaction of leukocyte integrins with counter-receptors on ECM components may provide anchoring and/or signalling functions for leukocytes allowing migration into and through the ECM (Elices, Osborn et al., 1990). Indeed, some in vitro studies have provided evidence that the interaction of integrins with collagen, fibronectin and laminin influences leukocyte motility during extravasation (Arencibia and Sundqvist,
Recently, in certain rodent inflammation models anti-VLA-4, but not anti-LFA-1, mAbs were able to block extravasation of T cells (Mackay and Imhof, 1993). This suggests that VLA-4 may be operating in preference to LFA-1 under some physiological circumstances and may reflect the importance of the β1 integrins in binding BM components during transendothelial migration.

1.5.1.3 Immunoglobulin superfamily

Many of the cell adhesion molecules in the immunoglobulin (Ig) superfamily, acting in concert with adhesion molecules from other families, are involved in important cell-cell adhesion events which control immune responsiveness. For example, members of the family include the antigen specific receptors of T and B cells, CD3, CD4, CD8, and the MHC class I and II molecules (Table 1.11). There are several extensive reviews on these molecules (Janeway, 1992; Keegan and Paul, 1992). Those members of the Ig superfamily which play a role in leukocyte-endothelium adhesion, particularly during inflammation, will be discussed further here (Fig. 1.10, Tables 1.11 and 1.12).

ICAM-1, 2 and 3

ICAM-1 (intercellular adhesion molecule-1) is an Ig-like adhesion molecule widely distributed on cells including ECs, lymphocytes, follicular dendritic cells, fibroblasts and epithelial cells (Dustin, Rothlein et al., 1986)). It is a ligand for LFA-1 (Marlin and Springer, 1987) and has been identified as the major integrin counter-receptor involved in inflammation leading to several pathological states. ICAM-1 is expressed constitutively, but at low levels, on resting EC and HEV but is markedly upregulated (predominantly on venular EC), by the cytokines IL-1 and TNFα (Pober et al., 1986). Under these circumstances, ICAM-1 expressed on EC binds both neutrophils and lymphocytes and may also be a ligand for Mac-1 as well as LFA-1 (Smith et al., 1988). Another cell adhesion molecule, which is homologous to ICAM-1 and a ligand for LFA-1, is ICAM-2 (Staunton et al., 1989). ICAM-2 is expressed constitutively by EC, and there have been no reports in the literature so far to indicate that its expression is upregulated by proinflammatory cytokines. Its role in cell adhesion reactions thus remains unclear at this stage. The third LFA-1 ligand, ICAM-3, found on lymphocytes, monocytes and neutrophils, has recently been cloned and found to be homologous to ICAM-1 and ICAM-2 (Fawcett et al., 1992). The low level of ICAM-3 expression on resting leukocytes has lead to the suggestion that it is potentially the most important ligand for LFA-1 in the initiation of
the immune response (Fawcett, Holness et al., 1992).

**VCAM-1**

VCAM-1 (vascular cell adhesion molecule 1) (Osborn et al., 1989) is minimally expressed on unstimulated EC, but is rapidly induced by LPS, TNFα or IL-1 (Bevilacqua, Stengtelin et al., 1989; Carlos, Schwartz et al., 1990; Graber et al., 1990). VCAM-1 binds mononuclear leukocytes but not neutrophils (Bevilacqua, Stengtelin et al., 1989; Carlos et al., 1990). This binding specificity can be explained by the restricted expression of the VCAM-1 counter-receptor, the β1 integrin VLA-4, which is found on monocytes and lymphocytes but not on neutrophils (Elices, Osborn et al., 1990). The properties of VCAM-1 make it a prime candidate for playing a central role in the recruitment of T cells into sites of inflammation (Damle, Klussman et al., 1992). Indeed, recent experiments with EAE, a VLA-4-dependent inflammatory disease model, demonstrated that mAbs directed to the VCAM-1-binding portion of VLA-4, and not to the fibronectin-binding portion, prevent disease (Yednock et al., 1992).

**CD31**

CD31 (previously known as PECAM-1 or endoCAM) (Albelda et al., 1991) is, like ICAM-1, a member of the Ig superfamily and is expressed by EC, platelets and most leukocytes, including lymphocytes (Muller et al., 1989; Stockinger et al., 1990). There is recent evidence to suggest that CD31, while probably mediating only weak binding interactions between lymphocytes and endothelium, is most important in its ability to trigger the adhesive functions of β1 and β2 integrins (Tanaka, Albelda et al., 1992). The localisation of CD31 at inter-EC junctions has also lead to the suggestion that it is involved in the transmigration of lymphocytes across endothelial layers (Albelda et al., 1990; Albelda, Muller et al., 1991). Two ligands for CD31 have been identified, CD31 itself which represents a homotypic interaction (Albelda, Muller et al., 1991) and certain GAGs such as heparan sulfate (Watt et al., 1993). The role of these two ligands in CD31 function is still unclear.

1.5.1.4 **Link protein associated family**

**CD44**

The only known cell adhesion molecule which is a member of the link protein associated family of molecules is CD44 (otherwise called HCAM, Hermes antigens, Pgp-1, ECMRIII). It is an acidic, sulfated protein with O- and N-linked polysaccharide side-chains (Jalkanen et al., 1987), expressed by a
variety of cell types including T cells, smooth muscle cells, epithelial cells, monocytes (Flanagan et al., 1989) and fibroblasts (Carter and Fearon, 1989). Cloning data have revealed structural similarities between the amino-terminal extracellular domain sequence of CD44 and cartilage link proteins and a related sequence found on proteoglycan core proteins (Goldstein et al., 1989). CD44 has been implicated in a number of functions. It is involved in lymphocyte binding to HEV possibly via recognition of hyaluronic acid (Jalkanen, Bargatze et al., 1987; Holzmann and Weissman, 1989). It also binds to the ECM components collagen types I and VI and fibronectin and has been proposed to link the fibroblast cytoskeleton with the ECM (Carter and Fearon, 1989). CD44 also appears to play an important role in leukocyte cell-cell adhesion and activation. For example, antibodies to CD44 have been shown to inhibit the CD2-LFA-3 interaction which normally leads to E-rosette formation between human lymphocytes and sheep erythrocytes (Shimizu et al., 1989). Furthermore, certain anti-CD44 mAbs dramatically augment the T cell proliferation induced by CD3 and CD2 mAbs (Shimizu, van Seventer et al., 1989). Similarly, the engagement of monocyte CD44, as well as LFA-3 and CD45, was also shown to induce the release of TNFα and IL-1β by monocytes (Webb, Shimizu et al., 1990). Some of the activities of CD44 also may be due to its ability to immobilise soluble triggering molecules such as MIP-1β (Tanaka et al., 1993).

1.5.2 The subendothelial basement membrane as a barrier to leukocyte extravasation

Once leukocytes have transmigrated through the vascular EC lining they are faced with the task of infiltrating the fibrous barrier of the subendothelial BM. The BM is highly discriminatory, controlling the passage of plasma proteins and other molecules from capillaries into tissues. For example, while Igs, complement proteins and some other plasma proteins are found in normal tissue, their concentration is significantly lower than that found in the blood. In fact, the largest Ig, IgM is not found outside of the blood circulation (Mims, 1982). If the BM forms a selectively permeable barrier for the passage of molecules from the vasculature to the tissues, it seems reasonable to suppose that it also provides a formidable barrier to the extravasation of leukocytes. Despite its obvious importance, this aspect of leukocyte extravasation is far less studied and understood than the processes of adhesion and transmigration described earlier.
Adhesion molecules, especially of the integrin family, have been shown to be important in mediating adhesion of leukocytes to ECM components, an important event in BM extravasation (see section 1.5.1) (Dustin and Springer, 1991; Shimizu et al., 1991). The latest extravasation models also suggest that hydrolytic enzymes may play an important role by 1) reversing adhesive interactions, 2) degrading BM components, (which may also release fragments with motility stimulating qualities) and 3) clearing a path for the extravasating leukocyte (Ratner, 1992). The suggestion that hydrolytic enzymes may play a role in leukocyte extravasation leads us to ask which enzymes are important, what is their origin and how are they expressed in order to bring about matrix solubilization. These issues are the subject of the following subsections, but before attempting to answer these questions, the composition and structure of the BM will be described

1.5.2.1 The basement membrane

BMs are a specialised type of ECM synthesised by ECs and epithelial cells (Pauli et al., 1983). They are organised, continuous ECM structures which form the outermost layer of blood vessels, underlying the basal surface of vascular EC, mesothelial and epithelial cells. BMs also surround muscles cells, Schwann cells, adipocytes and many of the body’s vital organs including the kidney (Vracko, 1974; Yurchenco and Schittny, 1990). The extensive crosslinking and low solubility of the BM components made the task of isolating and accurately characterising its constituents very difficult. However, the discovery in the late 1970’s of the Engelbreth-Holm-Swarm (EHS) murine tumour, which produced large amounts of BM macromolecules with a low degree of crosslinking, finally allowed detailed structural analysis of the building blocks of BM (Orkin et al., 1977). A major component of the BM which is important in delineating boundaries between tissues, is collagen type IV, which is found in no other type of ECM. Collagen type IV forms a flat polymer which binds heparan sulfate proteoglycans (HSPG) and other molecules unique to BM such as laminin, and entactin (nidogen), forming a barrier which is also somewhat more dense than normal ECM (Yurchenco and Schittny, 1990; Mosher et al., 1992). Type IV collagen, laminin and HSPG account for the greatest mass in BMs. Some other BM components include fibronectin, osteonectin (SPARC, BM40) and type VII procollagen (Timpl, 1989). The principle properties of the BM components are summarised in Table 1.13 and a schematic representation of the three dimensional structure of the three major components, collagen type IV, laminin and HSPG, is depicted in Fig. 1.13 (see also Fig. 1.14).
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Size ($M_r$)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IV collagen</td>
<td>550 000 - 600 000</td>
<td>Network formation&lt;br&gt;Binding to HSPG, laminin, entactin, cells</td>
</tr>
<tr>
<td>Proteoglycans (HSPG and CSPG)</td>
<td>130 000 - 1 000 000</td>
<td>Charged barrier.&lt;br&gt;Binding to laminin, collagen type IV, cells.&lt;br&gt;Binding to CD45, Mac-1.&lt;br&gt;Repositories for bFGF, TGFβ, other growth factors?&lt;br&gt;Activating bound growth factors</td>
</tr>
<tr>
<td>Laminin</td>
<td>1 000 000</td>
<td>Cell attachment and binding to collagen type IV, HSPG, entactin binding</td>
</tr>
<tr>
<td>Entactin (nidogen)</td>
<td>150 000</td>
<td>Binding to laminin, collagen IV,</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>450 000</td>
<td>Cell attachment, multiple binding abilities</td>
</tr>
</tbody>
</table>

HSPG, heparan sulfate proteoglycans; CSPG, chondroitin sulfate proteoglycans
Adapted from (Abrahamson, 1984)
Fig. 1.13 Schematic representation of the three dimensional structure of the three major components of BM - type IV collagen (black), laminin (red) and HSPG (blue). Type IV collagen and laminin form double polymer networks. HSPG complexes interact with laminin and collagen through its polyanionic side chains.

Adapted from Yurchenco and Schittny (1990)
Collagen Type IV

Laminin

Heparan Sulphate PG
Fig. 1.14

Scale diagrams of basement membrane macromolecules based on electron microscopy of rotary shadowed preparations.

a. Type IV collagen monomer
b. Type IV collagen tetramer. Networks of collagen IV may also form by crosslinking of tetramers at the large, terminal globular domains and by lateral associations along the arms
c. Heparan sulfate proteoglycan showing four heparan sulfate chains bound to a smaller core protein. Larger forms probably also exist
d. Laminin
e. Fibronectin
f. Entactin/nidogen with its characteristic dumbbell shape

Adapted from Abrahamson (1986) and Yurchenco and Schitity (1990)
1.5.2.1.1 Type IV collagen
Type IV collagen is a non-fibrillar, flexible, threadlike collagen occurring uniquely in BMs. It is composed of three \( \alpha \) chains arranged primarily as a triple helix (Yurchenco and Schittny, 1990). The amino-terminal triple helical ends of type IV collagen are capable of anti-parallel overlapping with the same segments from three other type IV molecules, meaning that each type IV molecule is capable of forming a tetramer (Timpl, 1989) (Fig. 1.14). The overlapped regions are further stabilised by inter- and intramolecular disulfide bonds and intermittent lateral (side-by-side) associations, as well as by associations with other BM components. The covalent crosslinking of multiple type IV molecules results in the formation of a lattice-like structural framework which confers structural stability and flexibility within BMs.

1.5.2.1.2 Proteoglycans
Proteoglycans are a heterogeneous family of macromolecules consisting of a core protein with covalently linked polyanionic glycosaminoglycan (GAG) chains (reviewed by (Kjellin and Lindahl, 1991)). Heparan sulfate proteoglycans (HSPG) are major components of the blood vessel wall (Yurchenco and Schittny, 1990) (Figs. 1.13 and 1.14). In large vessels they are mainly found in the intima and inner media, whereas in the microvasculature they are concentrated in the subendothelial BM where they support proliferating and migrating EC and stabilise the structure of the capillary wall (Schmidt et al., 1988). Heparan sulfate is by far the most predominant GAG found in most BM proteoglycans (Heremans et al., 1989), although small amounts of chondroitin and dermatan sulfates are also present (Lemkin and Farquhar, 1981; Ruoslahti, 1988) and chondroitin and dermatan sulfates actually predominate in BMs of the placenta (Brennan et al., 1984). Many proteoglycans of differing core size and with different heparan sulfate chain size and degrees of sulfation have been found and characterised (Paulsson et al., 1987). Molecular cloning techniques have so far revealed the existence of at least 16 different core proteins alone which have also been given trivial names based on their structure, biological activity or proposed function (Esko, 1991). For example, proteoglycans secreted into the ECM include aggrecan, versican, decorin, biglycan, fibromodulin, type IX collagen and BM proteoglycan (perlecan). Another class (including fibroglycan, syndecan, betaglycan, thrombomodulin, CD44, NG2 and glypican), are anchored to the plasma membrane. While the only proteoglycan core protein so far found to be unique to BMs seems to be BM
proteoglycan/perlecan (Engel, 1991; Noonan et al., 1991), a number of the core proteoglycans already characterised may reside also in the BM. Moreover, many other proteoglycans exist which have not been fully characterised or cloned (Esko, 1991).

The specific GAG chains associated with the core proteins impart some of the biological activities of the proteoglycans. These GAGs consist of a number of repeat disaccharide units in a linear arrangements. Heparan sulfate, and the structurally related heparin, are copolymers of two types of disaccharides. These are \( \text{GlcUA-GlcNAc} \) (where GlcUA is glucuronic acid and GlcNAc is N-acetylglucosamine) and \( \text{IdoA-GlcNS} \) (where IdoA is iduronic acid and GlcNS is sulfated glucosamine). The number of such disaccharides in a chain usually varies from 10-60. Segments of one or several disaccharides, containing one type of hexuronic acid, are interspersed with segments containing the other. During synthesis these chains undergo a series of modifications including N-deacetylation and N-sulfation of GlcNAc residues, and O-sulfation at position 3 or 6. O-sulfation also occurs on a large proportion of the IdoA at position 2, but not on GlcUA (Esko, 1991). Heparin and the heparan sulfates are unique GAGs in that they contain both N-sulfated and N-acetylated glucosamine residues. The extent of these modifications varies, giving rise to a potentially enormous structural heterogeneity. However constraints on the sites of action of the heparan sulfate biosynthetic enzymes means that the full potential for diversification does not occur (Lindahl et al., 1986). Such biosynthetic constraints also result in heparan sulfates with clusters of high sulfation interspersed with regions of low sulfation (Fransson et al., 1980; Roden, 1980; Gallagher et al., 1992). The high content of sulfate, and the presence of hexuronic acids, thus imparts a large negative charge to heparan sulfates, important in the provision of a charge barrier to the BM (Yurchenco and Schittny, 1990).

HSPGs were at first thought to be a passive structural element of BMs and the ECM, however it is now clear that their function is varied and complex due to their diversity of structure and promiscuity of binding. For example, some HSPGs are also found as integral components of the plasma membrane where they are capable of binding a low MW form of CD45 (Bradbury and Parish, 1991), as well as Mac-1, and basic FGF (Gallagher and Turnbull, 1992). Acidic and basic FGF, which belong to a family of heparan sulfate binding molecules, have been immunolocalized to, and isolated from the BM of a variety of tissues and have been demonstrated to interact within
these ECMs (Folkman et al., 1988; Vigny et al., 1988). Moreover, the binding of HS to bFGF is essential for the growth factor to mediate its biological activities (Rapraeger et al., 1991; Yayon et al., 1991). These findings indicate that HSPGs provide not only a physical barrier to extravasation, but that they may also influence the process of inflammation by other mechanisms.

1.5.2.1.3 Other BM components
Laminin, a major constituent of the BM, is a glycoprotein of cruciform structure with flexible rod like regions consisting of rows of EGF-like domains or $\alpha$-helical coiled coil regions (Yurchenco and Schittny, 1990; Engel, 1991) (Fig. 1.14). Many cell types interact with laminin, which has been found to influence cell attachment, spreading, growth and differentiation (Timpl, 1989). Its importance in influencing the supramolecular organisation of BMs is demonstrated by the fact that laminin is also able to provide a structural framework for BMs in the absence of collagen type IV (Yurchenco et al., 1992). Entactin (Fig. 1.14) also seems to be involved in the assembly of BMs. It has been implicated in recent studies as a link molecule between laminin and type IV collagen (Fox et al., 1991). The introduction of recombinant entactin into JAR choriocarcinoma cells, which synthesise type IV collagen, laminin and HSPG but not entactin, caused an increase in the deposition of type IV collagen and laminin (Chung and Durkin, 1990).

1.5.2.1.4 Other functions of BM components
Various other biologically significant roles have been attributed to BMs besides that of compartmentalization of tissues. BMs contribute to cell anchorage, the maintenance of cell polarity and control of cell migration (Albini et al., 1987; Yurchenco and Schittny, 1990; Mosher, Sottile et al., 1992). Many of these functions can be ascribed to the special constituents of the BM. For example, fibronectin, synthesised by proliferating cells in connective tissue, acts as a "temporary scaffolding" material and is expressed especially during angiogenesis. Laminin (Campbell and Terranova, 1988; Beck et al., 1990) also serves as a substratum for cell attachment and spreading and as a signal for cell migration (McCarthy et al., 1985). As outlined above, the HSPG constituents of the BM may bind adhesion molecules, and act as repositories and modulators of growth factors and possibly other biologically active molecules. It is clear from these examples that the BM serves as more than simply a dense, physical barrier to extravasating leukocytes, and that it has the capacity to regulate inflammation by a number of other important mechanisms.
1.5.2.2 BM degradation during inflammation

It was observed as early as the late 1940's that the growth of tumours into the surrounding connective tissue was associated with a change in the physical state of that connective tissue from that of a firm gel to one that was markedly less viscous (Gersh and Catchpole, 1949). It was postulated that this change resulted from the activity of depolymerising enzymes secreted at the edge of the tumour. By the mid-1960's electron microscopic studies had revealed that collagen degradation takes place in BMs at the peripheries of infiltrating ascitic tumours (Birbeck and Wheatley, 1965). The importance of hydrolytic enzyme expression during tumour metastasis has since been well established. Studies with tumours have provided clear evidence of the presence of degradative enzymes during metastasis and correlations have been established between enzyme levels and metastatic potential (Poole, 1973; Liotta et al., 1986; Karakiulakis, 1988; Liotta et al., 1991; Murphy et al., 1991; Pollanen et al., 1991).

Blood borne leukocytes arrested in capillaries, like metastatic tumour cells, must invade not only the endothelial layer, but also the subendothelial BM in order to gain access to the surrounding extravascular tissues. Leukocytes thus share with metastatic tumour cells the requirement to extravasate from the circulation into tissues and it is quite probable that many of the mechanisms are conserved between leukocytes and invading tumours. For example, cells involved in inflammation express hydrolytic enzymes capable of degrading many of the components of the ECM (Jessup and Dean, 1982; Naparstek et al., 1984; Matzner et al., 1985; Fridman, 1987; Pipoly and Crouch, 1987). The expression of some of these enzymes has also been found to be increased in some autoimmune diseases. For example, activated T cells specific for MBP were found to express high levels of heparanase activity in vitro which correlated with their ability to induce EAE (Naparstek, Cohen et al., 1984). Similarly, proteolytic and other lysosomal enzymes were found to be expressed at high levels in EAE induced CNS lesions in rodents (Marks, 1977; Massacesi, 1988) and in the joint tissue of rheumatoid arthritis patients in humans (Murphy, Docherty et al., 1991).

Since the BM and ECM are comprised of many different interacting components such as collagen type IV, fibronectin, laminin and HSPGs (Figs. 1.13, 1.14), destruction of the matrix by invading cells may require a variety of hydrolases which act either simultaneously or sequentially to solubilize the
matrix and permit penetration. In fact, studies have shown that successful penetration of the BM requires the combined action of proteases and heparanases (Bar-Ner *et al.*, 1985; Bar-Ner *et al.*, 1986). In facilitating cell movement, these enzymes may also play a dual role in the degradation of the ECM barrier and in the breaking of adhesive cell-matrix contacts (Chen, 1992; Ratner, 1992). Enzymes which have been characterised and implicated in cell movement include endoglycosidases such as heparanase (Nakajima *et al.*, 1983; Vlodavsky *et al.*, 1983; Bar-Ner, Kramer *et al.*, 1985), type IV collagenase (Liotta *et al.*, 1982) and proteases of the serine, cysteine, aspartyl and metalloprotease classes (Mignatti *et al.*, 1986; Ricoveri and Cappeletti, 1986; Kirchheimer *et al.*, 1990; Rozhin *et al.*, 1990). These enzymes are discussed in more detail below.

1.5.2.2.1 **Proteases**

A strong positive correlation has been shown between the expression of tumour cell proteases and tumour invasion (Liotta, Steeg *et al.*, 1991; Murphy, Docherty *et al.*, 1991; Pollanen, Stephens *et al.*, 1991). Proteases capable of dissolution of BM components include matrix metalloproteases (MMPs) such as collagenase and stromelysin, serine proteases such as plasminogen and its activators, the cathepsins, and some integral membrane proteases (Table 1.14).

Matrix metalloproteases (MMPs), which include collagenase, gelatinases and stromelysin, are secreted as zymogens whose activation by proteases such as plasminogen activator (PA) is required before BM degradation can be effected. MMPs are found in both latent and active forms complexed with tissue inhibitors (TIMPs) which regulate their activity (Murphy, Docherty *et al.*, 1991). Activation of MMPs has been shown to take place after the binding of the zymogen or their complexes with TIMPs on the plasma membrane (Zucker *et al.*, 1987; Ward *et al.*, 1991). Expression of various MMPs has been correlated with a number of biological processes including neovascularization (Moses and Langer, 1991) and proteolytic opening of the blood brain barrier (Rosenberg *et al.*, 1992) and is increased in highly metastatic tumour cells (Templeton and Stetler-Stevenson, 1991; Sato *et al.*, 1992), transformed cells (Chen *et al.*, 1991) and in inflammatory cells involved in rheumatoid arthritis (Murphy, Docherty *et al.*, 1991).

Some of the serine proteases, such as plasminogen activator, rather than effecting BM degradation directly, seem to mainly be responsible for
Table 1.14

Major membrane-associated or secreted BM-degrading proteases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mode of extracellular expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serine proteases</strong></td>
<td></td>
</tr>
<tr>
<td>Urokinase plasminogen activator</td>
<td>secreted, receptor bound (uPAR)</td>
</tr>
<tr>
<td>Tissue-type plasminogen activator</td>
<td>secreted, receptor bound</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>secreted, receptor bound</td>
</tr>
<tr>
<td>Elastase</td>
<td>secreted, ?</td>
</tr>
<tr>
<td>Thrombin</td>
<td>secreted, ?</td>
</tr>
<tr>
<td><strong>Matrix metalloproteases</strong></td>
<td></td>
</tr>
<tr>
<td>Collagenase (MMP1)</td>
<td>secreted, ?</td>
</tr>
<tr>
<td>Gelatinase (MMP2)</td>
<td>secreted, ?, bound to ECM (via FN-like collagen binding domain)</td>
</tr>
<tr>
<td>Gelatinase (MMP9)</td>
<td>secreted, ?, bound to ECM (via FN-like collagen binding domain)</td>
</tr>
<tr>
<td>Stromelysin (MMP3)</td>
<td>secreted, ?, bound to ECM (via collagen-binding site)</td>
</tr>
<tr>
<td><strong>Cathepsins</strong></td>
<td></td>
</tr>
<tr>
<td>Cathepsins B, H, L</td>
<td>secreted, cell-surface expression associated with glycosylation</td>
</tr>
<tr>
<td>Cathepsins C, J, K.</td>
<td>secreted, cell-surface expression associated with glycosylation</td>
</tr>
<tr>
<td><strong>Integrin membrane proteases</strong></td>
<td></td>
</tr>
<tr>
<td>CD10 or CALLA</td>
<td>integral, altered proteolytic processing, glycosylation</td>
</tr>
<tr>
<td>Meprins</td>
<td>integral, altered proteolytic processing, glycosylation</td>
</tr>
<tr>
<td>Hepsin</td>
<td>integral, altered proteolytic processing, glycosylation</td>
</tr>
</tbody>
</table>

Adapted from (Chen, 1992)
activating metalloproteases and plasminogen. Mononuclear phagocytes are the dominant source of plasminogen activator activity amongst leukocytes (Danø et al., 1985). In the well characterised urokinase plasminogen activator-receptor (uPA-uPAr) system (Novokhatny et al., 1992), endogenously secreted and externally added uPA binds to a specific receptor and allows the surface expression of uPA. Urokinase catalyzes the activation of the zymogen plasminogen to the active nondiscriminant protease, plasmin, by cleavage of a single peptide bond. Plasmin can directly degrade integral BM glycoproteins (eg. fibronectin, laminin), and may also activate procollagenases and degrade collagen within BM matrices (Salo et al., 1982). During recruitment, mononuclear phagocytes utilise the surface receptor bound uPA to focus plasmin activity in the immediate pericellular environment, thereby effecting sufficient localised ECM degradation to permit migration across tissue planes (Chen, 1992). Plasminogen activator inhibitors (PAIs), a family of serine protease inhibitors (serpins) which control the activity of plasminogen activators, have been divided into three genetically and immunologically distinct types (PAI-1, PAI-2 and protease nexin) (Kruithof, 1988). PAI-2 has been isolated from placenta, monocytes and the monocytic cell line U937 and inhibits uPA specifically and irreversibly. In contrast, PAI-1 inhibits both tPA and uPA. Although the precise biological role of these inhibitors is unknown, it has been suggested that they are involved in the control of local cell-mediated proteolysis during inflammation (Kruithof, 1988). Proteases and their inhibitors have been found to bind to the cell surface at different sites. Interestingly, PAI-1 is found in focal adhesions but not in pseudopodia, while BM is degraded by transformed cells at sites of BM contact with pseudopodia but not at focal adhesive sites (Chen, 1992). Therefore, during inflammation, the main purpose of the PAI system may be to release cells from the substratum during migration.

The cathepsins, which are lysosomal cysteine proteases, are normally associated with the lysosomal compartment rather than being expressed extracellularly. However, malignantly transformed cells exhibit an increased cell surface expression of the cathepsins, which can degrade BM components at both acidic and neutral pH (Kane and Gottesman, 1990; Buck et al., 1992). Interestingly, proteolytic processing and glycosylation of cathepsin B has been shown to be involved in its cell-surface expression and also in determining the optimum pH of action of the enzyme for hydrolysing ECM components (Hasnain et al., 1992; Saitoh et al., 1992).
In addition, integral membrane proteases including CD10 (a zinc metallo-endoproteinase), meprins (metallo-endopeptidases), hepsin (a membrane protease) and some others, have been isolated from a variety of tissues and tumour cells, (reviewed by (Chen, 1992)). It is not clear yet whether these proteases are relevant to inflammation, although it has been suggested that expression of this group of membrane proteases contributes to the localised degradation of the BM during invasion and metastasis.

1.5.2.2.2 GAG degrading enzymes

The ability of HSPGs to interact with ECM macromolecules such as collagen, laminin and fibronectin, and with different attachments sites on plasma membranes (Laterra et al., 1983; Heremans, Van der Schueren et al., 1989), suggests it plays a key role in the self assembly and insolubility of ECM components. Degradation of the HS side chains of proteoglycans may therefore result in the solubilization of the subendothelial BM. The existence of inherited defects in the degradation of the polysaccharide chains of proteoglycans leading to diseases such as mucopolysaccharidoses and mucolipidoses, has allowed the study of specific degradative enzymes and their substrates (Neufeld and Muenzer, 1989). These GAG degrading enzymes, which are mainly lysosomal, include exoglycosidases, which act in a stepwise manner to remove glycosyl groups, endoglycosidases (heparanases) of various specificities, and sulfatases. The heparanases are the least characterised, but are probably the most relevant enzymes with respect to BM degradation during tumour invasion and leukocyte extravasation due to their endo- rather than exoglycosidase activity.

In this context, heparanase activity was found to correlate with metastatic potential in mouse lymphoma (Vlodavsky, Fuks et al., 1983; Vlodavsky et al., 1988), melanoma (Nakajima, Irimura et al., 1983; Jin et al., 1990) and fibrosarcoma cells (Ricoveri and Cappeletti, 1986). Further evidence for the involvement of heparanases in tumour cell invasion and metastasis was provided when it was shown that there was a good correlation between the heparanase inhibitory activity of various heparin preparations and sulfated polysaccharides and their anti-metastatic activity (Coombe et al., 1987; Parish et al., 1987; Nakajima et al., 1988).

Heparanase activity has also been found to correlate with leukocyte activation. For example, the activation of lymphocytes with antigen results
in their release of heparanases which degrade components of the labeled ECM produced by cultured ECs (Vlodavsky et al., 1992). A close correlation was also observed between heparanase expression in vitro and the ability of activated MBP-specific T cells to produce EAE in vivo (Naparstek, Cohen et al., 1984). Paradoxically, the heparanase activity of MBP-specific T cells, measured by their ability to release HS degradation products from $^{35}$sulfate labeled ECM in vitro, was enhanced about 5 fold when the T cells were activated by MBP bound to the ECM (Naparstek, Cohen et al., 1984). Interestingly, heparanase activity could be demonstrated in lysates of both activated and nonactivated T cells in the absence of antigen, and also when protein synthesis was inhibited by cycloheximide (Fridman, 1987). Further, soluble antigen presented to antigen specific T cells induced heparanase activity in vitro within five minutes (Fridman, 1987). Together, these experiments seem to indicate that heparanases exist preformed in intracellular compartments and are rapidly expressed extracellularly upon T cell activation. Subsequent studies have shown that heparin, a potent heparanase inhibitor, is able to inhibit infiltration of T cells in autoimmune lesions (Willenborg et al., 1989), DTH reactions, and allografts (Cohen et al., 1987; Lider et al., 1989). Neutrophils (Matzner et al., 1990) and macrophages (Savion et al., 1987) have also been shown to express heparanase activity in vitro which is heparin inhabitable. Degranulation of neutrophils was not necessary in order to induce heparanase release, which was increased in the presence of chemotactic stimuli (Matzner, Vlodavsky et al., 1990). Collectively these studies support the concept that the extracellular expression of heparanases plays an important role in the degradation of subendothelial BMs by leukocytes.

1.5.2.2.3 Localisation of BM degrading enzymes

During extravasation, the processes of cell-matrix adhesion and degradation of the BM probably occur, not in isolation from each other, but as a coordinated process. There is evidence that this process involves adhesive foci, where relatively high concentrations of adhesion molecules and their BM counter-receptors are localised, and cell-surface extensions or pseudopodia which invade the BM/ECM and where extensive degradation and integrin expression occurs (Mueller and Chen, 1991). Whether membrane-associated degradative enzymes become concentrated at these invading pseudopodia or not is unclear, but in vitro studies strongly suggest that, like the adhesion molecules, proteases and heparanases are expressed at the cell surface. For example, in tumours the subcellular localisation of cathepsin B, cathepsin L
and β-N-acetylglucosaminidase was found to change from lysosomes to the plasma membrane with increasing metastatic potential (Sloane et al., 1986; Rozhin et al., 1989). Similarly, cathepsin B was found to be localised to the cell surface of the invasive bladder carcinoma cell line EJ, but was confined to the lysosomes in its equivalent non-invasive cell line, or normal bladder epithelium (Weiss et al., 1990). Furthermore, plasma membranes isolated from the invasive cell line, but not from the non-invasive cell line, were able to degrade purified ECM components (Weiss, Liu et al., 1990).

Studies of biochemically defined membrane fractions of normal cells and tumour cells also indicated that the active forms of BM-degrading enzymes are associated with the plasma membrane. For example, in an in vitro BM permeability assay, the ability to permeabilize the BM was dependent upon intact tumour cells, with tumour cell extracts, lysates and supernatants being inactive (Parish et al., 1992). Another well characterised example of cell-surface expression of degradative enzymes is the urokinase plasminogen activator-receptor (uPA-uPAr) system (Novokhatny, Medved et al., 1992) where endogenously secreted and externally added uPA binds to a specific (55 - 60 kD) receptor found on many tumour cells and normal cell types. This system allows extravasating cells to focus plasmin activity to the immediate vicinity of BM degradation with minimal damage to surrounding endothelium and stroma. Thus, if sufficient BM degradation to allow migration of extravasating leukocytes is to occur, there must be a) clustering of degradative enzymes at focal adhesion and migration sites, and b) cell receptors recruiting the intracellular and secreted proteases and heparanases to the cell surface (Chen, 1992).

1.5.2.2.4 Role of the phosphomannosyl recognition system in BM degradation
Most of the glycosidases (including heparanases), and some of the proteases implicated in BM degradation, such as the cathepsins, are lysosomal enzymes (Kaiser, 1980). Lysosomes contain over 50 different hydrolytic enzymes which function in the intracellular digestion of some cellular components such as lipids, mucopolysaccharides, glycogen and proteins. The individual activities of these enzymes has been reviewed in detail (Barret, 1969) and the ability of lysosomal enzymes to degrade elements of the BM has been studied in several experimental systems (Briozzo et al., 1988; Maciewicz et al., 1989; Weiss, Liu et al., 1990). Thus it has been proposed that lysosomal enzymes may play a role in the degradation of BMs
during inflammation (Kaiser, 1980). While it has been recognised that cell surface expression is important in BM degradation (Chen, 1992), the precise mechanism by which many degradative enzymes are recruited to the cell surface remains undefined. However, a recognition system which may be involved in their presentation at the cell surface is the phosphomannosyl recognition system (reviewed by (Kornfeld, 1987; Kornfeld, 1990; Kornfeld, 1992). Lysosomal enzymes are synthesised in the RER where an oligosaccharide precursor (consisting of 3 glucose, 9 mannose and 2-N acetyl glucosamine residues) is co-translationally transferred from a dolichol carrier onto the asparagine residue of an Asn-X-Ser/Thr sequon in the protein (Fig. 1.15). The oligosaccharide is then trimmed of three of the glucose and one of the mannose residues. Following trimming, the protein is moved by vesicular transport to the Golgi apparatus where two more mannose residues are removed and the 1-2 terminal mannose residues are phosphorylated in the 6-position. These mannose 6-phosphate (M6P) residues then constitute the specific recognition marker for lysosomal enzymes which is recognised by the mannose phosphate receptor (MPR) glycoprotein. Mammalian cells express two MPRs. The larger of the two receptors (300 kD) is cation independent while the smaller receptor (46 kD) is known as cation dependent, since it requires divalent cations for its activity (Chao et al., 1990). The MPR binds to the M6P ligand with high affinity and transports lysosomal enzymes to the acidified prelyosomal compartment where it dissociates. Some lysosomal enzymes, complete with their M6P recognition marker, also escape to the outside of the cell. The large MPR is also found on the cell surface where it has been found to bind and pinocytose any secreted lysosomal enzymes (Kornfeld, 1987; Chao, Waheed et al., 1990; Kornfeld, 1990). Since MPRs are found on the cell surface, an important mechanism exists whereby lysosomal enzymes may be held at the cell surface. Such a model would explain the extracellular solubilization of BMs by lysosomal enzymes which has been shown to occur during tumour metastasis. A mechanistic parallel may be applicable to leukocytes, which share with metastatic cells the ability to extravasate from the circulatory system.

The proposed model of lysosomal enzyme capturing by MPR has important implications for the design of anti-metastatic and anti-inflammatory drugs. Anti-uPA antibodies, for example, which prevented binding of secreted uPA to its specific cell surface receptor, inhibited tumour metastasis in a chorioallantoic membrane metastasis model by 40-80% (Ossowski and Reich, 1983). Inhibitors of MPR also exist. The discovery of the MPR
Fig. 1.15
Schematic representation of the pathway of lysosomal enzyme targeting to lysosomes. Lysosomal enzymes are synthesised in the rough endoplasmic reticulum (RER) and glycosylated. The proteins are translocated to the Golgi where the oligosaccharide moieties are processed before two mannose residues are phosphorylated. Most lysosomal enzymes bind via the two mannose phosphate residues to a mannose phosphate receptor (MPR) and are translocated to an acidified prelysosomal compartment.

Once in the acidified compartment, the lysosomal enzymes dissociate from the MPR and the enzyme is transferred to a lysosome. The MPR then recycles back to the Golgi to pick up more enzyme. Some enzymes are secreted, but are recaptured by the MPR on the cell membrane.

Adapted from Kornfeld (1987)
actually coincided with the discovery that phospho-mannose containing yeast mannans and M6P itself were potent competitive inhibitors of β-glucuronidase pinocytosis (Kaplan et al., 1977). Evidence also exists to suggest that cell-surface located MPRs contain bound lysosomal enzymes capable of degrading the ECM. M6P inhibited the turnover of extracellular $^{35}$S-labeled proteoglycans by human fibroblast cells in culture, whereas, in an experiment with I-cells (which lack lysosomal enzymes carrying the M6P marker), no difference was seen in the turnover of proteoglycans with or without added M6P (Roff et al., 1982). Furthermore, when lysosomal enzymes derived from normal human fibroblasts were added to the I cells, M6P inhibitable degradation of the labeled ECM occurred (Roff, Wozniak et al., 1982). If the lysosomal enzymes were active in solution, then the addition of M6P would have enhanced, rather then impaired, the degradation of the labeled ECM. Thus, these data suggest that cell-surface MPR present lysosomal enzymes, thereby allowing them to degrade extracellular proteoglycans (Kornfeld, 1992).

It was subsequently found that M6P was a potent inhibitor of EAE (Willenborg and Parish, 1989) and adjuvant induced arthritis in the rat (Willenborg et al., 1992). Mannose, or other sugar phosphates which were not structurally analogous to M6P failed to inhibit disease (Parish et al., 1990). In further support of this model, castanospermine, an inhibitor of glycoprotein processing which causes the formation of aberrant lysosomal enzyme recognition markers, was also able to inhibit EAE (Willenborg, Parish et al., 1989) and adjuvant induced arthritis (Willenborg, Parish et al., 1989).

In summary, there is much evidence to support the proposal that both proteases and heparanases may be important in BM degradation during leukocyte extravasation. Furthermore, during recruitment to an inflammatory site, leukocytes probably utilise cell surface receptors to these enzymes in order to focus degradative activity in the immediate pericellular environment. The phosphomannosyl recognition system has been proposed as a mechanism of recruiting and displaying lysosomal enzymes at the cell surface, a process which may be important in the lysosomal enzyme degradation of the subendothelial BM during the migration of leukocytes to an inflammatory focus.
1.6 Autoimmunity, inflammation and disease

1.6.1 Historical background

Historically, the concept of autoimmunization seems to have progressed through three stages (Schwartz and Syamal, 1989). The first stage, which was basically non-acceptance, is epitomised by Ehrlich’s Latin description "Horror autotoxicus". Though Ehrlich did not believe that autoimmunity was impossible, his Latin was misconstrued to mean just that, and most of the scientific community thenceforth rejected the concept of autoimmunity, if not openly, then by neglect, for the next 50 years (Schwartz and Syamal, 1989). Despite an accumulation of evidence, doubts about the existence of autoimmune disease were not finally eliminated until the discovery, in 1959, of the first animal model of a spontaneous autoimmune disease in the New Zealand Black (NZB) mouse (Bielschowsky et al., 1959). Many other animal models have since been found (Table 1.15).

By 1949 there was sufficient evidence for autoimmunity to provoke McFarlane and Fenner to examine mechanisms of self-non-self discrimination in the immune system (Burnet and Fenner, 1949). Their astute predictions of tolerance to self antigens based on Owen’s studies of dizygotic cattle twins’ acquired tolerance of foreign antigens crossing the placenta (Owen, 1945) seemed at odds with the concept of autoimmunity. In order to justify the paradox, Burnet postulated that autoimmunity arose when forbidden clones, or autoreactive lymphocytes, escaped clonal deletion or suppression in the thymus (Burnet, 1969). However, the concept that autoimmunity is an abnormal state is no longer completely valid. This most recently emerging paradigm of autoimmunity suggests that the normal, healthy immune system generates self-recognising and potentially autoaggressive T and B cells (Schwartz and Syamal, 1989; Kumar and Sercarz, 1991). Autoimmunity is then regulated to prevent immune pathologies, or has a place in the normal functioning of the healthy body. As an example, when the human erythrocyte nears the end of its normal life span of 120 days, it exposes a transmembrane protein (band 3), which elicits an autoantibody that binds to senescent erythrocytes and may help in their elimination (Kay, 1984; Lutz et al., 1987). This concept leads to a more clear distinction between autoimmunity, an inherent property of the normal immune system, and autoimmune disease, the culmination of a pathological process. The immunologist’s attention is shifted, therefore, from self
### Spontaneous autoimmune diseases in laboratory animals

<table>
<thead>
<tr>
<th>Disease</th>
<th>Animal model</th>
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<tbody>
<tr>
<td><strong>Organ specific autoimmune diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Autoimmune thyroiditis</td>
<td>Obese strain (OS) chickens</td>
</tr>
<tr>
<td>Type I diabetes</td>
<td>Nonobese diabetic (NOD) mouse</td>
</tr>
<tr>
<td></td>
<td>C57Bl/Ks-db/db mouse</td>
</tr>
<tr>
<td></td>
<td>BB rat</td>
</tr>
<tr>
<td><strong>Systemic autoimmune diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Systemic lupus erythematosis</td>
<td>NZB mouse</td>
</tr>
<tr>
<td></td>
<td>(NZBxNZW) F1 mouse</td>
</tr>
<tr>
<td></td>
<td>(NZBxSWR) F1 mouse</td>
</tr>
<tr>
<td></td>
<td>MRL-lpr/lpr mouse</td>
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<tr>
<td></td>
<td>MRL-+/- mouse</td>
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<tr>
<td></td>
<td>BXSB mouse</td>
</tr>
<tr>
<td></td>
<td>C3H/He/J-gld/gld mouse</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>Tight skin (TSK) mouse</td>
</tr>
<tr>
<td></td>
<td>White leghorn chicken</td>
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</tbody>
</table>

Adapted from Schwartz and Datta (1989)
recognition to self regulation, in an attempt to understand autoimmune pathologies (Kumar and Sercarz, 1991).

1.6.2 Autoimmune disease pathology
As summarised in Table 1.16, there is a wide range of autoimmune diseases with different sites of action and pathological features. However, since this thesis will be dealing with novel anti-inflammatory compounds that have already been demonstrated to inhibit T cell dependent adjuvant induced arthritis and EAE (an animal model of CNS inflammation resembling multiple sclerosis (MS)) (Raine, 1985), it is appropriate in this section to briefly summarise the important clinical features of rheumatoid arthritis (RA) and MS in humans. Furthermore, these two autoimmune diseases are thought to be excellent examples of T-cell mediated tissue-specific damage.

1.6.2.1 Rheumatoid arthritis
Rheumatoid arthritis (RA) is an example of a systemic autoimmune inflammatory disorder. It is characteristically chronic, lasting weeks, months or even years, prevalent (affecting 1-2% of population) and often disabling (Brooks, 1992). Although its principle manifestation is arthritis, RA is not confined to the joints - vasculitis caused by immune complexes can involve skin, eyes and lungs. In the current models of synovitis, T cells orchestrate the local inflammatory response following stimulation by an arthrotropic agent, such as a virus (Zeigler et al., 1989), bacterial fragments, autoantigens such as type II collagen (Tarkowski et al., 1989; Klareskog and Olsen, 1990), proteoglycan or MHC molecules (Janossy et al., 1981).

Antigen-specific T cells, which predominate in the initial inflammatory lesion, move into the synovium via postcapillary venules, especially through those with high endothelium. Cytokines such as IL-1 and TNFα then induce the expression of adhesion molecules such as ICAM-1, E-selectin and LFA-3 on the surface of endothelium and HEV, increasing the ability of T cells to bind and migrate through vascular endothelium (Hale et al., 1989). While all T cells express ligands for ICAM-1 and LFA-3, (LFA-1 and CD2 respectively), they are found in significantly greater amounts on mature memory helper T cells (CD4+, CD45RO subset) (Sanders et al., 1988) which represent the highest proportion of T cells in the inflamed synovium. According to current models, once in the synovium T cells clonally expand, elaborate cytokines which recruit other cells from the circulation and stimulate B cells, resident macrophage-like cells and fibroblast-like cells of the synovium. The synovial cells also become activated, expressing surface MHC molecules and
Table 1.16

Principle clinical and experimental autoimmune diseases

<table>
<thead>
<tr>
<th>Organ specific autoimmune diseases</th>
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<tbody>
<tr>
<td><strong>Endocrine system</strong></td>
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<tr>
<td>Autoimmune (Hashimoto’s) thyroiditis</td>
</tr>
<tr>
<td>Hyperthyroidism (Grave’s disease, thyrotoxicosis)</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus (insulin dependent or juvenile diabetes)</td>
</tr>
<tr>
<td>Insulin resistant diabetes</td>
</tr>
<tr>
<td>Autoimmune adrenal insufficiency (Addison’s disease)</td>
</tr>
<tr>
<td>Autoimmune oophoritis</td>
</tr>
<tr>
<td>Autoimmune orchitis</td>
</tr>
<tr>
<td><strong>Haematopoietic system</strong></td>
</tr>
<tr>
<td>Autoimmune haemolytic anaemia (warm autoantibody type)</td>
</tr>
<tr>
<td>Autoimmune haemolytic anaemia (cold agglutinin disease)</td>
</tr>
<tr>
<td>Paroxysmal cold haemoglobinuria</td>
</tr>
<tr>
<td>Autoimmune thrombocytopenia</td>
</tr>
<tr>
<td>Autoimmune neutropenia</td>
</tr>
<tr>
<td>Pernicious anaemia</td>
</tr>
<tr>
<td>Pure red cell anaemia</td>
</tr>
<tr>
<td>Autoimmune coagulopathies (circulating anticoagulants)</td>
</tr>
<tr>
<td><strong>Neuromuscular system</strong></td>
</tr>
<tr>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td>Autoimmune polyneuritis</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
</tr>
<tr>
<td>Pemphigus and other bullous diseases</td>
</tr>
<tr>
<td><strong>Cardiopulmonary system</strong></td>
</tr>
<tr>
<td>Rheumatic carditis</td>
</tr>
<tr>
<td>Goodpasture’s syndrome (pulmonary haemorrhage, nephritis)</td>
</tr>
<tr>
<td>Postcardiotomy syndrome (Dressler’s syndrome)</td>
</tr>
<tr>
<td><strong>Systemic autoimmune diseases</strong></td>
</tr>
<tr>
<td>Systemic lupus erythematosis</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Sjogren’s syndrome (keratitis, parotitis, arthritis)</td>
</tr>
<tr>
<td>Polymyositis</td>
</tr>
<tr>
<td>Scleroderma (progressive systemic sclerosis)</td>
</tr>
</tbody>
</table>

Adapted from Schwartz and Datta (1989)
elaborating cytokines, prostaglandins and collagenase (Firestein et al., 1990; Firestein and Zvaifler, 1990).

Recently the role of T cells in the pathogenesis of RA has been questioned on the basis that T cells in the inflamed synovium lack characteristics of activation. For example, there is a relatively low production in the synovium of T cell cytokines such as IL-2 and IFNγ compared with the macrophage cytokines TNFα and GM-CSF (Firestein and Zvaifler, 1990). Zvaifler and Firestein also reported low evidence of T cell clonal expansion and a lack of dramatic effects of immunosuppressants on RA (Firestein and Zvaifler, 1990). However these observations may be explained, at least in part, by the detection of TGFβ in vitro (Kehrl et al., 1986), and in the synovial fluid of RA patients (Lotz et al., 1990), and the fact that TGFβ has potent down-regulatory effects on T cell activity (Kehrl, Wakefield et al., 1986).

Clearly, cytokines are not the only soluble factors contributing to inflammation in RA. B cells infiltrating the synovium produce Igs, some of which are IgG rheumatoid factors which bind to other IgG molecules in the joint to form immune complexes. It is not clear whether the B cell Ig production is driven by CD4+ T cells or by polyclonal activation not requiring T cells (Solovera et al., 1988). However, the resultant immune complexes are capable of activating the complement cascade, leading to increased vascular permeability and recruitment of more inflammatory cells including neutrophils (Zvaifler, 1983). Invading neutrophils, in turn, release proteases, oxygen free radicals, prostaglandins and other products of the lipoxygenase pathway (Henson, Henson et al., 1992; Klebinoff, 1992). By this stage the joint is showing all the classic symptoms of inflammation including swelling, pain and heat. These symptoms result from a combination of synovial hypertrophy, tissue oedema, angiogenesis, infiltration with inflammatory cells and increase in the volume of synovial fluid. A chronic granulomatous lesion forms, invading and eroding the joint components. This encroaching vascular pannus, with its destruction of bone and cartilage, is one of the major immunopathological features of rheumatoid arthritis.

1.6.2.2 Multiple sclerosis

Multiple Sclerosis (MS) (reviewed by (Hafler and Weiner, 1989)) is the most common of a number of neurological diseases in which autoimmune pathogenesis is implicated. It is a chronic, often relapsing disease wherein demyelination of nerve cells of the brain and spinal cord results in
neurological symptoms culminating in paralysis or coma. The role of autoimmunity is suggested by characteristic histological features of the lesions, MHC-linked susceptibility and similarity to the autoimmune disease model, EAE (Arnon, 1981).

EAE is a useful animal model for studying MS. This acute inflammatory disease of the CNS can be induced by an injection of brain or spinal cord tissue with adjuvant into different laboratory animals including mice, rats and primates (Weigle, 1980; Arnon, 1981). Perivascular infiltration of the CNS follows, with a resultant hind leg paralysis in mice and rats occurring about 2 weeks following the challenge. Histologically in MS there are infiltrates of CD4+ and CD8+ T cells and macrophages in the CNS (Waksman, 1985). Similarly, although antibodies to CNS components are produced in EAE, the disease is T cell mediated and can be transferred by T cells sensitised to MBP. The sensitised cells are CD4+, but they initiate the inflammatory reaction by recruiting both CD4+ and CD8+ effector cells into the brain (Weigle, 1980; Arnon, 1981). In the CNS, glial cells express MBP on their surface, and are induced to express high levels of Class I and II MHC antigens as a result of cytokines expressed by the infiltrating mononuclear cells (Arnon, 1981). These events, and the increased expression of MHC class II molecules by ECs of CNS blood vessels (McCarron et al., 1986), act to perpetuate the inflammation and demyelination.

The lesions of MS may be mediated by T cells that become sensitised to autoantigens present in the white matter of the CNS. MBP has been a prime suspect for a number of years, but T cells specific for MBP or other common CNS proteins have not been consistently found in MS, and recently other structures, such as proteolipid protein (PLP) have emerged as other important encephalitogens (Whitham et al., 1991). However, T cell clones reactive to MBP have been isolated not only from diseased, but also from healthy individuals and animals (Burns et al., 1983; Tournier-Lasserve et al., 1988). Considering the low incidence of spontaneous MS and EAE, activation of these autoimmune encephalitogenic T cell clones is a rare event. Molecular mimicry is one possible mechanism whereby the pathogenic T cell responses are stimulated (Fujinami and Oldstone, 1985; Yamada et al., 1990). Nevertheless, the causative agent(s) of MS is not known despite extensive research for many years, although a number of viruses have been suggested (Hafler and Weiner, 1989).
1.6.3 Mechanisms of autoimmune disease

Inflammation and tissue injury characterise a wide variety of diseases. The causes of autoimmune disease are still unclear, although infectious agents have often been claimed to be implicated in the breakdown of tolerance to self antigens (see below). A significant association has also been found between some human autoimmune diseases and certain MHC class II alleles. Indeed, the only molecular alteration that has been specifically linked to an autoimmune disease has been found within the MHC molecule. For example, such associations have been found in IDDM and its corresponding animal model, the NOD mouse, as well as in RA and pemphigus vulgaris (Gasser et al., 1973; Happ et al., 1988; Todd et al., 1988). In human IDDM, susceptibility is associated with an alteration in amino acid 57 of the DQβ chain. In the NOD mouse, an alteration is found in the same position of the I-Aβ chain, the murine equivalent of DQβ. In the shared epitope hypothesis (Janossy, Panayi et al., 1981), a structure around amino acid 70 in the β chain of the HLA-DR molecule is important for RA susceptibility. It is possible that when the altered MHC presents exogenous antigen, it elicits a T cell response that mimics an autoantigen (Strominger, 1986). Alternatively, disease-linked class II MHC molecules may present an autoantigen as if it were a foreign antigen.

There is evidence that some microbial antigens share regions of amino acid sequence homology with mammalian proteins and can play a role in the development of autoimmune disease (Inman et al., 1987; Winfield, 1989). How such a microbial polypeptide, imitating a self-peptide, escapes the normal rules of tolerance to provoke an immune response is still unclear, but pathogenic relationships between microbial antigens and immune injury have been demonstrated. For example, adjuvant arthritis, which is characterised by a T cell dependent destruction of joint cartilage, can be induced in susceptible strains of rats by injection of *Mycobacterium tuberculosis* (Van Eden et al., 1985). Significantly, there is a structural relationship between the mycobacterial peptidoglycans and proteoglycans in joint cartilage. A nonapeptide (residues 180-188) from a *M. tuberculosis* antigen contains the epitope recognised by T cells mediating adjuvant arthritis as well as by T cells from patients with RA (van Eden et al., 1988). Heat shock proteins are also known to evoke autoimmune responses in a number of autoimmune diseases including RA and systemic lupus erythematosus (SLE) (reviewed by Cohen (Cohen, 1991; Res et al., 1991). For example, a 65 kD *M. tuberculosis*
antigen shares sequence homology with human intracellular heat shock proteins, and this relationship may lead to loss of tolerance through molecular mimicry (Winfield, 1989).

Viruses have also been implicated in certain autoimmune diseases. For example, Epstein Barr virus has been incriminated in RA (Britton, 1982) and coxsackie viruses have been implicated in Type I diabetes mellitus (Ahmad and Abraham, 1982). If these, and other infectious agents do play a role in the aetiology of autoimmune disease, they may do so by antigenic mimicry (Inman, Chiu et al., 1987) as described above, by polyclonal activation of B cells, or by causing direct damage to the organ or tissue resulting in the release of autoantigens (Neu et al., 1987; Neu et al., 1987).

1.6.4 Therapeutic approaches to autoimmune disease
The treatment of autoimmune diseases still presents a great challenge to physicians. Since the causes of autoimmune disease are unknown, therapies for autoimmune diseases can only be aimed at suppressing the signs and symptoms of the disease. There are three general categories of therapeutic agents currently used in the treatment of these diseases: 1) non-steroidal anti-inflammatory drugs (NSAIDs) and low dose corticosteroids which decrease inflammation and pain; 2) systemic immunosuppressive drugs and 3) disease modifying anti-rheumatic drugs (DMARDs), which appear to abate progression transiently in certain patients, but which possess neither non-specific anti-inflammatory nor systemic immunosuppressive properties. None of these agents affect the long term outcome of disease. Although there are now available an increasing number of drugs in each of these categories, with subtle differences in their effects, all of these drugs have significant side effects.

Although still in the experimental stages, there are a number of new anti-inflammatory drugs which have novel modes of action. These include anti-idiotypes, anti-adhesive (eg. anti-VCAM-1, anti-ICAM-1 - see also sections 1.5.1.3 and 1.6.5.1), cytokine synthesis inhibitors and inhibitors of BM degradation. This section will outline the effects of the major anti-inflammatory drugs and their mechanisms of action.
1.6.4.1 Non-steroidal anti-inflammatory drugs

The non-steroidal anti-inflammatory drugs (NSAID) are very commonly prescribed. Americans, for example, consume 16,000 tonnes of aspirin tablets per year (Weissman, 1991). Australia also has one of the highest NSAID prescription rates in the world (Brooks, 1992). The major mechanism of action of NSAIDs is the inhibition of cyclooxygenase activity, and therefore the synthesis of prostaglandins (Vane, 1971). Indeed, the rank order of anti-inflammatory efficacy of a number of different NSAIDs is directly linked to their effectiveness in inhibiting prostaglandin synthesis in vitro (Higgs, Moncada et al., 1980). The inhibition of prostaglandin synthesis relieves some of the symptoms of inflammation such as pain, oedema and hyperalgesia. Despite the symptomatic relief offered by prostaglandin inhibitors, NSAIDs, especially at higher concentrations, can actually lead to an increase in the migration of inflammatory leukocytes. In a carrageenan-induced model of inflammation, the leukocyte influx was shown to increase by up to 70 per cent when certain NSAIDs were administered (Higgs, Moncada et al., 1980). Furthermore, there are a lot of data suggesting that the NSAIDs have other modes of action besides prostaglandin inhibition (Forrest and Brooks, 1988) (Table 1.17). For example, a number of the NSAIDs also inhibit lipoxygenase enzymes, thus decreasing the production of leukotrienes (Siegel et al., 1979). NSAIDs also interfere with some membrane associated processes such as the NADPH oxidase activity in neutrophils (Biemond et al., 1986). Other neutrophil functions such as homotypic adhesion (Minta and Williams, 1985) and hydrogen peroxide generation (Abramson et al., 1985) are also inhibited by some NSAIDs.

Unfortunately, all of the NSAIDs have adverse reactions (reviewed by (Brooks and Day, 1991; Steadman, 1992), Table 1.18). The most commonly encountered problems with these drugs concern gastrointestinal complications such as dyspepsia, and less frequently, peptic ulceration. This is probably because they block the synthesis of prostaglandins which are required to regulate overproduction of gastric acids and to synthesise the stomach’s mucus barrier. In fact, it has been reported that in the United States, the syndrome of NSAID-associated gastropathy accounts for at least 2,600 deaths and 20,000 hospitalisations each year in patients with RA alone (Fries et al., 1989). Adverse effects on renal function, skin complaints, and perturbation of normal blood clotting and platelet aggregation are also common side effects of the NSAIDs (Brooks and Day, 1991).
Table 1.17

Processes influenced by NSAIDs

<table>
<thead>
<tr>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin synthesis</td>
</tr>
<tr>
<td>Leukotriene synthesis</td>
</tr>
<tr>
<td>Superoxide generation</td>
</tr>
<tr>
<td>Lysosomal enzyme release</td>
</tr>
<tr>
<td>Neutrophil aggregation and adhesion</td>
</tr>
<tr>
<td>Cell membrane functions</td>
</tr>
<tr>
<td>NADPH oxidase activity</td>
</tr>
<tr>
<td>Phospholipase C activity</td>
</tr>
<tr>
<td>Transmembrane anion transport</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>Uptake of arachidonic acid</td>
</tr>
<tr>
<td>Lymphocyte function</td>
</tr>
<tr>
<td>Rheumatoid factor production</td>
</tr>
<tr>
<td>Cartilage metabolism</td>
</tr>
</tbody>
</table>

Adapted from Brooks and Day (1991)
Table 1.18
Adverse reactions of major anti-inflammatory drugs

<table>
<thead>
<tr>
<th>Corticosteroid hormones</th>
<th>Nonsteroid Anti-inflammatory Agents</th>
<th>Disease modifying anti-rheumatic drugs</th>
<th>Immunosuppressants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>Diarrhoea</td>
<td>Nausea</td>
<td>Increased susceptibility to infection</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>Nausea</td>
<td>Anorexia</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>Glucose intolerance</td>
<td>Epigastric pain</td>
<td>Abdominal cramps/pain</td>
<td>Tremor</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>Heartburn</td>
<td>Renal disorders</td>
<td>Impaired renal function</td>
</tr>
<tr>
<td>Menstrual irregularities</td>
<td>Constipation</td>
<td>Skin reactions</td>
<td>Hepatic dysfunction</td>
</tr>
<tr>
<td>Muscle wasting</td>
<td>Abdominal cramps/pain</td>
<td>Immediate hypersensitivity/autoimmune phenomena</td>
<td>Gastrointestinal disturbances</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>Indigestion</td>
<td>Retinopathy</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Pathological fractures of long bones</td>
<td>Peptic ulcer</td>
<td>Haematopoietic disturbances</td>
<td></td>
</tr>
<tr>
<td>Glaucoma</td>
<td>Renal disorders</td>
<td>Central nervous system disturbances</td>
<td></td>
</tr>
<tr>
<td>Peptic ulceration, perforation and haemorrhage</td>
<td>Indigestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mental disturbances</td>
<td>Epigastric pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convulsion</td>
<td>Constipation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suppression of growth in children</td>
<td>Dizziness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggravation or precipitation of diabetes Mellitus</td>
<td>Dizziness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased susceptibility to infection</td>
<td>Dizziness</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data from Gallin (1992) and cited references
1.6.4.2 **Corticosteroids**

Glucocorticosteroids (glucocorticoids), due to their profound multiple effects on the inflammatory and immune responses, relieve all of the early symptoms of inflammation such as erythema, hyperemia, oedema and hyperalgesia (DiRosa *et al.*, 1985). Corticosteroids also have profound effects on the production and function of many mediators of inflammation including Igs, complement components, arachidonic acid metabolites, histamine and cytokines, which further affect inflammatory processes such as capillary dilatation and leukocyte migration into inflammatory foci (reviewed by (Goldstein *et al.*, 1992)). Glucocorticoids also have multiple profound effects on the cellular components that modulate the inflammatory and immune cascades (Goldstein, Bowen *et al.*, 1992). The major anti-inflammatory actions of glucocorticoids have been attributed to their effects on neutrophils and monocytes. The effects of glucocorticoids, which are too numerous to be described here in detail, are summarised in Table 1.19. While the net effect of glucocorticoids on human inflammatory and immune responses is suppression, their broad, non-specific effects and associated, sometimes life-threatening side effects, limits their use to severe or life-threatening situations (Table 1.18).

1.6.4.3 **Disease modifying antirheumatic drugs**

The widespread use of NSAIDs in the community, and the consequent morbidity of gastropathies such as peptic ulceration has led to concern about their use, even in chronic inflammatory situations such as RA. This has led to the emergence of drugs which seem to modify RA, but with a milder spectrum of side effects (reviewed by Smith and Ahern (Smith and Ahern, 1992)). Unlike NSAIDs, disease modifying anti-rheumatic drugs (DMARDs) are assumed to influence the disease process and slow its progression. Included in the group of DMARDs, which are now mostly used to treat RA, are gold compounds, anti-malarial drugs, D-penicillamine and sulfasalazine. All of these compounds have been shown in clinical trials to be more effective than a placebo (Ward *et al.*, 1983; Felson *et al.*, 1990), however the mechanisms of action of these agents is still unclear. Evidence suggests that most DMARDs act by local immuno-suppression, although each appears to have relatively characteristic modes of action at the level of preferential inhibition of the function of certain cells involved in chronic inflammation. Generally these compounds are weak immunomodulatory agents and tend to provide relatively temporary treatment due to their high toxicity, low efficacy (Wolfe, 1990) and drug resistance (Wollheim, 1988).
Table 1.19

Effects of glucocorticoids on inflammation and immunity

<table>
<thead>
<tr>
<th>Humoral: Eicosanoids:</th>
<th>Inhibit production and release in vitro (less consistent findings in vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>Inhibit generation and release in basophils and mast cells</td>
</tr>
<tr>
<td>Ig Production</td>
<td>In vivo, inhibit polyclonal production and decrease specific Ig synthesis particularly IgG and IgA subtypes; in vitro, effect dependent upon culture conditions and assay system used</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Inhibit production of IL-1, IL-2, IL-3, IL-6, IL-8, IFNγ, TNFα, CSF and plasminogen activator</td>
</tr>
<tr>
<td>PAF</td>
<td>Inhibition in many cell types</td>
</tr>
</tbody>
</table>

**Cellular**

| Phagocytic cells | Alteration of the distribution pattern resulting in peripheral neutrophilia and neutropenia at inflammatory sites; deplete monocytes from peripheral blood; prevent chemotaxis directly, inhibit phagocytosis and pinocytosis; suppress oxidative antimicrobial function; decrease cytotoxic function, decrease cytokine production; change surface receptor characteristics and alter cellular interactions; inhibition of release of elastase, collagenase, plasminogen activator, prostaglandins, but not release of lysosomal enzymes; no effect on antigen presentation |
| Neutrophils       | Induction of moderate neutrophilia; altered maturation of granulocytes; inhibition of neutrophil influx into inflammatory sites due to reduced margination and inhibition/total suppression of chemotaxis; blockage of plasminogen activator production by neutrophils; altered superoxide generation; altered synthesis of selected peptides; suppression of endocytosis |
| Eosinophils       | Depletion of peripheral eosinophils; decreased adherence and chemotaxis |
| Basophils, mast cells | Peripheral basophil depletion; inhibition of local mast cell proliferation; decrease histamine and specific granule release; deplete histamine content in cells; change cell-surface marker density |
| Lymphocytes       | Inhibition of antigen induced proliferation, alteration of the mitogenic response (depending upon the signal given), suppression of production of most lymphokines, inhibition of cell-cell interactions |
| Platelets         | Inhibition of platelet aggregation |
| Fibroblasts        | Inhibition of arachidonate release |
| Endothelial cells | Inhibition of arachidonic acid metabolite release, blockage of activation |

Adapted from Goldstein et al (1992)
Several studies have shown that about 70% of patients stop treatment with most DMARDs within 2 years (reviewed by (Felson, Anderson et al., 1990; Wijnands et al., 1990)). Risk factors associated with DMARDs vary depending upon the drug (Table 1.18). The major toxic manifestations of gold therapy involve the skin (eg. rashes) (Penneys et al., 1974) and mucous membranes, the kidney (eg. proteinuria) (Singh et al., 1991) and the haematopoietic system (eg. thrombocytopenia) (Madhok et al., 1985). The most common adverse reactions to antimalarial therapy are gastrointestinal, however a variety of central nervous system symptoms and rashes have also been reported (Stillman, 1981). Approximately two thirds of patients treated with D-penicillamine develop adverse reactions which include mucocutaneous reactions, gastrointestinal disturbances (eg. nausea, anorexia and abdominal pain), thrombocytopenia and nephropathy (Singh, Fries et al., 1991).

1.6.4.4 **Immunosuppressants**

Most immunomodulatory agents used in the treatment of autoimmune diseases cause non-specific suppression of the immune system (Reviewed by (Gourley et al., 1992)). Many of these agents were developed specifically for chemotherapy of malignant diseases. While this class of therapeutic agents may have higher efficacy than more conventional drugs, it also has high toxicity which can result in serious side effects (Table 1.18). Cyclophosphamide, for example, which has been used in the treatment of RA, was associated with a large increase in the risk of malignancy (Kahn et al., 1979) as well as a number of other debilitating side effects. Cyclosporin A has been used with some success in the treatment of RA (Yocum et al., 1988), but again, side effects such as hypertension and renal impairment can result. Methotrexate, an inhibitor of dihydrofolate reductase, has been used extensively in severe psoriasis, psoriatic arthritis and rheumatoid arthritis. Adverse effects of methotrexate therapy include bone marrow suppression and resultant leukopenia and thrombocytopenia, ulcerative stomatitis, nausea, vomiting, diarrhoea (from loss of intestinal epithelium), dermatitis and nephrotoxicity to name a few (Gourley, Seldin et al., 1992). In general, due to the severe side effects of this class of drugs, the use of these agents is reserved for life threatening or debilitating inflammatory manifestations of disease.
1.6.5 Novel inhibitors of inflammation

The large list of side effects associated with even the most commonly used anti-inflammatory agents (Table 1.18) highlights the need for improved therapeutic agents for autoimmune diseases. These agents should be more specific, with lower toxicities. As mentioned earlier (section 1.6.4) there are theoretically a number of novel approaches for designing more specific anti-inflammatory compounds which block the adhesion, transmigration and extravasation of leukocytes through the blood vessel wall at sites of inflammation. In this section I will limit discussion of these novel agents to three compounds which are believed to inhibit the BM passage of leukocytes, and which will be examined in some detail in this thesis, namely sulfated polysaccharides (SPS), phosphosugars and the glycoprotein processing inhibitor, castanospermine (CS).

1.6.5.1 Sulfated polysaccharides

Based on our understanding of leukocyte entry into inflammatory sites, SPS could potentially act as anti-inflammatory agents at two levels. First, they could interfere with cell adhesion by preventing selectin-anionic carbohydrate recognition, and second, they could inhibit BM degradation by acting as heparanase inhibitors. In fact, studies with metastatic tumour cells have supported the latter possibility. SPS were shown to have anti-metastatic activity in rodents, the activity being directly attributable to their ability to prevent the passage of tumour cells through the endothelium and underlying BM of blood vessels (Coombe, Parish et al., 1987). Non-anticoagulant species of heparin have similarly been shown to markedly reduce disease severity in EAE and in adjuvant induced arthritis in the rat, apparently by inhibiting lymphocyte entry into inflammatory sites (Willenborg and Parish, 1988; Lider, Baharav et al., 1989; Willenborg, Parish et al., 1992). Willenborg et al's experiments with EAE, using adoptively transferred MBP specific T cells, demonstrated that the effects of the SPS was on the effector, or inflammatory, phase of the response. Histological examination of spinal cord tissue taken 8 days after cell transfer showed extensive perivascular and meningeal inflammatory lesions in control rats and a total absence of inflammatory lesions in treated rats. Based on the available evidence SPS may inhibit inflammation either at the adhesion or extravasation stages of leukocyte entry into inflammatory sites. However, anti-metastatic SPS were shown to act by inhibiting tumour cell-derived heparanases (endoglycosidases) (see section 1.5.2) which degrade the heparan sulfate side
chains of blood vessel BM and thereby assist tumour cell extravasation (Parish, Coombe et al., 1987; Nakajima, Irimura et al., 1988).

In support of the importance of heparanases in tumour growth and metastasis, in very early studies Carr and co-workers (Carr, 1963) were able to inhibit the subcutaneous growth of a transplantable tumour by using, together or singly, specific inhibitors of the enzymes β-glucuronidase (saccharo-1,4-lactone) and β-N-acetylglucosaminidase (2-acetamido-2-deoxygluconolactone). Inhibition of tumour growth was suggested as being due to an inhibition of these glycosidases which might normally be involved in the degradation of hyaluronic acid and chondroitin sulfate of the ECM, although it is also possible that these enzymes were required during angiogenesis, which is necessary to support the growth of solid tumours.

It has also been shown that, like tumour cells, the ability of activated T-cells to penetrate the ECM and migrate to target tissues is related to the expression of an endoglycosidase that specifically degrades heparan sulfate moieties of the proteoglycan scaffolding of the ECM (Naparstek, Cohen et al., 1984; Savion et al., 1984). Accordingly, heparin has been shown to inhibit the activity of T lymphocyte heparanase in vitro and in vivo (Lider, Baharav et al., 1989). Interestingly, it has been found that some heparanases are unable to degrade heparin. This suggests that heparin, and some other SPS may be potent inhibitors of heparanases due to their ability to act as noncleavable substrates for these enzymes, occupying and blocking their active sites (Parish, Willenborg et al., 1990) (Fig. 1.16).

Alternatively, inhibition of inflammation by SPS may be at the level of adhesion. It has already been shown that the blocking of interaction between adhesion molecules and their counter-receptors can inhibit inflammation. Targeting the LFA-1/ICAM-1 interaction, it was found that a cocktail of mAbs to these adhesion molecules could confer indefinite survival on fully incompatible cardiac allografts in mice (Isobe et al., 1992). Further, antibodies to the VCAM-1-binding portion of VLA-4 prevented disease progression of EAE (Yednock, Cannon et al., 1992). Since selectins interact with anionic carbohydrate ligands it is possible that SPS block selectin-mediated adhesion of leukocytes to endothelium (Parish et al., 1984; Parish and Snowden, 1985; Brenan and Parish, 1986). Further work was required to resolve this issue at the time of my thesis commencement.
Fig. 1.16 Model for the mechanism underlying the anti-inflammatory effects of sulfated polysaccharides.
1.6.5.2 Phosphosugars

It is quite likely that some of the enzymes involved in the degradation of BMs during leukocyte extravasation are of lysosomal origin. As described in section 1.5.2 (Fig. 1.15), lysosomal enzymes are synthesised in the RER where they are glycosylated, and a specific M6P recognition marker is attached. The MPR, which recognises terminal M6P residues on lysosomal enzymes transports the enzymes to lysosomes and also recaptures lysosomal enzymes that have been secreted. The MPR-lysosomal enzyme interaction has been extensively studied and shown to be inhibited by exogenous M6P (Fischer et al., 1982; Steiner and Rome, 1982; Varki and Kornfeld, 1983). Parish and co-workers (Parish, Willenborg et al., 1990) hypothesised that these MPR/lysosomal enzyme interactions may provide a means of presenting ECM degradative enzymes at the cell-surface, thus aiding the passaging of leukocytes across the subendothelial BM. They further reasoned that M6P might have anti-inflammatory properties by being able to deplete leukocytes of cell surface bound lysosomal enzymes (Fig. 1.17). They tested this hypothesis and found that M6P could effectively inhibit EAE and adjuvant induced arthritis in the rat (Willenborg and Parish, 1989; Willenborg, Parish et al., 1992). It seems unlikely that M6P interferes with selectin mediated cell adhesion as selectins have a very low affinity for M6P both in vitro (Stoolman and Rosen, 1983; Stoolman et al., 1984; Cairns et al., 1986; Stoolman et al., 1987) and in vivo (Weston and Parish, 1991). These results also tend to support the hypothesis that lysosomal enzymes are involved in leukocyte extravasation. Moreover, because of the highly specific activity of M6P, it is potentially an effective anti-inflammatory which should be free of the numerous side effects encountered with other anti-inflammatory drugs currently used.

1.6.5.3 Glycoprotein processing inhibitors

In the process of glycoprotein synthesis, the core oligosaccharide chain consisting of Glc3 Man9 (GlcNAc)2 is transferred from a lipid carrier to asparagine residues on polypeptides. The oligosaccharide subsequently undergoes a number of enzymatic processing steps by glucosidases and mannosidases to produce the lysosomal enzyme M6P-recognition marker (Section 1.5.2, Fig. 1.15). The oligosaccharide processing required for the formation of this recognition marker is an obligatory stepwise process. If any one of the oligosaccharide trimming enzymes is inhibited, then further processing of the glycoprotein ceases, and the subsequent formation of the phosphorylated lysosomal enzyme recognition marker does not occur (Elbein,
**Mannose 6-Phosphate**

**Fig. 1.17** Model for the mechanism underlying the anti-inflammatory effects of mannose 6-phosphate (M6P)
In the light of above-described results with M6P, inhibitors of glycoprotein processing may similarly act as anti-inflammatory agents. Castanospermine (CS) is a recently described alkaloid of the indolizidine class first isolated from the seeds of the Australian legume *Castanospermum australie* (Hohenschutz *et al.*, 1981). It is a potent inhibitor of glucosidase-I, an oligosaccharide trimming enzyme involved in the formation of N-linked carbohydrate structures on glycoproteins (Sasak, 1985). Castanospermine treatment would also result in the formation of aberrant carbohydrate moieties on the lysosomal enzymes which lack the M6P marker and therefore are not recognised by the MPR (Elbein, 1987). Consequently the cell would be unable to target lysosomal enzymes to the lysosomes, capture lysosomal enzymes that have been released by the cell, or express these enzymes on the cell surface via the MPR (Fig. 1.18).

In support of the hypothesis that expression of lysosomal enzymes is important in the enzymic penetration of inflammatory cells into tissues, CS was found to inhibit EAE in rats (Willenburg, Parish *et al.*, 1989). Histology taken from CS treated animals provided some insights into the mechanism of inhibition of EAE. Unlike M6P and heparin treated rats, CS treated rats were found to have numerous CNS lesions. These lesions were characteristically intense, but inflammatory cells were found to be tightly aggregated around the margins of vessels, with very few mononuclear cells entering the brain parenchyma compared to control animals. Electron microscopy revealed that mononuclear cells had crossed the endothelium, but subsequently accumulated between the ECs and the subendothelial BM (C. Parish, personal communication). Following these experiments it was proposed that the transferred T cells may have had sufficient preformed lysosomal enzymes to allow normal migration across the CNS endothelium into the parenchyma, whereas the recruitment of host monocytes was prevented due to the depletion of lysosomal enzymes from these cells (Parish, Willenburg *et al.*, 1990).

1.7 Aims of this study

A number of reports have demonstrated the potent anti-inflammatory activity of the SPS, heparin, and have suggested that the anti-inflammatory effects of this compound are due to the inhibition of the endoglycosidase, heparanase. Following evidence that heparanase and a number of other BM-degrading enzymes are of lysosomal origin (Kaiser, 1980; Nakajima,
Fig. 1.18 Model for the mechanism underlying the anti-inflammatory effects of castanospermine (CS)
Irimura et al., 1983), and that lysosomal enzymes can be expressed on the cell surface via MPR, (von Figura and Voss, 1979; Fischer, 1980), (reviewed by (Kornfeld, 1987; Kornfeld, 1990), Willenborg et al showed that the phosphosugar, M6P, and CS, an inhibitor of N-linked oligosaccharide processing, are both effective inhibitors of EAE and adjuvant arthritis (Willenborg and Parish, 1989; Willenborg, Parish et al., 1989; Willenborg, Parish et al., 1992). The major aim of this thesis was to test the hypothesis, proposed by Parish et al (1991), that the anti-inflammatory effects of CS and M6P are due to their ability to displace lysosomal enzymes from the leukocyte cell surface, thereby preventing the degradation of subendothelial BM and ultimately impeding the ability of leukocytes to transmigrate into tissues.

1.7.1 Anti-inflammatory effects of SPS, M6P and CS
The anti-inflammatory activity of SPS has been fairly well documented previously. However, the anti-inflammatory effects of CS and M6P have only recently been reported. Thus, an initial study was conducted to confirm the previous findings that SPS, M6P, and CS are effective inhibitors of inflammation. The two models studied were allograft rejection and entry of leukocytes into the peritoneal cavity following an inflammatory stimulus. Since the working hypothesis is that these compounds inhibit leukocyte transmigration, the models used in this study were chosen on the basis of their ability to provide data on the level of infiltration of leukocytes, so that comparisons between control and treated animals could be made.

1.7.2 Effects of SPS, M6P and CS on degradation of the subendothelial BM
SPS, M6P and CS have been shown to inhibit inflammation and prolong allograft survival. However the only direct evidence for the cell-surface expression hypothesis of Parish et al was in the histology of CNS lesions of EAE rats, where CS appeared not to be affecting leukocyte-EC adhesion and transmigration events, but appeared to be inhibiting the subsequent passage of monocytes through the subendothelial BM. Thus, these studies were unable to provide direct evidence of the effect of these compounds on BM-degrading enzymes expressed by the extravasating leukocytes. The major aim of the present study was to address the hypothesis that these inhibitors prevent degradation of the ECM.
Before the anti-inflammatory compounds were tested for their effects on BM degradation, a preliminary study was conducted to investigate the comparative potential of the major cellular components of inflammation, both resting and activated, to degrade the subendothelial BM. This aspect of my thesis also included a comparative analysis of venular and arterial EC in terms of their ability to express adhesion molecules or degrade the subendothelial BM before and after cytokine activation. Following this analysis, the major cells involved in inflammation were tested for their ability to degrade the ECM in the presence of the anti-inflammatory compounds heparin, M6P and CS.

Leukocytes, endothelium and the subendothelial BM are intimately involved in the inflammatory response, and any insights that can be gained into these cell-cell and cell-ECM interactions will be of considerable importance in understanding a number of clinical disorders including autoimmune pathologies. Clarification of the roles and relative importance of leukocyte and EC degradation of the BM, and the effects of these novel anti-inflammatory compounds which are thought to perturb this vulnerable point in the process of inflammation, may ultimately lead to new, more specific ways of treating human disease.
Chapter 2

Materials and methods

2.1 Animals
Specific pathogen free BALB/c (H-2^d) and CBA/H (H-2^k) male mice were obtained from the John Curtin School of Medical Research Animal Breeding Establishment, The Australian National University, Canberra, and were used at 9-12 weeks of age.

2.2 Tumour Cell lines
The murine mastocytoma P815 was maintained by serial passage in tissue culture medium F15 (Eagle's Minimum Essential Medium, Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat inactivated foetal calf serum (FCS) (Flow Laboratories, Rickmansworth, UK). 13762 MAT, a rat metastatic mammary adenocarcinoma cell line, was cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS.

2.3 Antibodies
An E-selectin mAb (clone 4D10, Rat IgG2a) was obtained from Immunotech, S.A. (Cedex, France), an ICAM-1 mAb (WEHI-CAM-1, IgG2a) was a gift from Dr Andrew Boyd, Walter and Eliza Hall Institute, Melbourne, Australia and a VCAM-1 mAb was a gift from Prof. J. Harlan, University of Washington, Seattle. Fluorescein conjugated Sheep F(ab)\_2 Anti-mouse Ig was obtained from Silenus Laboratories, (Melbourne, Australia).

2.4 Activating factors and cytokines
PMA (Sigma Chemical Company, St. Louis, MO) was added to a concentration of 50 ng/ml (95 nM). N-formyl-Met-Leu-Phe (fMLP) was purchased from Sigma Chemical Company (St. Louis, MO) and used at a concentration of 100 nM. Monocyte Chemotactic and Activating Factor (MCAF, recombinant human, Genzyme Corp., Cambridge MA) was used at a concentration of 50 ng/ml. TNF\(\alpha\) (recombinant human TNF\(\alpha\), 2.2 x 10^6 U/mg, Asahi Chemical Co, Tokyo Japan) was used at a concentration of 2.5 ng/ml. IL-1\(\alpha\) (recombinant IL-1\(\alpha\) purified from E. coli, 150 x 10^6 U/mg, Hoffmann-LaRoche Inc., Nutley NJ) was used at a concentration of 0.2 ng/ml. IL-8 (recombinant human -77 amino-acid, Biosource International, Westlake Village, CA) was used at 100 ng/ml. IFN\(\gamma\) (recombinant human,
Garamycin (gentamycin) was obtained from Schering Corp. (Kenilworth, NJ). Gelatin, trypsin and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Medium 199 contained Earle’s salts.
Boehringer Ingelheim Pty Ltd, NSW, Australia) was used at 200 U/ml.
4HPE (4-hydroxy-pentadecenal) was a gift from Prof. H. Esterbauer,
(Institut für Biochemie der Universität, Graz) and was used at a
concentration of 20 nM.

2.5 Anti-inflammatory compounds
CS was isolated and purified in our laboratory from the seeds of the legume
Castanospermum australe following a previously described procedure
(Hohenschutz, Bell et al., 1981). M6P, heparin (bovine intestinal mucosa)
and fucoidan were purchased from Sigma Chemical Company (St. Louis,
MO). Fucoidan, rather than heparin, was the chosen SPS for in vivo studies
because of its low anticoagulant effects whereas heparin was used for in vitro
experiments. CsA was a gift from the Hunter Valley Transplantation Unit,
John Hunter Hospital, Newcastle, Australia.

2.6 Preparation of endothelial cells (EC)
Human umbilical vein ECs were obtained from umbilical veins by the method
of Jaffe (Jaffe et al., 1973), with some minor modifications. After cutting off
all areas of the cord with clamp or syringe marks, one end of the cord was
cannulated with a size 10 foley catheter (Boston & Pacific Co., Boston, MA).
The vein was perfused with 100 ml PBS to wash out the blood. After
clamping the other end of the vein with forceps, collagenase, type IV
(Worthington Biochemical Corporation, Melbourne, Australia) at 50 U/ml
was infused into the vein. The umbilical vein was then incubated at 37°C in
a shaking water bath for 15 mins. After incubation, the ECs were flushed
from the cord by perfusion with 20 ml of medium 199 (Flow Laboratories,
Irvine, UK) containing 20% FCS and garamycin 60 U/ml, into a 50 ml plastic
tube. The cells were centrifuged at 250 x g for 10 minutes and the pellet
resuspended with 5 ml fresh medium containing garamycin (60U/ml), 4 mM
glutamine (Sigma, St. Louis, MO), 13.5 U/ml heparin (David Bull
Laboratories, Melbourne, Australia) and 100 µl/ml endothelial cell growth
supplement (ECGS) (Sigma, St. Louis, MO). The cells were placed into a
gelatinised (0.1%) plastic 25 cm² tissue culture flask (Corning Glass Works,
Corning, NY) and incubated at 37°C under 5% CO₂. The medium was
replaced with fresh medium plus additives twice per week. Once confluent,
the cells were passaged using 1% trypsin-EDTA. Cells from passage 5 - 7
were used in most experiments.
Human umbilical artery ECs were obtained from umbilical arteries using the same method as for HUVECs but with some minor modifications. After cannulation and infusion of the artery with approximately 5 ml of collagenase (50 U/ml) (Worthington Biochemical Corporation, Melbourne, Australia) the cord was lightly massaged and incubated at 37°C in a shaking water bath for 10 mins. After incubation, the arterial ECs were flushed from the cord by perfusion with 20-30 ml of Hanks balanced salt solution (Cytosystems, Sydney, Australia) into a 50 ml plastic tube. FCS was added to the perfusate to 20% (v/v) and the cells were centrifuged at 1 000 x g for 10 minutes. The pellet was resuspended with 5 ml fresh medium (as for HUVECs), the cells placed into a gelatinised (0.2%) plastic 25 cm² tissue culture flask (Corning Glass Works, Corning, NY) and incubated at 37°C under 5% CO₂. Following isolation of the arterial EC, the culture conditions were as described for HUVECs.

Bovine corneal ECs were prepared according the method of MacCallum (MacCallum et al., 1982) with some minor modifications. Adult bovine eyes were obtained from cattle within 2 hours of slaughtering. After removal of adhering extraocular tissue, the eyes were soaked in a solution of 70% alcohol, 0.5% hibitane for 5 mins. Under sterile conditions, the eyes were then rinsed in medium 199 with double strength antibiotics before excision of the cornea. The corneal endothelium was soaked in 0.05% trypsin and 0.02% EDTA for 20min. at room temperature. The cornea was then rubbed gently with the back of a curved scalpel (Feather Safety Razor Co. Ltd, Japan). The dislodged ECs were aspirated with a pipette and cultured in a 25 cm² tissue culture flask with medium 199 containing 20% heat inactivated FCS and garamycin (60 U/ml) under 5% CO₂. Cells were checked regularly for fibroblasts, which were removed if necessary by flushing gently with trypsin for one minute.

2.7 Preparation of human blood cells
Mononuclear and polymorphonuclear leukocytes were simultaneously isolated from human peripheral blood by centrifugation of diluted blood on Polymorphprep™ (Nycomed Pharma A.S., Oslo, Norway). Fifteen to 20 ml of fresh whole blood anticoagulated with 10 U/ml heparin (Commonwealth Serum Laboritories, Melbourne Australia) was obtained by venipuncture from human volunteers who denied taking any drugs during the previous 2 weeks. The blood was diluted to 50% with phosphate buffered saline (PBS) and divided into 8 ml aliquots in 10 ml flat bottomed plastic test tubes. The
blood was then underlayed with 2-3 ml of Polymorphprep™ and centrifuged for 27 min. at 268 x g. After centrifugation, mononuclear and polymorphonuclear leukocytes were aspirated from their respective bands and washed 3 times in PBS. Cells were incubated in 0.83% NH₄Cl at 37°C for 10 minutes to lyse erythrocyte contaminants. Adherent cells were removed from the mononuclear cell fraction by incubation at 37°C for 1 hr in Hank's balanced salt solution with 5% FCS on plastic tissue culture flasks or by incubation for 2 hrs at 37°C on fibronectin coated, gelatinised tissue culture flasks in PBS plus 5% FCS. At this point, non adherent cells (primarily lymphocytes) were decanted from the flasks. Adherent cells (enriched for monocytes) were detached by incubation with 5 mM EDTA in PBS:medium 199 (1:1) plus 5% FCS for 10 min. at 37°C, followed by agitation of the flask. These cells were then washed twice with PBS. Finally, all cells were resuspended to 2 x 10⁶ cells/ml in RPMI medium 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS. Cytocentrifuge smears were prepared by centrifuging 10⁵ cells in 80 µl of 50% FCS onto glass slides, air drying and fixing immediately. Neutrophils and mononuclear cell preparations were greater than 95% pure by Diff-Quick staining (Lab Aids, Narrabeen, Australia) and non-specific esterase staining (Yam et al., 1971) and were greater than 95% viable by trypan blue exclusion. Greater than 80% of the monocyte preparation expressed non-specific esterase.

ConA activated T cells were a kind gift from Dr H. Warren and were prepared as previously described (Warren and Skipsey, 1991). U937, a macrophage cell line (CRL1593), was obtained from the American type Culture Collection (Rockville, MD) and was cultured in RPMI medium 1630 (Gibco, Grand Island, NY).

Platelets were prepared from venous blood obtained from healthy adult volunteers who were non-smokers and who had denied taking any drugs for at least two weeks previously. Blood for platelet isolation was collected into 1/7 volume acid citrate-dextrose solution (2.5 g sodium citrate, 1.5 g citric acid and 2 g dextrose in 100 ml of water) and platelet-rich plasma prepared by centrifugation at 100 x g for 15 min. at room temperature. The platelet-rich plasma was removed and the platelets sedimented at 300 x g for 15 min. at room temperature. The platelets were washed three times in a modified Tyrode's solution containing bovine serum albumin (3.0 g/l) and prostaglandin I₂ (PGI₂) (3µg/ml). The washed platelets were finally resuspended in the
modified Tyrode's solution without PGI2 and kept in a stoppered syringe at 37°C for 30 min. to allow the effect of PGI2 to wear off.

2.8 Preparation of radio-labelled ECM
Bovine corneal endothelial cells were seeded onto 3.5 cm plastic tissue culture dishes (Kayline, Camden Park, Australia) in medium 199 with additives (see section 2.5) as well as 5% dextran T40 (Pharmacia, Uppsala, Sweden). At days 4-5, and 8-9 (by which time the cells were confluent), the medium was changed and 35SO4 (as H2SO4, Dupont/NEN, Boston MA) was added at 40 µCi/ml. For 3H-labelled ECMS, 3H (as glucosamine hydrochloride, D-(6-3H(N)), Dupont/NEN, Boston MA) was added at 10 µCi/ml. After isotopic labeling, the endothelial cell monolayer was rinsed twice in PBS and lysed by the addition of 20 mM NH4OH for 5 minutes at room temperature. The NH4OH treatment was repeated before washing twice in PBS. Then 0.5% Triton X-100 in PBS was added and the dishes rocked for a further 30 min. at room temperature, rinsed twice with PBS and then air dried. Dishes not used immediately were stored under PBS plus 0.1% azide at 4°C. Stored ECMs were rinsed four times with PBS before using to remove all traces of azide.

2.9 Degradation of 35SO4 labelled ECM
Leukocytes and endothelial cells were added directly to the labelled ECM at a density of 10^6 cells per dish in 1.0 ml of medium, as indicated, containing 10% FCS. Platelets were added at a density of 10^8 cells per dish in 1.0 ml of medium. The cells or platelets were then incubated at 37°C under 10% CO2 for 24 hours unless otherwise indicated. In some experiments the 35SO4-labeled GAG side-chains were released from the ECMs by treating the latter with 1 ml of 0.5M NaOH for 18 hr at 37°C in a humidified atmosphere. In the case of nitrous acid cleavage for molecular weight determination, GAGs were exposed to 0.24M NaN02 in 1.8 M acetic acid for 80 min. at room temperature (Lindahl et al., 1973). Papain and chondroitinase ABC treatments were as described previously (Wasteson, 1971; Roden et al., 1972; Parish, Coombe et al., 1987).

2.10 Chromatography
Culture supernatants from ECM degradation experiments were centrifuged at 13,000 x g for 5 min. in an Heraeus Biofuge centrifuge (Heraeus Sepatech GmbH, Kalkberg, Germany). Supernatants were then subjected to gel filtration chromatography utilising a Superose-6 column on an automated FPLC chromatography system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The flow rate for the mobile phase (PBS) was 0.25 ml/min. 0.5 ml
fractions were counted for radioactivity in 3.0 ml Ready safe™ scintillation fluid (Beckman Instruments Inc., Fullerton, CA) using a Packard Tricarb β-scintillation counter (Model 1900CA, Packard Instrument Company, Meriden, CT). Heparan sulfate mol. wt. standards (mol. wts 30,000; 16,000; 10,000; 6,700 and 3,100 Da) were a gift from Dr C. Freeman.

2.11 Immunofluorescent flow cytometry
Cells were treated with saturating concentrations of mAb, washed and incubated with sheep anti-mouse Ig directly conjugated with fluorescein. Incubations were in 96 well V-shaped plates using 10^5 ECs/well. All incubations were for 30 min on ice, and cells were washed three times in between the different incubations. Cells were either fixed with paraformaldehyde (1%) and analysed within 1 day, or analysed immediately. Samples analysed for fluorescence on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA) using Lysys software and analysed for median fluorescence intensity units (FIU) using Lysys software. Median FIU values were compared with control samples incubated with the fluoresceinated anti mouse Ig reagent.

2.12 Administration of anti-inflammatory compounds
Since CS and M6P have relatively short serum half lives, all of the compounds were dissolved in saline and administered by miniosmotic pumps (Alza Corp, Palo Alto, CA) which were implanted subcutaneously. The pumps released the drugs at a rate of 1 µl/hr or 0.5 µl/hr over a period of 1 or 2 weeks respectively. Drug concentrations were as indicated in the text. Control mice were implanted with miniosmotic pumps containing saline to control for any pump induced effects on inflammation (Willenborg, Parish et al., 1992). CsA was dissolved in pure olive oil at a concentration of 50 mg/ml and administered subcutaneously (60 mg/kg) one day before immunisation with the P815, and again 5 days after immunisation.

2.13 Tumour allografts
In tumour allograft experiments 3.0 x 10^6 P815 cells suspended in 100 µl of saline were injected subcutaneously into the shaved flank of CBA/H mice. Tumour size was determined at daily intervals using vernier callipers (Mitutoyo, Tokyo, Japan) with a dial gauge (360°=5.0 mm) and expressed as the average of two measurements of the tumour at right angles to each other.
2.14 Thyroid allografts
BALB/c mice were treated with cyclophosphamide (300 mg/kg) at 4 and 2 days prior to thyroidectomy. The thyroids were cultured for 3 weeks in 95% O₂ and 5% CO₂ as described previously (Lafferty et al., 1976). Thyroids were transplanted under the kidney capsule of thyroidectomized CBA/H mice (H-2k) and graft acceptance monitored at 3 weeks by visual inspection of the graft by laparotomy. Grafts were allowed to remain in the mice for at least 8-10 weeks before rejection of the grafts was elicited. Rejection of the thyroid allografts could be effectively triggered by the i.p. transfer of 2.5 x 10⁷ P815 tumour cells (Warren and Pembrey, 1986) or 2.5 x 10⁷ BALB/c (H-2d) derived spleen cells. Following elicitation of rejection, grafts were removed and fixed in formalin for histological examination.

2.15 Measurement of cytotoxic activity
Spleen cells were assayed for their cytotoxic activity on the P815 tumour cell target by methods described previously (Prowse et al., 1983). Cytotoxic activity on ⁵¹Cr-labeled target cells is expressed as cytotoxic units (CU), which adjusts the percentage of specific lysis to a value linearly related to the number of effector cells (Davidson, 1977). One CU is defined as the amount of activity that causes the specific lysis of one target cell during a 4 hr incubation period.

2.16 Assessment of graft rejection
Graft rejection was assessed by histological examination of 4µm sections taken at 18 section intervals through the entire graft (H and E stained). Each section was scored on an arbitrary scale from 1 to 7 as previously described (Warren and Pembrey, 1986) and the average score calculated for each graft. Briefly: score 1: focal point of mononuclear cells (MNC), score 2: less than 25% MNC infiltration, score 3: 25-50% MNC infiltration, score 4: 50-75% MNC infiltration, score 5: 75-100% MNC infiltration, score 6: total destruction of the graft, score 7: scar tissue only remaining (Fig. 2.1).

2.17 Elicitation of peritoneal exudates
Dehydrated fluid thioglycollate medium (Difco Laboratories, Detroit, MI) was reconstituted at 59.6 g/l in boiling water, sterilised and left in the dark to mature for at least 6 weeks. Peritoneal exudates were elicited by the injection i.p. of 2 ml per mouse of the sterile medium. Alternatively, mice were primed for an antigen-induced peritoneal exudate by injecting a 1:1 emulsion of Freund’s complete adjuvant (FCA, Difco Laboratories, Detroit,
Fig. 2.1
Histological analysis of rejecting thyroid grafts. Grafts were scored according to the degree of mononuclear cell (MNC) infiltration and tissue destruction. The scores, as indicated, are: 0: intact graft; 1: focal point of MNC; 2: <25% MNC; 3: 25-50% MNC; 4: 50-75% MNC; 5: 75-100% MNC; 6: total graft destruction; 7: scar tissue. The parathyroid, where present, is indicated by an arrow.

From: Warren and Pembrey (1986)
MI) and saline subcutaneously (50 µl per site) in four separate sites per mouse. The exudate was induced 1 week or more later upon i.p. injection of 25 µg of purified protein derivative of tuberculin (PPD, Commonwealth Serum Laboratories, Melbourne, Australia) in 500 µl saline. The exudate was extracted by peritoneal lavage using a total of 10 ml of ice-cold magnesium/calcium-free phosphate buffered saline (PBS), pH 7.0, in three rinses. Cells were pelleted by centrifugation at 200 x g for 10 minutes, washed twice in PBS and resuspended in RPMI 1640 culture medium (Grand Island Biological Co., Grand Island, NY) containing 10% FCS prior to cell counting.

2.18 Assay of cell type and number in peritoneal exudates
Peritoneal exudate cells were mixed with the vital stain trypan blue and counted on a haemocytometer. Cytocentrifuge smears were prepared by centrifuging $10^5$ cells in 80 µl of 50% FCS onto glass slides. Smears were then air dried, fixed and stained by Diff-Quick stain (Lab Aids, Narrabeen, Australia). Neutrophil, macrophage and monocyte/small mononuclear cell content of exudates was determined microscopically (Olympus microscope model BH-2) from the smears by using an ocular grid and categorising and counting the number of cells in at least 5 fields (magnification x 200) for each sample.

2.19 Statistics:
Sample variables were analysed by their group means (Student’s t test). P values less than 0.05 were considered statistically significant.
Chapter 3

Effects of the anti-inflammatory compounds castanospermine, mannose-6-phosphate and fucoidan on allograft rejection and elicited peritoneal exudates

3.1 Introduction

Experimental autoimmune encephalomyelitis (EAE) and adjuvant induced arthritis in the rat were recently shown to be inhibited by the glycoprotein processing inhibitor castanospermine (CS), sulfated polysaccharides (SPS) and the simple sugar phosphate, mannose-6-phosphate (M6P) (Cohen, Lider et al., 1987; Willenborg and Parish, 1988; Willenborg, Parish et al., 1989; Willenborg, Parish et al., 1992). SPS and CS were also found to prolong skin and renal allograft survival (Lider, Baharav et al., 1989; Grochowicz et al., 1990). The ability of activated T-cells and macrophages to penetrate the subendothelial extracellular matrix (ECM) and migrate to target tissues is reported to be related to the activity of certain lysosomal enzymes (Naparstek, Cohen et al., 1984; Savion, Vlodavsky et al., 1984), suggesting that degradation of components of the ECM is important in allowing activated leukocytes to cross the vascular endothelium during inflammation. The anti-inflammatory activity of SPS has been thus attributed to its ability to inhibit lysosomal endoglycosidase enzymes or heparanase (Cohen, Lider et al., 1987; Lider, Baharav et al., 1989). There is some evidence to suggest that CS and M6P also prevent the extravasation of leukocytes into an inflammatory focus by inhibiting degradation of the ECM during inflammation (Naparstek, Cohen et al., 1984; Savion, Vlodavsky et al., 1984; Willenborg, Parish et al., 1989; Parish, Willenborg et al., 1990). Lysosomal enzyme targeting and expression are dependent upon their interaction with specific mannose-6-phosphate receptors (MPR) (Kornfeld, 1987), and are thus potentially sensitive to glycoprotein processing inhibitors and M6P which can interfere with these interactions.

The purpose of this study was to examine the anti-inflammatory effects of CS, M6P and the SPS, fucoidan, in three different models of inflammation with the goal that such studies may also increase our understanding of the mode of action of these compounds. In the first model, rejection of permanently accepted thyroid allografts vascularized by host endothelium was triggered by the transfer of donor strain stimulator cells. The second
model used mastocytoma allografts, also vascularized by the host endothelium. The third model used thioglycollate induced and antigen induced elicitation of peritoneal leukocytes. In two of the models these anti-inflammatory agents had moderate effects on leukocyte accumulation, although there were subtle differences in the efficacy of the three compounds.

### 3.2 Results

**3.2.1 Effect of CS, M6P and fucoidan on thyroid allograft rejection:** Rejection of cultured BALB/c (H-2^d^) thyroids permanently accepted in CBA/H (H-2^k^) mice could be effectively triggered by the i.p. transfer of 2.5x10^7 P815 (H-2^d^) tumour cells (Warren and Pembrey, 1986) or BALB/c spleen cells. Rejection triggered by P815 was maximum between day 10 (80% MNC infiltration, n=2) and day 13 (100% MNC infiltration or scar tissue, n=2) following immunisation, at the time when the splenic CD8+ cytolytic response was greatest. In 9 different experiments the cytotoxic activity of day 10-12 immune spleen cells was 99±28 x 10^2 Cytotoxic Units/10^6 cells (mean±SE).

Rejection triggered by BALB/c spleen cells was more rapid than that triggered by P815 with substantial graft destruction by day 7 (50-60% MNC infiltration, n=6) and complete destruction by day 10 (100% MNC infiltration or scar tissue, n=6) (Fig. 3.1). In contrast to the strong splenic cytolytic response generated in response to P815 immunisation, immunisation with BALB/c spleen cells gave little or no response at day 10, corresponding to the time of total graft destruction. These data suggest that factors other than the generation of a splenic CD8+ cytotoxic T cell response are important in determining the final outcome of graft rejection.

In the experiments described in this study, based on the preliminary studies outlined above, allografts were examined histologically for MNC infiltration at 7 days following BALB/c spleen cell immunisation and 11 days following P815 immunisation. With the experiment presented in Table 3.1, P815 injection resulted in 100% MNC infiltration of the allografts (score of 6.0±0.1) in all recipients by day 11, whereas spleen cell immunisation resulted in a significantly lower MNC infiltrate by day 7 (score of 4.8±0.4). Cyclosporin A (CsA) treatment inhibited P815 induced allograft rejection although substantial MNC infiltration was still observed in all grafts (score 4.2±0.2).
Fig. 3.1
Histology of rejecting thyroid allografts showing reduction in leukocyte infiltration after treatment with CS, M6P and fucoidan. A: Intact graft from unimmunized animal, B: graft showing MNC infiltration 11 days following P815 injection, C: MNC infiltration 7 days following BALB/c spleen cell injection, D: Immunization as in C, but with M6P treatment at 100mg/kg/day, E: Immunization as in C, but with CS treatment at 200mg/kg/day, F: Immunization as in C, but with fucoidan treatment at 100mg/kg/day,
In contrast, unimmunised animals exhibited little or no influx of MNC in the thyroid grafts (score 0.4±0.5) (Table 3.1).

CS, M6P and fucoidan treatment of mice immunised with donor strain (H-2d) spleen cells resulted in lower levels of leukocyte infiltration than controls, with mean scores from 25% to 36% lower than saline treated control mice, i.e., approximately 50% mononuclear cell infiltration in the treated animals compared to around 75% mononuclear cell infiltration in the control mice (Table 3.2).

Where acute rejection of the thyroid grafts was elicited by P815 (H-2d) tumour cells, no significant differences were observed between the saline treated mice, and those treated with CS, M6P and fucoidan (Table 3.2). However, when the 3 compounds were re-tested for their effects on P815 induced graft rejection in the presence of the immunosuppressant CsA, CS, M6P and fucoidan reduced the extent of leukocyte infiltration by 30%, 22% and 17% respectively compared to mice treated with CsA alone (Table 3.2, Fig. 3.1).

3.2.2 Effect of CS, M6P and fucoidan on tumour allograft rejection
As an additional measure of the ability of the three compounds to inhibit allograft rejection, CS, fucoidan and M6P were examined for their ability to inhibit the rejection of a tumour (P815) allograft in CBA/H mice. Following a subcutaneous injection of P815 cells in the flank of mice the progress of the tumour was monitored over 14 days. The tumour increased in size from a mean diameter (+SE) of 2.0 ± 0.46 mm on day 4 to 6.2 ± 0.66 mm on day 7 post injection. After day 7 the graft gradually reduced in size to 1.1 ± 0.36 mm on day 14. Treatment with M6P, CS or fucoidan commenced 3 days following immunisation so that any possible immunosuppressive effects of the compounds could be minimised. Neither M6P, CS nor fucoidan produced a significant change in tumour growth and rejection when compared with saline controls (Fig. 3.2).

3.2.3 Effect of CS, M6P and fucoidan on the leukocyte infiltration into the peritoneal cavity
In the models described above, the effects of CS, M6P and fucoidan may have been manifested either through an effect on the induction of the immune effector cells or on leukocyte migration. To help distinguish between these two possibilities we next studied the effects of CS, M6P and fucoidan on
Table 3.1
Ability of different stimuli to elicit rejection of thyroid allografts\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histological score\textsuperscript{b}</th>
<th>Mean score ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>0.2, 0.5, 0.0, 0.2</td>
<td>0.4±0.5</td>
</tr>
<tr>
<td>Spleen cells (2.5 x 10\textsuperscript{7} i.p.)\textsuperscript{c}</td>
<td>5.9, 4.5, 4.7, 4.2</td>
<td>4.8±0.4</td>
</tr>
<tr>
<td>P815 (2.5 x 10\textsuperscript{6} i.p.)</td>
<td>6.0, 6.1, 6.0, 6.0</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td>P815+CsA\textsuperscript{d}</td>
<td>4.9, 4.0, 4.0, 4.0</td>
<td>4.2±0.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Cultured thyroids from BALB/c mice placed under the kidney capsule of CBA/H recipients

\textsuperscript{b} Grafts were scored as described in the Materials and Methods, the scores indicating the degree of leukocyte infiltration into the graft.

\textsuperscript{c} Spleen cells were donor (BALB/c) strain

\textsuperscript{d} CsA (60mg/kg) was injected subcutaneously 1 day prior to and again at 5 days post P815 injection.
Table 3.2
Reduction in leukocyte infiltration into thyroid allografts after treatment with CS, M6P and fucoidan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>Spleen cells (2.5 x 10^7)</th>
<th>P815 (2.5 x 10^6)</th>
<th>P815+CsA&lt;sup&gt;b&lt;/sup&gt; (2.5 x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td></td>
<td>4.8±0.4 (0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0±0.1 (0)</td>
<td>4.2±0.2 (0)</td>
</tr>
<tr>
<td>CS</td>
<td>200</td>
<td>3.1±0.1 (36)*</td>
<td>6.0±0.1 (0)</td>
<td>3.0±0.3 (30)*</td>
</tr>
<tr>
<td>M6P</td>
<td>100</td>
<td>3.6±0.1 (25)*</td>
<td>6.0±0.0 (0)</td>
<td>3.3±0.2 (22)*</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>100</td>
<td>3.6±0.2 (25)*</td>
<td>6.0±0.1 (0)</td>
<td>3.5±0.1 (17)*</td>
</tr>
</tbody>
</table>

<sup>a</sup> Histological score of grafts as in Table 1. Values in brackets represent percent reduction in leukocyte infiltration induced by drug treatments. Significant reductions in infiltrate compared with control animals indicated by * (P<0.05).

<sup>b</sup> CsA (60mg/kg) was injected s.c. 1 day prior and again at 5 days post P815 injection.
Effect of (A) M6P (40 mg/kg/day) and (B) CS (200 mg/kg/day) or fucoidan (40 mg/kg/day) on the rejection of tumour (P815) allografts in CBA/H mice. Drug administration (miniosmotic pump) was commenced 3 days following subcutaneous injection of the tumour. Control in (A) represents mouse with no pump implanted.
thioglycollate elicited peritoneal exudates. In this model we examined qualitative and quantitative changes in leukocyte infiltration into the peritoneal cavity induced by thioglycollate and by specific antigen.

Within 24 hours following i.p. thioglycollate injection there was a 40-100 fold increase in leukocytes in the peritoneal cavity from a resting level of approximately $10^6$ resident cells. The cellular composition of the elicited peritoneal exudate varied with time, neutrophils representing a substantial proportion of the exudate at 24 hr, and macrophages/MNC being the predominant cell type by 48 hr. By day 5 virtually all of the cells in the peritoneum were mononuclear cells.

When mice were implanted with pumps containing CS, M6P or fucoidan 24 hr prior to the injection of thioglycollate there was a significant inhibition in leukocyte infiltration compared to saline treated controls (Fig. 3.3). Overall CS was found to be the most effective inhibitor, giving sustained inhibition of leukocyte infiltration over the 108 hr time period of the experiments. In contrast, the effects of M6P and fucoidan were more transient with the effect of M6P on leukocyte influx abrogated by 108 hr (Fig. 3.3B). The analysis of the effects of CS, M6P and fucoidan on the cellular composition of the peritoneal exudates at 36 hr revealed that all three compounds significantly reduced the proportion of neutrophils relative to the other cell types (Table 3.3).

CS and fucoidan, but not M6P, also affected the antigen induced influx of leukocytes into the peritoneal cavity. In these experiments mice were inoculated subcutaneously with Freund’s complete adjuvant (FCA), followed by an i.p. injection of PPD 1-2 weeks later. This treatment resulted in a 2-3 fold increase in the number of leukocytes in the peritoneal cavity 24-48 hr following PPD injection compared to controls. Here CS and fucoidan were able to inhibit transmigration of leukocytes into the peritoneal cavity by 63% and 35% respectively compared to controls (Fig. 3.4). In contrast, M6P treatment did not result in a significant change in leukocyte infiltration. Microscopic examination of cytocentrifuge smears revealed that the elicited cells were predominantly macrophages and other mononuclear cells with no significant qualitative differences in leukocyte populations between the treated and the control animals (Fig. 3.4).
Fig. 3.3
Ability of CS (200 mg/kg/day), M6P (40 mg/kg/day) and fucoidan (40 mg/kg/day) to inhibit entry of thioglycollate elicited leukocytes into the peritoneal cavity. Drug administration was commenced 1 day prior to thioglycollate injection using 7 day miniosmotic pumps. Each value is the mean ± SE (n=5) of leukocytes counted in the peritoneal cavity. Significant reduction in exudates compared with saline controls are indicated by * (P<0.05) and ** (P<0.005).
Table 3.3

Effect of CS, M6P and fucoidan on different leukocyte populations in thioglycollate elicited peritoneal exudates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mφ</th>
<th>MNC</th>
<th>Neut.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>52 ± 4.5</td>
<td>8 ± 1.0</td>
<td>40 ± 2.5</td>
</tr>
<tr>
<td>CS</td>
<td>70 ± 3.4**</td>
<td>11 ± 3.2</td>
<td>19 ± 1.5**</td>
</tr>
<tr>
<td>M6P</td>
<td>65 ± 6.2</td>
<td>20 ± 4.9*</td>
<td>15 ± 3.4**</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>61 ± 5.1</td>
<td>11 ± 1.7*</td>
<td>28 ± 2.4**</td>
</tr>
</tbody>
</table>

*Peritoneal exudates obtained from animals 36 hr following thioglycollate injection and corresponding to the 36 hr samples in Fig. 2A. Drug doses as in Fig. 2. Mφ = macrophages, Neut. = neutrophils and MNC = mononuclear cells. Significant change in leukocyte population compared with saline control indicated by * (P<0.05), ** (P<0.005).
Fig. 3.4
Ability of CS (200 mg/kg/day), M6P (40 mg/kg/day) and fucoidan (40 mg/kg/day) to inhibit entry of PPD elicited leukocytes into the peritoneal cavity of mice. Drugs were administered by minisomatic pumps as in Fig. 3.3. Each value is the mean ± SE where n=6, (2 experiments in triplicate). Significant reduction in exudates compared with saline controls indicated by * (P<0.05).

The dotted lines represent the range of leukocyte numbers in the peritoneal cavity of control mice not injected with PPD.
3.3 Discussion
The results of this study show that CS and fucoidan can inhibit the rejection of thyroid allografts. These results support earlier work by Grochowicz et al. (Grochowicz, Hibberd et al., 1990), Cohen et al. (Cohen, Lider et al., 1987) and Lider et al. (Lider, Baharav et al., 1989) showing that CS and heparin prolong renal, and skin allograft survival. Moreover, this work provides the first evidence that M6P may also inhibit thyroid allograft rejection. Previous studies reported the inhibition of EAE and adjuvant arthritis by CS and M6P (Willenborg, Parish et al., 1989; Willenborg, Parish et al., 1992) and presented histological evidence that CS inhibited leukocyte migration through the vascular basement membrane. Here, CS, M6P and fucoidan were shown to inhibit thioglycollate elicited peritoneal exudates, thus supporting the hypothesis that these compounds inhibit leukocyte extravasation. CS and fucoidan, but not M6P were also shown to have moderate effects on leukocyte accumulation in the peritoneal cavity in a DTH model of inflammation.

The mechanism by which these compounds act to inhibit such inflammation is not fully understood, however recent evidence suggests that these compounds inhibit leukocyte extravasation by preventing the solubilization of vascular ECM (Willenborg, Parish et al., 1992). The ability of activated leukocytes to migrate through the vascular ECM is thought to be dependent on degradative lysosomal enzymes such as the endoglycosidase, heparanase (Naparstek, Cohen et al., 1984; Fridman, 1987). These lysosomal enzymes possess a polysaccharide moiety with terminal M6P residues which bind to specific receptors for M6P (MPR) (von Figura and Hasilik, 1986). This enzyme-receptor interaction normally enables the transport of nascent active enzymes from their site of synthesis to the lysosome, but also allows the binding and expression of lysosomal enzymes on the cell surface (Neufeld et al., 1975). M6P inhibits lysosomal enzyme-MPR binding (Kaplan, Achord et al., 1977) and CS, which is an inhibitor of glycoprotein processing, inhibits the formation of functional M6P recognition markers on lysosomal enzymes and prevents their normal targeting and expression. The disruption of this phosphomannosyl recognition system may deplete leukocytes of cell-surface degradative enzymes and thus inhibit leukocyte migration through the vascular ECM. Sulfated polysaccharides, such as fucoidan and heparin, act as non-cleavable substrates for endoglycosidases and thus prevent degradation of the heparan sulfate scaffolding in vascular ECM (Cohen, Lider et al., 1987).
The thyroid allograft model allowed testing of the compounds in a CD8+ cytotoxic T cell dependent rejection (elicited by P815 injection) and non CD8+ cytotoxic T cell dependent rejection (elicited by donor strain spleen cell injection). In the initial experiments none of the compounds inhibited CD8+ cytotoxic T cell mediated rejection, whereas all of the compounds inhibited non-CD8+ cytotoxic T cell dependent rejection. However a comparison of the saline controls for each experiment showed that the magnitude of the rejection response following P815 immunisation was much greater than that following donor strain spleen cell immunisation. Where CsA was used to reduce the severity of the P815 induced rejection, CS, M6P and fucoidan were shown to effect a significant reduction in leukocyte infiltration into the grafts. CS, M6P and fucoidan were thus shown to be sensitive to the magnitude of the rejection response, rather than to qualitative differences in the immune response resulting from immunisation with P815 compared to spleen cells. The lack of effect of CS, M6P and fucoidan on the rate of P815 tumour allograft rejection might similarly be explained by the strong immune response induced by P815. Clearly, the rejection response in these particular models was extremely vigorous. It is important, therefore, to note that while the effects of the three compounds do not appear dramatic, even the potent immunosuppressant, CsA was only able to reduce the clinical scores by 2 points.

Since the peritoneal exudate model precluded the need for induction of effector cells as in the allograft models, the inhibition of inflammation observed in the presence of CS, M6P and fucoidan must have been primarily a result of its action on the effector phase of inflammation (leukocyte migration) rather than on the induction of immune effector cells. Nevertheless, subtle differences between the three compounds were observed. While all three compounds inhibited thioglycollate elicited peritoneal exudates, M6P did not inhibit migration in the PPD elicited exudate. It is possible that M6P in this model exerted its effects primarily on neutrophils. In the thioglycollate model, during early stages, neutrophils were the preponderant infiltrating cell type. M6P could inhibit these exudates in the early stages, but could not inhibit leukocyte infiltration in the MNC dominated DTH model. Cytocentrifuge smears of the thioglycollate induced exudates revealed that the M6P effect was primarily due to a reduction in the relative numbers of neutrophils. Further, the effect of M6P (as well as fucoidan) was more pronounced early and became less pronounced at later times when a higher proportion of cells
in the exudate were MNC. These observations raise the possibility that MNC and macrophages may have a different mode of entering the peritoneum compared to neutrophils. MNC probably participate in immune surveillance of the peritoneum and may move into the peritoneal cavity via specialised sites in the vascular bed of the mesentery. It is possible that this migration does not require the expression of lysosomal enzymes. However during the acute early phase of inflammation, neutrophils, in order to enter the peritoneum, may be required to degrade vascular ECM. During extravasation into the peritoneal cavity, neutrophils would therefore be more sensitive to compounds which inhibit the cell-surface expression of degradative enzymes.

CS was found to have longer lasting effects than M6P and fucoidan in preventing thioglycollate elicited leukocyte migration into the peritoneal cavity. While the effects of M6P are probably quite specific - acting on the phosphomannosyl recognition system at the cell surface - CS may have a broader range of effects. For example, since CS is an inhibitor of oligosaccharide processing, CS may exert some of its anti-inflammatory properties by altering the synthesis of adhesion molecules. ICAM-1, for example, is heavily glycosylated, and in vitro studies have shown that CS inhibits ICAM-1 mediated endothelial cell-lymphocyte adhesion (Renkonen and Ustinov, 1991). Interference with the expression of any of the cell adhesion molecules could influence the inflammatory process.

Clearly, since there are legitimate concerns with even the most commonly used anti-inflammatory drugs, the investigation of new drugs with potential as more specific treatments with less side effects are a high priority. This study has shown that CS, sulfated polysaccharides and even M6P, inhibit thyroid allograft rejection, and that these compounds may work by inhibiting leukocyte migration. Since M6P has previously been shown to affect the interaction of lysosomal enzymes with the MPR, these results provide further support for the notion that cell-surface expression of ECM hydrolysing enzymes plays an important role in leukocyte extravasation, and that compounds which inhibit this process may emerge as an important new class of anti-inflammatory agents.
3.4 Summary
The glycoprotein processing inhibitor castanospermine (CS), the monosaccharide M6P as well as some sulfated polysaccharides (SPS) have been shown to inhibit inflammation in rat models of EAE and adjuvant induced arthritis. Here, the anti-inflammatory effects of these agents has been further explored in murine models of allograft rejection and elicitation of peritoneal exudates. CS, M6P and the SPS, fucoidan partially inhibited rejection of permanently accepted thyroid allografts induced by the i.p. injection of donor strain \((H-2^d)\) spleen cells with a reduction in leukocyte infiltration by 25-36%. However none of these agents reduced the more extensive leukocyte infiltration induced by the i.p. injection of P815 \((H-2^d)\) unless recipient mice were pre-treated with the immunosuppressant, CsA. Elicitation of peritoneal exudates by thioglycollate was inhibited by CS, M6P and fucoidan with sustained leukopenia being induced by CS. In contrast, CS and fucoidan, but not M6P, inhibited antigen elicited peritoneal exudates. These results suggest that CS, M6P and the SPS fucoidan exhibit subtle differences in their anti-inflammatory activity but probably inhibit inflammation at the level of leukocyte extravasation.
Chapter 4

Comparative analysis of the ability of leukocytes, endothelial cells and platelets to degrade the subendothelial basement membrane: Evidence for cytokine dependence and detection of a novel sulfatase

4.1 Introduction
A key aspect of inflammation is the adhesion and extravasation of leukocytes through the blood vessel wall prior to entry into inflammatory sites. In order to leave the circulation and enter a focus of inflammation in the tissue, circulating leukocytes must adhere to the luminal surface of endothelial cells and then actively pass through the endothelium. Recent studies have shed considerable light on the nature of these leukocyte adhesive interactions with endothelial cells (EC) and indeed with the subendothelial basement membrane (BM) (reviewed in section 1.5). However, comparatively little is known about the extravasation process. In particular, although the subendothelial BM represents a major barrier for leukocyte entry, relatively little is known about how this barrier is solubilized prior to leukocyte passage, except that it is presumably with the aid of a battery of degradative enzymes (Savion, Vlodavsky et al., 1984; Furie et al., 1987; Ratner, 1992 Vlodavsky, 1992). In contrast, tumour cells, which share with leukocytes the capacity to travel through, and extravasate from, the circulatory and lymphatic systems, have been studied extensively with respect to subendothelial BM degradation. Studies of tumour cell invasion and metastasis have provided ample evidence that increased expression of degradative enzymes correlates with metastatic potential (Karakiulakis, 1988). Enzymes capable of degrading BM components found to be elevated in metastatic tumours were proteases (Tryggvason et al., 1987), heparanase (Nakajima, Irimura et al., 1983); Vlodavsky, 1983; Bar-Ner, 1985; Parish, 1987) and type IV collagenase (Liotta, Thorgeirsson et al., 1982). It is reasonable to suppose that mechanisms of invasion are conserved, and that there are mechanistic parallels between leukocytes and metastatic tumour cells in the invasion of solid tissue. For example, it has been shown that neutrophils (Matzner, Bar-Ner et al., 1985; Pipoly and Crouch, 1987; Matzner et al., 1992), T lymphocytes (Naparstek, Cohen et al., 1984; Fridman, 1987; Vlodavsky, Eldor et al., 1992), B-lymphocytes (Laskov et al., 1991), monocytes (Jessup and Dean, 1982; Sewell, 1989),
macrophages (Savion, Vlodavsky et al., 1984; Savion, Disatnik et al., 1987), EC (Herron, 1986) and platelets (Gordon, 1975; Eldor et al., 1987), like tumour cells, express enzymes capable of degrading the BM.

Investigators of BM degradation during invasion and metastasis have examined the degradation of purified components of BM or ECM by tumour cell hydrolytic enzymes (reviewed by (Jones and DeClerck, 1982)). However such macromolecules do not occur as pure components in nature, but rather as complex supramolecular aggregates stabilised by a variety of chemical crosslinking interactions (Yurchenco and Schittny, 1990). These stabilising interactions may change the susceptibility of ECM components to degradation by tumour cell- or leukocyte-derived hydrolytic enzymes. ECMs have also been found to bind biologically active molecules, such as tissue type plasminogen activator (Eldor, Bar-Ner et al., 1987), which may further influence the solubilisation of ECM. For this reason, research in invasion and metastasis has shifted from studies with isolated macromolecules toward more natural substrates which more closely reflect the in vivo situation (Bar-Ner, Kramer et al., 1985).

These ECMs, which contain collagen type IV and type III (with smaller amounts of types I and V), proteoglycans (chiefly HS, but with smaller amounts of CS and DS), laminin, nidogen, fibronectin and elastin, closely resemble BM in vivo in molecular composition (section 1.5.2) (Gospodarowicz et al., 1980; Vlodavsky et al., 1980). Leukocytes, as well as platelets and endothelial cells, were investigated to determine the major enzymes expressed by these cells which were capable of solubilizing the ECM heparan sulfate proteoglycans (HSPG), with the goal of gaining a greater insight into how these cells might cooperate in BM solubilization to facilitate leukocyte extravasation. The second aim was to investigate the effects of some relevant pro-inflammatory cytokines on the degradation of ECM by these cells.

4.2 Results
4.2.1 ECM degradation assay
The studies described in this chapter on the extravasation of leukocytes focus on the ability of cells to degrade HSPG in a basement membrane (BM)-like ECM produced by bovine corneal EC cultured on tissue culture dishes. The use of dextran T40 in the EC culture medium helps to polarise the secretion of ECM to the basal side of the endothelial monolayer, where it is
firmly attached to the entire area of the tissue culture dish (Vlodavsky, Fuks et al., 1983). This means that the EC layer can be denuded while leaving the underlying ECM layer intact and free of cellular debris (Gospodarowicz, Delgado et al., 1980; Vlodavsky, Liu et al., 1980).

The ability of leukocytes, EC and platelets to degrade the ECM was studied by allowing these cells to interact with an ECM metabolically labeled with $^{35}$SO$_4$ (Fig. 4.1). Newly synthesised ECM incorporates $^{35}$SO$_4$ preferentially into proteoglycans and sulfated glycoproteins (Nakajima, Irimura et al., 1988; Vlodavsky, Eldor et al., 1988). The products of labeled ECM degradation released into the supernatant were analysed by gel filtration and, using HS standards, the approximate mol. wt. of these products provided evidence of the type of enzymes that were utilised by the cells to degrade the ECM (Fig. 4.1). It has already been shown that the interaction of the metastatic rat mammary adenocarcinoma cell line (13762 MAT) with $^{35}$SO$_4$ labeled ECM results in the formation of high- and low-mol. wt. degradation products, particularly fragments of HS chains (Parish, Coombe et al., 1987). The same MAT cell line and NaOH were used to degrade my $^{35}$SO$_4$ labeled ECM, and the approximate mol. wt. of the major radioactive peaks was determined by gel filtration chromatography. From Fig. 4.2 it can be seen that some high mol. wt. material was eluted in the void volume of the column ($K_{av} < 0.2$). This peak (region I), which represents predominantly intact HSPG (Table 4.1), could be obtained by incubating the labeled ECM with medium, with or without 10% FCS, for 24 hours at 37°C and could generally also be observed following the culturing of most cell types with the ECM. Treatment of ECM with 1M NaOH releases intact HS side chains, via $\beta$-elimination, from their core proteins. A characteristic peak (region II, approx. $K_{av} = 0.35$) was obtained with NaOH treatment of $^{35}$SO$_4$ labeled ECM which represents intact HS side chains (Table 4.1). Detection of peaks in regions I and II would usually suggest the presence of protease activity (Table 4.1). Region III (approx. $K_{av} = 0.5-0.7$) characteristically contained a very broad peak since it represents a heterogenous population of HS degradation products. The MAT tumour cells, which have high heparanase activity, produced considerable amounts of degradation products in this region (Fig. 4.2). That these low mol. wt. labeled products were indeed heparanase-mediated degradation products of HS side chains was confirmed by their sensitivity to de-amination with nitrous acid (Lindahl, Blackstrom et al., 1973) and resistance to digestion with papain and chondroitinase ABC (Wasteson, 1971; Roden, Baker et al., 1972) (data not shown). It has also
Fig. 4.1
Schematic representation of the assay system used to quantitate degradation of radiolabeled endothelial ECM by different populations of cells.
Fig. 4.2
Fractionation of degradation products released from $^{35}$SO$_4$-labeled ECM by gel filtration on a Superose-6 FPLC column. The column was standardized using fragments released by either 1M NaOH incubation (24hr, 37°C) or $10^6$ mammary adenocarcinoma 13762 cells (24hr, 37°C). The position of the major classes of degradation products (I-IV) is indicated.

Table 4.1
Properties of different regions detected in FPLC elution profiles

<table>
<thead>
<tr>
<th>Region</th>
<th>Mol. wt (Da)$^a$</th>
<th>Predominant HS fragments</th>
<th>Predominant enzymic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.5-2x10$^6$</td>
<td>HS proteoglycan</td>
<td>Proteases</td>
</tr>
<tr>
<td>II</td>
<td>0.4-1x10$^5$</td>
<td>Free HS chains</td>
<td>Proteases</td>
</tr>
<tr>
<td>III</td>
<td>0.3-3x10$^4$</td>
<td>Large HS chain fragments</td>
<td>Endoglycosidases (heparanases)</td>
</tr>
<tr>
<td>IV</td>
<td>0.2-1x10$^3$</td>
<td>HS oligo/mono saccharides, free sulfate</td>
<td>Exoglycosidases, sulfatase</td>
</tr>
</tbody>
</table>

$^a$ Based on mol. wt of HS standards run through Superose-6 FPLC column.

Mol. wts 30,000 Da and 6,700 Da were determined using HS standards. $V_o$ and $V_t$ were determined using blue dextran (mol. wt. $2x10^6$) and $^{35}$SO$_4$ respectively.

Based on mol. wt of HS standards run through Superose-6 FPLC column
previously been shown that similar gel filtration profiles occur whether the ECM was produced by corneal or vascular endothelial cells (Vlodavsky, Fuks et al., 1983). Finally, a fourth peak (region IV) at Vt represents labeled free sulfate, sulfated oligosaccharides or sulfated monosaccharides released from the ECM. Release of these degradation products from the ECM would imply sulfatase or exoglycosidase activity respectively (Table 4.1).

4.2.2 Comparative abilities of leukocytes, HUVEC and platelets to degrade ECM

As stated above, one of the aims of this study was to investigate the comparative potential of the major cells involved in inflammation to degrade the subendothelial BM and to determine the major enzymes expressed by each cell-type during solubilization of the ECM. Such information would provide greater insight into how these cells might cooperate in order to bring about leukocyte extravasation. Since it was not possible to conduct all of the experiments on the same batch or age of $^{35}$SO$_4$-labeled ECM, the total radioactivity released into the incubation medium was standardised, for each batch of ECMs, against the total radioactivity released from each ECM. In initial studies each cell type was examined for its ability to degrade the ECM either in a nonactivated state or following activation by the phorbol ester, PMA. The interaction with ECM of all cell types examined resulted in an increase in the release of radioactive ECM components above the medium control (Fig. 4.3). Different cell types varied in their ability to release radiolabeled material from the ECM, HUVECs and platelets being at least 2-3 times more active than leukocytes (Fig. 4.3), whereas of the leukocytes the monocytes were the least active. Furthermore, with each cell type, activation with PMA enhanced ECM degradation two- to three-fold over the non-stimulated levels, a notable exception being monocytes, where no increased degradation was observed.

The $^{35}$SO$_4$-labeled degradation products released from the ECM by each cell type were then determined by fractionating the ECM supernatants on a Superose-6 FPLC gel filtration column. As described above, the FPLC elution profile could be subdivided into 4 major regions representing 4 types of degradation products (Table 4.1). The data obtained from these fractionation studies were analysed in two ways. First, the percentage of degradation products in each region was calculated (Table 4.2) and second, the ratio of radioactivity above the medium control for each region was determined (Table 4.3). The former calculation gives an estimate of the predominant
Fig. 4.3
Ability of human leukocytes, human umbilical vein endothelial cells (HUVECs) and platelets to release radioactivity from $^{35}$S-labeled ECM. In each case, $10^6$ cells or $10^8$ platelets were incubated with ECM for 24hr at 37°C in the presence or absence of 50ng/ml PMA. Data mean +/- SE of at least 3 experiments. Monocytes represent pooled data from peripheral blood monocytes and the monocytic cell line U937 as both cell populations gave similar results.
### Table 4.2

Degradation of $^{35}S_{04}$-labelled ECM by leukocytes, EC and platelets\(^a\)

<table>
<thead>
<tr>
<th>Cell/Treatment</th>
<th>Percent released radioactivity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region I</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>17 ± 8.5</td>
</tr>
<tr>
<td>Neutrophils + PMA</td>
<td>51 ± 7.8</td>
</tr>
<tr>
<td>Monocytes(^c)</td>
<td>9 ± 4.3</td>
</tr>
<tr>
<td>Monocytes + PMA(^c)</td>
<td>31 ± 8.8</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes + PMA</td>
<td>15 ± 3.0</td>
</tr>
<tr>
<td>HUVECs</td>
<td>31 ± 7.9</td>
</tr>
<tr>
<td>HUVECs + PMA</td>
<td>44 ± 4.6</td>
</tr>
<tr>
<td>Platelets</td>
<td>10 ± 3.7</td>
</tr>
<tr>
<td>Platelets + PMA</td>
<td>10 ± 2.0</td>
</tr>
</tbody>
</table>

\(^a\) Fractionation by gel-filtration on a Superose-6 FPLC column of products released by different treatments (see Fig. 4.3). Properties of different regions are described in Table 4.1.

\(^b\) Data expressed as percentage of released radioactivity above medium control in each peak ± SE of mean.

\(^c\) Monocyte data represents pooled data from peripheral blood monocytes and the monocytic cell line U937.
Table 4.3

Relative quantities of ECM fragments released from $^{35}$S0$_4$-labeled ECM by leukocytes, HUVECs and platelets$^a$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Region I</th>
<th>Region II</th>
<th>Region III</th>
<th>Region IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.2 ± 0.2</td>
<td>2.5 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>Neutrophils + PMA</td>
<td>3.1 ± 0.5</td>
<td>4.8 ± 0.4</td>
<td>3.9 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>Monocytes$^c$</td>
<td>-</td>
<td>-</td>
<td>1.2 ± 0.2</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td>Monocytes+ PMA$^c$</td>
<td>1.8 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.9 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>4.2 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocyte+ PMA</td>
<td>1.8 ± 0.4</td>
<td>3.4 ± 0.7</td>
<td>9.4 ± 3.0</td>
<td>-</td>
</tr>
<tr>
<td>HUVECs</td>
<td>4.3 ± 1.3</td>
<td>4.9 ± 0.8</td>
<td>5.3 ± 0.3</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>HUVECs + PMA</td>
<td>16.7 ± 3.5</td>
<td>9.0 ± 1.5</td>
<td>9.8 ± 2.1</td>
<td>11.4 ± 2.2</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.7 ± 0.5</td>
<td>-</td>
<td>9.1 ± 4.4</td>
<td>-</td>
</tr>
<tr>
<td>Platelets + PMA</td>
<td>0.7 ± 0.3</td>
<td>-</td>
<td>13.8 ± 2.0</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Experimental procedures as in Fig. 4.3 and Table 4.2. Properties of different regions described in Table 4.1.

$^b$ Data expressed as ratio of released radioactivity of the preparation versus medium control +/- SE of mean. Ratios not included when < 10% of total radioactivity released by a cell population in a particular region.

$^c$ Monocyte data represents pooled data from peripheral blood monocytes and the monocytic cell line U937.
degradation products generated by each treatment and the latter, an estimate of the relative release of each degradation product above the medium control by the various cell types. Representative elution profiles for each cell type are also depicted (Fig. 4.4), although the data in Tables 4.2 and 4.3 represent pooled results and therefore allow estimates of significant differences to be made.

Based on the pooled data in Tables 4.2 and 4.3 it is clear that different cell populations differed markedly in the degradation products which they released from the labeled ECMs. For example, nonstimulated monocytes and HUVECs produced substantial amounts of low mol. wt. degradation products detected in region IV, lymphocyte degradation products appeared predominantly in regions II and III, whereas products released by platelets appeared almost exclusively in region III. In some cases, PMA stimulation markedly altered the type of degradation products being produced by cells. PMA inhibited the release of region IV products by monocytes but greatly enhanced release of region I products by neutrophils and HUVECs. Each cell type will be considered in more detail below.

In the case of nonstimulated neutrophils, the predominant degradation products were in regions II and III, suggesting protease mediated release of free HS chains and some heparanase activity (Fig. 4.4a, Tables 4.2 and 4.3). PMA stimulation resulted in the appearance of region I degradation products (i.e., intact proteoglycans), with modest increases in region II and III products, implying induction of increased protease and heparanase activity.

Nonstimulated human lymphocytes, like neutrophils, did not release intact proteoglycans above medium control levels (Table 4.3, Fig.4.4b). However nonstimulated lymphocytes, in contrast to neutrophils, expressed relatively high levels of heparanase activity, as indicated by a high proportion of low mol. wt. (Kav < 0.5) degradation products (Tables 4.2, 4.3 and Fig. 4.4b). Lymphocyte heparanase activity increased approximately two-fold when the cells were incubated on ECMs in the presence of PMA. Moreover, PMA stimulated lymphocytes released substantial amounts of high mol. wt. material from the labeled ECM (Region I, Table 4.3 and Fig. 4.4b). Similar results were obtained with nonstimulated and PMA stimulated ConA-activated T lymphocytes (data not shown).
Fig. 4.4

Representative elution profiles on a Superose-6 FPLC column of degradation products released from $^{35}$SO$_4$-labeled ECM by $10^6$ leukocytes, $10^6$ HUVECs and $10^8$ platelets. Treatments (24hr, 37°C) are medium control (………….), cells or platelets alone (———) and cells or platelets in the presence of 50 ng/ml PMA (--------). The position of regions I - IV is indicated, the properties of these regions being described in Table 4.1.
In all of my studies peripheral blood monocytes and the monocytic cell line U937 gave similar degradation results, hence the pooling of data in Tables 4.2 and 4.3 and Fig. 4.3. Figs 4.4c and 4.4d are representative elution profiles and show that incubation of non-activated monocytes or U937 cells on radiolabeled ECM for 24 hours resulted mainly in the release of substantial amounts of labeled products of very low mol. wt. eluting in region IV at the column Vt. In terms of total release, stimulation of the cells with PMA did not result in a significant increase in release of labeled products from the ECM (Fig. 4.3). However, PMA stimulation of monocytes and U937 generally resulted in a dramatic reduction in products eluting in region IV. The PMA induced reduction in very low mol. wt. products was offset, in part, by a slight increase in products eluting in regions I-III (Fig. 4.4c and 4.4d, Tables 4.2 and 4.3).

Tables 4.2, 4.3 and Fig. 4.4e show that incubation of $^{35}$SO$_4$-labeled ECM with non-stimulated HUVECs resulted in the release of labeled degradation products eluting in all of the major regions (I-IV) at levels substantially above the medium control levels. Interestingly, about 20% of the released radioactivity also eluted in a distinct peak in region IV (Table 4.2 and Fig. 4.4e). PMA stimulated HUVEC released substantially greater amounts of ECM degradation products in all of the major mol. wt. regions (Tables 4.2 and 4.3). However, this increase was more marked in regions I and IV, where a 4-5-fold increase was observed. The passage number of the HUVECs appeared to have no affect on the ECM degradation results obtained, similar results being observed with HUVECs from passages 2-11 although, routinely, passage 5-7 HUVECs were used.

In order to determine whether the release of radioactivity into region IV by HUVECs and monocytes was as a result of an exoglycosidase or a sulfatase (Table 4.1), $^3$H-glucosamine-labeled ECM (prepared by incubating cultured EC in the presence of $^3$H-glucosamine as described in Materials and Methods) were used in place of the $^{35}$SO$_4$-labeled ECMs. It was found that >80% of the $^3$H incorporated into these ECMs was HS associated. Incubation of stimulated or non-stimulated HUVECs with the $^3$H-labeled ECMs resulted in the release of labeled material eluting entirely in regions I-III, with a virtual absence of radioactivity eluting in region IV (Fig. 4.5). A similar result was obtained with the U937 monocytic line (data not shown). Furthermore the $^3$H-glucosamine labeled HS chains had approx. 5-fold higher specific activity than the $^{35}$SO$_4$-labeled HS chains. This data therefore provides clear
Fig. 4.5
Degradation of $^{35}$SO$_4$ (---) or $[^3]$H]-glucosamine (- - - - - - -) labeled ECM by HUVECs. Labeled ECM was incubated (24 hr, 37°C) with $10^6$ PMA (50 ng/ml) stimulated EC, the incubation medium collected and fractionated by gel filtration on a Superose-6 FPLC column. The tritium CPM were 4-fold higher than indicated on the graph.
evidence that the degradation products released by HUVECs and monocytes appearing in region IV represent free $^{35}\text{SO}_4$ rather than low mol. wt. oligosaccharides or monosaccharides, indicating that a sulfatase is produced by these two cell types. It should be noted that HUVEC also released $^3\text{H}$-labeled degradation products detected in regions II-III of the FPLC elution profiles which probably represent desulfated HS chains (Fig. 4.5). The significance of this observation requires further investigation.

In contrast to leukocytes and HUVECs, the interaction of washed platelets with labeled ECM (Fig. 4.4f) resulted in high levels of heparanase activity, with 70% of the labeled degradation products eluting in the HS fragment region (region III). Stimulation of platelets by PMA resulted in a substantial increase in degradation, but did not result in a qualitative change in the elution profile (Tables 4.2 and 4.3, Fig. 4.4f). Similar results to PMA were obtained when the platelets were stimulated with thrombin (0.1 U/ml) (results not shown).

4.2.3 Effect of cytokines on ECM degradation by leukocytes and EC

PMA, which bypasses normal signal transduction pathways by directly activating protein kinase C (Blumberg, 1988), was shown to be able to upregulate ECM degradation by most cell types tested. However during inflammation cytokines, chemoattractants and other inflammatory mediators are the major physiological activators of these cells and should be more relevant in influencing the events leading to leukocyte extravasation and BM degradation. Thus, a number of cytokines which are known to regulate inflammation were tested for their ability to enhance ECM degradation by leukocytes and HUVEC.

Degradation of labeled ECM by neutrophils was markedly enhanced by the chemokine IL-8, resulting in the release of mainly high mol. wt. degradation products (Fig. 4.6). Similarly, the combination of the cytokines IL-1 and TNF caused a 2-fold increase in the release of high mol. wt. degradation products (Fig. 4.6). Unlike PMA, however, none of the cytokines used enhanced heparanase activity. Also, 4-Hydroxy-pentadecenal, an aldehydic end-product of lipid peroxidation which is a potent neutrophil chemoattractant (Curzio et al., 1986), as well as fMLP, induced slightly enhanced release of high mol. wt. degradation products by neutrophils (data not shown). In contrast, TNF and IL-1 slightly reduced the ability of lymphocytes to release labeled ECM products in regions I-III, (Fig. 4.7). IL-8, on the other hand, was
Fig. 4.6
Degradation of sulfate-labeled ECM by cytokine-activated human neutrophils. Labeled ECM was incubated (24 hr, 37°C) with $10^6$ TNF (2.5 ng/ml) and IL-1 (0.2 ng/ml) or IL-8 (100 ng/ml) stimulated neutrophils, the incubation medium collected and fractionated by gel filtration on a Superose-6 FPLC column.
Degradation of sulfate-labeled ECM by cytokine-activated human lymphocytes. Labeled ECM was incubated (24 hr, 37°C) with $10^6$ TNF (2.5 ng/ml) and IL-1 (0.2 ng/ml) or IL-8 (100 ng/ml) stimulated lymphocytes, the incubation medium collected and fractionated by gel filtration on a Superose-6 FPLC column.
Fig. 4.8

Ability of different cytokines to stimulate degradation of $^{35}$SO$_4$-labeled ECM by $10^6$ HUVECs, degradation products being separated by gel filtration on a Superose-6 FPLC column. Treatments (24hr, 37°C) are medium control ( ), HUVEC alone (---), HUVEC + 50 ng/ml PMA (-----) and HUVEC + cytokine (- - - - - - -) as indicated. Cytokine concentrations used were TNF (2.5 ng/ml), IL-1 (0.2 ng/ml), IL-8 (100 ng/ml) and IFN$\gamma$ (150 U/ml). PBMC-CM represents PBMC conditioned medium used at 1/2 dilution.
TNF and IL-1

IL-8

IFNγ

PBMC-CM
able to modestly stimulate lymphocyte protease and heparanase activity (Fig. 4.7).

The cytokines IFNγ and TNF + IL-1, like PMA, did not significantly increase peripheral blood monocyte and U937 degradation of labeled ECM. Indeed, like PMA, these cytokines only affected monocyte sulfatase activity. Table 4.4 summarises the effects of the cytokines tested on monocyte sulfatase activity. It can bee seen that TNF + IL-1 inhibited endogenous monocyte sulfatase activity, IFNγ had a partial inhibitory effect, whereas treatment with the chemokine Monocyte Chemoattractant and Activating Factor (MCAF) resulted in a substantial (2.7 fold) increase in sulfatase activity.

IL-1 or TNF alone were unable to stimulate HUVEC degradation of the ECM (data not shown), whereas IL-1 and TNF combined did stimulate HUVEC to degrade ECM, resulting in a substantial increase in region I degradation products but only a slight increase in regions II-IV (Fig. 4.8a). IL-8 selectively enhanced the sulfatase activity of HUVECs (Fig. 4.8b). In contrast, IFNγ did not stimulate ECM degradation and, in fact, inhibited endogenous HUVEC sulfatase activity (Fig. 4.8c). Whilst the upregulation of HUVEC degradative potential by TNF + IL-1 appeared significant, the increase in degradation over control levels was somewhat less dramatic than that stimulated by PMA (Fig. 4.8a). Also, unlike IL-8, the combination of TNF and IL-1 did not appear to markedly stimulate HUVEC sulfatase activity. Only conditioned medium from peripheral blood mononuclear cells (PBMC) was able to stimulate EC to degrade ECM to a similar extent as PMA (Fig. 4.8d). Even though the nature of the soluble factor or factors in this PBMC supernatant responsible for enhancing the degradative capacity of HUVEC above that of TNF + IL-1 was not defined, this result demonstrates that mononuclear cell derived factors may play a significant role in stimulating EC degradation of the ECM in vivo.

4.2.4 Degradation of ECM by different HUVEC populations
The HUVEC data were particularly interesting 1) because the levels of degradation were surprisingly large compared to that of leukocytes, and 2) because of the striking sulfatase activity expressed by these cells which has not been previously reported in the context of BM degradation. As mentioned in section 1.4.7, EC potentially play a central role in inflammation due to their strategic location, their ability to express adhesion molecules upon activation, their ability to synthesise BM, and their role in angiogenesis. EC
Table 4.4

Effect of cytokines and PMA on the sulfatase activity of monocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Sulfatase activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>50 ng/ml</td>
<td>20</td>
</tr>
<tr>
<td>TNF+</td>
<td>2.5 ng/ml</td>
<td>0</td>
</tr>
<tr>
<td>IL-1</td>
<td>0.2 ng/ml</td>
<td>0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>50 U/ml</td>
<td>40</td>
</tr>
<tr>
<td>MCAF</td>
<td>50 ng/ml</td>
<td>270</td>
</tr>
</tbody>
</table>

a Sulfatase activity as determined by the release of very low mol. wt. (region IV - Fig. 4.2, Table 4.1) radioactive degradation products from $^{35}$SO$_4$-labeled ECM by $10^6$ control, PMA, or cytokine-stimulated monocytes following incubation (24hr, 37°C) with the labeled ECM. Data represents pooled data from peripheral blood monocytes and the monocytic cell line U937.

b Data expressed as percentage of region IV radioactivity compared with medium control (0%) and untreated monocyte control (100%)
have been reported to possess an active ECM degrading potential as part of their role in angiogenesis during wound healing (Folkman and Brem, 1992). Since the wound-healing/angiogenesis response is triggered, in part, by the perturbation of cell-cell interactions, it is logical to suppose that the EC angiogenic response might be favoured when the cells are in suspension. Recruitment of leukocytes during inflammation, on the other hand, can occur in the presence of intact vascular endothelium.

To determine whether HUVEC monolayers differ from HUVEC in suspension in their ability to degrade ECM, HUVECs were allowed to metabolically label their own ECM with $^{35}$SO$_4$. Instead of denuding the ECM, the HUVECs were allowed to remain on the labeled ECM. In this way, the degradation of ECM by a confluent endothelial cell monolayer could be examined. As described in Materials and Methods, these HUVEC monolayers were rinsed free of ECGF and heparin, cultured for 24 hours, and rinsed again before examining the incubation medium for radioactive products of degradation. Fig. 4.9 shows that incubation of nonactivated HUVEC monolayers on their own labeled ECM resulted in little or no high to low mol. wt. labeled products of ECM degradation in the incubation medium (regions I-II), but a significant peak (region IV) representing HUVEC sulfatase activity was detected. PMA activation of the HUVEC monolayers markedly enhanced the release of free $^{35}$SO$_4$ and caused the release of small amounts of high mol. wt. labeled products. Thus, HUVEC monolayers behave differently to HUVEC cell suspensions in their degradation of the ECM.

To ensure that the $^{35}$SO$_4$ released into the supernatant was of BM origin, unlabeled HUVECs were allowed to form a confluent monolayer on $^{35}$SO$_4$-labeled ECM of bovine corneal EC origin. Following rinsing with fresh basic medium, culturing for 24 hours, and rinsing again, these HUVEC monolayers were examined for ECM degradative potential. Again, PMA stimulation of the HUVEC monolayers resulted in high levels of sulfatase activity as indicated by the virtually exclusive release of free $^{35}$SO$_4$ in region IV (data not shown). That the ECM degradation product appearing in region IV was indeed free $^{35}$SO$_4$, was confirmed by an experiment incubating HUVEC monolayers with bovine corneal EC-derived $^3$H-glucosamine-labeled ECM. In this experiment, $^3$H-labeled degradation products were not found in region IV of the gel filtration elution profile (Fig. 4.10). Furthermore, there was little or no release of $^3$H-labeled material from the ECM following PMA stimulation of the HUVEC monolayers whereas HUVECs in suspension did release higher
Fig. 4.9

Representative profile of HUVEC monolayer degradation of its own ECM. The intact HUVEC monolayer was incubated (24 hr, 37°C) with its $^{35}$SO$_4$-labeled ECM in the presence (---) or absence (-----) of PMA (50ng/ml). The incubation medium was collected and fractionated by gel filtration on a Superose-6 FPLC column.
Fig. 4.10

Degradation of $^3$H]-glucosamine-labeled ECM by HUVEC monolayers (---) or $10^6$ HUVEC added to the ECM as a suspension (-----). Labeled ECM was incubated (24 hr, 37°C) with the cells in the presence of 50 ng/ml PMA, the incubation medium collected and fractionated on a Superose-6 FPLC column.
mol. wt. ³H-labeled material detected in regions I-III following PMA stimulation (Fig. 4.10).

4.3 Discussion

4.3.1 Comparative analysis of the ability of leukocytes, endothelial cells and platelets to degrade the subendothelial basement membrane

While a number of separate studies have revealed that leukocytes possess ECM degrading capabilities (section 1.5), the comparative abilities of each cell type to degrade the ECM has not previously been closely examined. In this study, ³⁵SO₄-labeled, EC-derived ECMs were used to examine the ability of leukocytes, EC and platelets to degrade a physiological subendothelial BM. These experiments confirmed that leukocytes, EC and platelets have the capacity to degrade the subendothelial BM in vitro. Further, the results of this study provide evidence that both nonstimulated and PMA stimulated HUVEC and platelets possess the greatest potential for solubilizing the ECM, with levels of degradation 2-5 fold higher than that of the leukocytes tested. Of the other circulating cells of the immune system, neutrophils and lymphocytes were shown to degrade the ECM to a much greater extent than monocytes, which showed very little degradation of the ECM compared to the medium control. These results are significant because they indicate that in spite of the emphasis placed on leukocytes as the sole participants in the penetration of BM during inflammation, ECs themselves may play an important role. It has already been demonstrated that EC can not only synthesise BM components, but can also degrade BM components and rapidly reorganise BM structure in a manner that alters biological reactivity (de Groot et al., 1987). The comparatively high levels of released radioactivity resulting from the interaction of EC with labeled ECM, shown here, further support the notion that EC directly participate in the process of leukocyte extravasation by preparing their BM for invasion.

It is also possible that the experimental conditions of this study favoured an angiogenic, rather than an inflammatory, response by the EC. There is evidence that the cell-cell contacts which occur in the normal vasculature between ECs themselves and, for example between ECs and pericytes, are important in maintaining vascular EC in a quiescent, non-proliferating state (Haudenschild et al., 1976; Klagsbrun and D'Amore, 1991). During
angiogenesis EC, stimulated by angiogenic substances, are known to increase their production of plasminogen activator and collagenases (Liotta, Thorgerirsson et al., 1982; Gross et al., 1983; Unemori et al., 1990), facilitating degradation of the parent vessel BM. The EC then migrate, as individual migratory cells, through the degraded BM of the parent vessel toward the angiogenic stimulus (reviewed by (Folkman and Brem, 1992)). Thus, the high levels of BM degradation mediated by EC which began in suspension, over those which began as monolayers might be suggestive of an EC angiogenic response rather than a typical inflammatory response. Experiments with HUVEC monolayers, which showed a markedly different degradation pattern, seemed to confirm this notion. However it is still important to note that neovascularisation is one of the major vascular responses in chronic inflammation, and may be necessary to sustain chronic inflammation (Folkman and Brem, 1992). Indeed, chronic inflammation is so frequently accompanied by neovascularization, that until recently the two processes have been inseparable. For example macrophages, which usually dominate the cellular infiltrate of chronic inflammatory sites, secrete both angiogenic and inflammatory products (reviewed by (Nathan, 1987; Sunderkotter, Goebeler et al., 1991)) releasing FGFs, TGF\(\alpha\) and angiotropin. EC migration is also augmented by mast cells (Azizkhan et al., 1980). Therefore, since the EC angiogenic response is an important part of the inflammatory response, it is also possible that the true degradative capacity of EC during inflammation has not been overestimated in this study.

The relatively low levels of expression of hydrolytic enzymes by leukocytes, as demonstrated in this study, may also be due to their activation requirements. PMA or cytokines alone may not be sufficient to trigger optimum leukocyte expression of BM degrading enzymes. Extravasating leukocytes may require signals (either alone, or in addition to cytokines) from activated ECs to trigger their full degradative potential in much the same way that adhesion molecule expression is induced. Such signals from EC may be in the form of adhesion molecules (such as CD31 (Tanaka, Albelda et al., 1992)), or cytokines presented to extravasating leukocytes by EC (Tanaka, Adams et al., 1993). Since such signals were not provided in this in vitro system, the degradative potential of leukocytes may have been underestimated. Alternatively, extravasating leukocytes may express BM-degrading enzymes in a polar, or directed fashion as has already been shown to occur with cytokines (Poo et al., 1988; Kupfer et al., 1991). Mononuclear phagocytes, for example, have been identified as the dominant source of
plasminogen activator activity amongst leukocytes (Danø, Andreason et al., 1985). During recruitment, monocytes utilise the surface receptor bound uPA to focus plasmin activity in the immediate pericellular environment, thereby effecting sufficient localised BM degradation to permit migration across tissue planes (Chen, 1992). Such directed expression of hydrolytic enzymes would require only small amounts of the enzymes and result in minimal damage to, and thus low levels of radioactivity released from, the ECM.

A possible role of platelets in tumour metastasis has been outlined in other studies (Tanaka et al., 1986; Vlodavsky, Eldor et al., 1992). Their role in leukocyte extravasation is less clear, but in the light of their high degradative potential, it is clearly worth further investigation. HSPG are known to sequester growth factors in the ECM (Wight et al., 1992) and release of the growth factor-HSPG complex could occur by the action of heparanases. The expression of platelet heparanase may therefore be more relevant to wound healing, rather than playing a direct role in inflammation. It is also significant that platelet heparanase, unlike leukocyte heparanase, is able to degrade cell surface HS (Hennes et al., 1988; Vlodavsky, Eldor et al., 1992). It is therefore possible that platelet heparanases may regulate leukocyte-EC adhesive interactions prior to leukocyte extravasation.

Another important point arising from this study is that the nature of the degradation products is cell specific, indicating that different leukocytes have unique profiles of degradative enzymes. For example, lymphocytes and platelets expressed mainly heparanase activity. In contrast, activated neutrophils expressed predominantly protease activity. In these experiments, monocytes and U937 cells expressed only sulfatase in significant amounts while HUVECs, whether stimulated or nonstimulated, expressed high levels of all three major enzymes. Moreover, while both lymphocytes and platelets appeared to express mainly heparanase activity, there were differences in their respective gel filtration elution profiles, with the platelet activity resulting in overall lower mol. wt. degradation products. This could mean either that the platelet heparanase is less specific in its degradation of HS, (i.e., it can cleave HS chains at more sites than the lymphocyte enzyme), or that it was simply more active than the lymphocyte heparanase, resulting in more extensive HS degradation during the assay period.
It should also be noted that degradation of the ECM may be sequential, and that the high activity of an enzyme acting later in the cascade may mask, in this assay, the apparent activity of an earlier acting enzyme. For example Bar-Ner (Bar-Ner, Kramer et al., 1985; Bar-Ner, Mayer et al., 1986) found that heparanase-mediated degradation of ECM-bound HSPG could be stimulated 5-20 fold by various serine proteases, suggesting that a proteolytic activity helps to expose the HS side chains to the heparanase. Thus, if platelets actually possess proteolytic enzymes, their true level of activity may have been underestimated in this experimental system due to the rapid degradation by platelet heparanase of any intact HSPG released by platelet proteases. Similarly, depending upon when de-sulfation of HS occurs, heparanase activity by ECs may be masked by their sulfatase activity, i.e., HS fragments may be rapidly desulfated and therefore remain undetected in region III of the gel filtration elution profile. Experiments with 3H-glucosamine-labeled ECMs suggest that this is indeed the case (Fig. 4.5). However, studies with monocytes and HUVEC monolayers suggest that the sulfatase can act independently of any other enzymic activity.

While the full spectrum of hydrolytic enzymes of some cell types may have been skewed in this experimental system, clearly there are still differences between the cell types. Neutrophils, for example, expressed very little heparanase, and the sulfatase activity of HUVEC and monocytes was a striking property of these cells. Since there is a suggestion that proteases and heparanases act synergistically in the sequential degradation of ECM, and that EC play an active role in leukocyte extravasation, it is possible that the profile of degradative enzymes of each cell type reflects the degree of interaction that it requires with EC for extravasation to occur.

4.3.2 Evidence of cell type-specific effects of PMA and cytokines
In the course of studying the comparative abilities of leukocytes, EC and platelets to degrade BM, evidence arose of cell type-specific effects of PMA and cytokines. Of interest, for example, was the finding that IL-1 plus TNF effected an increase in neutrophil and HUVEC degradation of the ECM, but inhibited lymphocyte ECM degradation. Similarly, MCAF, caused a large increase in monocyte sulfatase activity while PMA or IL-1 plus TNF actually suppressed monocyte sulfatase activity. IL-8, which has been reported to be a specific activator of neutrophils and T cells (Matsushima and Oppenheim, 1989), was found to cause an increase in ECM degradation not only by neutrophils and lymphocytes, but also by HUVECs. A large number of
chemokines and other cytokines have already been discovered and characterised, and clearly, these experiments represent only a preliminary study. There is a distinct possibility that other cytokines, not examined here, are more effective inducers of leukocyte hydrolytic enzyme expression. For example, IL-8 isolated from T lymphocytes or monocytes is a mixture of two IL-8 polypeptides (Hebert, Luscinskas et al., 1990). This study used IL-8 isolated from EC, which has been shown to be predominantly the longer chain form, whereas the shorter form has been shown to be 2-3 times more potent as a promoter of neutrophil degranulation and chemotaxis and 10 times more potent as an inhibitor of neutrophil adhesion to IL-1β activated HUVEC (Hebert, Luscinskas et al., 1990). Thus, other cytokines, growth factors and chemoattractants, or combinations of these, are likely to be involved in the differential triggering of leukocyte and EC degradation of ECM during inflammation and their role in leukocyte extravasation during inflammation clearly merits further investigation. The ability of PBMC conditioned medium to induce HUVECs to degrade the ECM in a similar manner to PMA, a response not achieved by several pro-inflammatory cytokines (i.e., IL-1 + TNF, IL-8, IFNγ) highlights this point.

Previous studies, especially by Vlodavsky and co-workers (reviewed in (Vlodavsky, Eldor et al., 1992)), have utilised labeled EC-derived BM to study the ability of leukocytes, as well as EC and platelets, to degrade subendothelial BM. However, these have generally been separate studies of each cell type, and have not directly compared the relative ECM degradative potential of the different cells. In general, the results of this study confirm earlier reports on lymphocytes and platelets (Savion, Vlodavsky et al., 1984; Vlodavsky, Eldor et al., 1992), namely that both of these cells express substantial heparanase activity. In contrast, there are significant differences between the present study and that of Vlodavsky and co-workers (Matzner, Bar-Ner et al., 1985) who reported high heparanase activity in neutrophils. Previous studies by Vlodavsky and co-workers (Godder et al., 1991) also found that cultured intact ECs, including HUVECs, possessed low protease and heparanase activity and that this activity was not changed when the cells were exposed to PMA and other agents. The discrepancies between these and my studies may be accounted for, in part, by the culturing of cells on labeled ECMs with medium containing 10% FCS in my experiments compared to the use of serum free medium in those of Vlodavsky et al. The harsher serum-free culture conditions may have induced neutrophil degranulation and perturbed HUVEC activation by PMA.
On the other hand, although macrophages have been reported to contain significant protease and heparanase activity (Savion, Vlodavsky et al., 1984; Savion, Disatnik et al., 1987; Sewell, 1989) peripheral blood monocytes have not been examined previously for their ability to degrade a labeled ECM.

4.3.3 Detection of a novel sulfatase
Perhaps one of the most interesting findings of this study concerns evidence for the existence of a unique sulfatase expressed by ECs and monocytes. Studies by Dawes (Dawes, 1990) have shown that cultured EC bind and desulfate heparin, suggesting that the major route of catabolism of administered heparin in man involves initial desulfation by the action of sulfatases, followed by depolymerisation of the resultant carbohydrate chains. As described in section 1.5, the N-and O-sulfate residues of HSPG confer a highly anionic character to the HS side chains. Within BMs this anionic charge is important in controlling entry and diffusion of charged macromolecules and also in stabilising interactions between different components of the BM (Sewell, 1989; Yurchenco and Schittny, 1990). A sulfatase would be expected, therefore, to reduce this negatively charged barrier and thus allow greater physical interaction between leukocytes and ECM, and may also increase the accessibility of BM components to degradation by hydrolytic enzymes. Indeed, it is conceivable that the action of a sulfatase may so alter BM characteristics as to render it penetrable by invading leukocytes without further need for degradation by heparanases and proteases. Thus, the expression of a sulfatase by EC supports the hypothesis that EC play an important role in preparing their ECM for leukocyte extravasation. In further support of this hypothesis, the EC sulfatase was found to be upregulated by inflammatory cytokines, and activated EC monolayers, which are more representative of their in vivo counterparts, were also found to express sulfatase activity.

In this study, monocytes were also found to possess a sulfatase, and their degradation of ECM was mainly due to this enzymic activity. Interestingly, PMA was shown to down-regulate sulfatase expression by monocytes isolated from human peripheral blood, and by the monocytic cell line U937, with the monocyte chemokine MCAF inducing a dramatic increase in sulfatase activity. Phorbol esters cause U937 (Yahalom et al., 1988) and other monocytic leukemia cells (Asseffa et al., 1993) to differentiate into macrophage-like cells, and has been shown to decrease monocyte adhesion to unstimulated EC (Kamp et al., 1989). In an inflammatory situation, BM
degradative capacity should be more relevant to circulating monocytes than to macrophages, which have already transmigrated. It is therefore possible that PMA, which promotes differentiation of monocytes, may also act to down-regulate sulfatase activity, consistent with the cell's reduced requirement to extravasate.
4.4 Summary

The subendothelial BM is regarded as an important barrier to the entry of leukocytes into inflammatory sites. This study compares the ability of the major cellular components of inflammation, namely leukocytes, platelets and EC, to degrade a $^{35}$SO$_4$-labeled subendothelial ECM and assesses the effect of PMA and various pro-inflammatory cytokines on this degradative activity. The products of degradation of $^{35}$SO$_4$-labeled ECM HSPGs were analysed by FPLC gel filtration chromatography and four major mol. wt. species were identified. These products corresponded to intact HSPG (I), free HS chains (II), HS chain fragments (III) and oligo/monosaccharides/free sulfate (IV). Degradation products I and II were indicative of protease activity, product III arose from endoglycosidase (heparanase) activity and product 4 suggested exoglycosidase and/or sulfatase activity.

In terms of ECM degradation, HUVECs and platelets were the most active, with PMA stimulation further enhancing the degradative activity of these two cell types. However, platelets exhibited predominantly heparanase activity whereas the HUVEC degradation products suggested a range of enzymic activities, namely proteases, heparanases and sulfatases. Interestingly, EC in suspension expressed these 3 enzymic activities whereas confluent EC monolayers only exhibited sulfatase activity, suggesting that the former situation might represent an angiogenic response. In the case of leukocytes, neutrophils and lymphocytes degraded the ECM to a much greater extent than monocytes. Each cell type differed in the predominant enzymic activities they expressed, i.e., heparanase by lymphocytes, protease, and to a lesser extent, heparanase by neutrophils and sulfatase by monocytes. PMA stimulation was shown to have differential effects on these enzymic activities.

Some pro-inflammatory cytokines were found to be cell type specific in their effects on ECM degradation. Thus, IL-1 + TNF enhanced neutrophil and HUVEC degradation of the ECM but inhibited lymphocyte ECM degradation. In contrast, the chemokine IL-8 enhanced ECM degradation by neutrophils, lymphocytes and HUVECs. Of particular interest was the unique sulfatase activity expressed by EC and monocytes which was induced in HUVEC by TNF + IL-1 and IL-8, whereas in monocytes the sulfatase activity was exclusively induced by the chemokine MCAF.
Collectively, the results of this study show that leukocytes differ markedly in the enzymes they express to degrade the BM during extravasation and that PMA and cytokines are cell-type specific in their induction of hydrolytic enzyme activity. These results also indicate that EC may have an important role, not only in the recruitment of leukocytes, but also in the preparation of vascular BM for extravasation. The compromised BM integrity resulting from sulfatase activity is likely to be important in allowing greater physical interaction between leukocytes and ECM, increasing the accessibility of BM components to degradation by hydrolytic enzymes.
Chapter 5

Differential effects of the anti-inflammatory compounds heparin, mannose-6-phosphate and castanospermine on degradation of the vascular basement membrane by leukocytes, endothelial cells and platelets.

5.1 Introduction

Hydrolytic enzymes secreted by activated leukocytes have been implicated in the degradation of the subendothelial basement membrane (BM) (Vettel et al., 1991; Vlodavsky, Eldor et al., 1992) and may facilitate the extravasation of circulating leukocytes into foci of inflammation. For example, the ability of activated lymphocytes sensitised to myelin basic protein (MBP) to penetrate the central nervous system (CNS) and cause experimental autoimmune encephalomyelitis (EAE) (Naparstek, Cohen et al., 1984) has been shown to correlate closely with their expression of an endoglycosidase (heparanase) capable of degrading the heparan sulfate (HS) side chains of the proteoglycan scaffolding of subendothelial BM (Naparstek, Cohen et al., 1984).

Evidence that leukocyte degradative enzymes, such as heparanases, may be of critical importance in allowing activated leukocytes to breach vascular barriers has prompted the investigation of inhibitors of these enzymes as potential antimetastatic and anti-inflammatory compounds. Certain sulfated polysaccharides, such as heparin, have since been shown by a number of studies to be effective anti-inflammatory agents, inhibiting EAE (Willenborg and Parish, 1988; Lider, Baharav et al., 1989), preventing delayed-type hypersensitivity reactions (Chelmicka-Szone and Arnason, 1972; Sy et al., 1983) and in prolonging allograft survival (Lider, Baharav et al., 1989). The antimetastatic and anti-inflammatory effects of heparin were, at first, largely assumed to be due to the anti-coagulant properties of these molecules. However subsequent studies revealed that heparin depleted of anticoagulant activity retained its antimetastatic and anti-inflammatory properties and that heparanase inhibition was probably the major mode of action (Sy, Schneeeberger et al., 1983; Parish, Coombe et al., 1987; Nakajima, Irimura et al., 1988; Willenborg and Parish, 1988; Lider, Baharav et al., 1989).
There is also evidence to suggest that heparanases, and other hydrolytic enzymes involved in the degradation of BM components, may be of lysosomal origin (Kaiser, 1980; Nakajima, Irimura et al., 1983). Several studies have shown that lysosomal enzymes can be expressed on the cell surface via mannose phosphate receptors (MPR), these receptors usually being involved in targeting newly formed lysosomal enzymes to the lysosomes (von Figura and Voss, 1979; Fischer, 1980), (reviewed by (Kornfeld, 1987; Kornfeld, 1990), (see section 4.3.2.3). Thus it is theoretically possible that the degradation of BM by lysosomal enzymes can occur extra-cellularly (Poole, 1973; Weiss, 1978; Weiss, Liu et al., 1990) via the cell surface presentation of these enzymes bound to MPR in a concentrating mechanism analogous to that described for the uPA-uPAr system (Chen, 1992; Novokhatny, Medved et al., 1992) (section 4.3.2.1). In support of this hypothesis, recent studies have shown that the phosphosugar, M6P, and castanospermine (CS), an inhibitor of N-linked oligosaccharide processing, are both effective inhibitors of EAE and adjuvant arthritis (Willenborg and Parish, 1989; Willenborg, Parish et al., 1989; Willenborg, Parish et al., 1992). Willenborg and co-workers suggested that the anti-inflammatory effects of CS and M6P may be due to their ability to inhibit the MPR-lysosomal enzyme interaction which is critical for the intracellular transport and cell-surface presentation of lysosomal enzymes. In their model it was proposed that M6P, which is a potent inhibitor of the lysosomal enzyme-MPR interaction, would displace lysosomal enzymes from the cell surface, whereas CS would prevent the correct formation of the M6P marker, thus preventing its attachment to the MPR. In a similar study described in chapter 3, the sulfated polysaccharide fucoidan, M6P and CS were shown to partially inhibit allograft rejection and elicitation of peritoneal exudates. Based on the results of this study it was suggested that these three compounds inhibit inflammation at the level of leukocyte extravasation. The present work was undertaken to test the extravasation inhibition hypothesis and determine whether heparin, M6P and CS inhibit the degradation of subendothelial ECM by the cellular components of the inflammatory response.

5.2 Results
In this study, heparin, M6P and CS were examined for their ability to prevent in vitro degradation of a $^{35}$SO$_4$-labeled ECM by leukocytes, EC and platelets. As described in chapter 4, the products of labeled ECM degradation released into the culture supernatants were analysed by gel filtration chromatography and the FPLC elution profiles were subdivided into 4 major
regions representing 4 types of degradation products (Table 4.1). Using HS standards, the approximate mol. wt. of these products was determined, providing evidence of the type of enzymes that were utilised by the cells to degrade the ECM. Thus, the highest mol. wt. material eluted in the void volume of the column (K_{av} < 0.2, region I), and represented predominantly intact HSPG (Table 4.1). A characteristic peak (region II, approx. K_{av} = 0.35), which was obtained following NaOH treatment of ^{35}SO_{4} labeled ECM, represented intact HS side chains (Table 4.1). Detection of peaks in regions I and II was reasoned, therefore, to be indicative of protease activity (Table 4.1). As described in chapter 4, the appearance of a heterogeneous population of HS degradation products eluting in region III (approx. K_{av} = 0.5-0.7), was indicative of heparanase activity (Fig. 4.2). Finally, a fourth peak (region IV) at V_{t} was shown in chapter 4 to contain very low mol. wt. ECM degradation products, with the presence of free sulfate implying the presence of a sulfatase (Table 4.1). This assay is, therefore, able to provide useful information not only about the level, but also the types of hydrolytic enzymes utilised by the cells tested. The assay is thus a useful means for determining the effects of the above-mentioned anti-inflammatory compounds on the ability of activated leukocytes, ECs and platelets to degrade the subendothelial BM.

As reported in chapter 4, after incubation of PMA-activated neutrophils with ^{35}SO_{4}-labeled ECM, the predominant degradation products were in regions I, II and III, suggesting protease mediated release of free HS chains and some heparanase activity (Fig. 4.4a, Tables 4.2 and 4.3). Fig. 5.1a is a representative elution profile, and shows that heparin (100 µg/ml) was able to effectively inhibit the appearance of degradation products in regions II and III, indicating the inhibition of heparanase. However, M6P (1 mg/ml) and CS (1 mg/ml) did not significantly inhibit ECM degradation by neutrophils (Figs 5.1b and c).

PMA-stimulated human lymphocytes, in contrast to neutrophils, expressed relatively high levels of heparanase activity, as indicated by a high proportion of low mol. wt. degradation products in region III of the FPLC elution profiles (Fig. 5.2). Lymphocyte heparanase activity was reduced markedly in the presence of heparin (Fig. 5.2a). Indeed, the appearance of low mol. wt. degradation products (region III) was reduced to below nonstimulated lymphocyte levels, with a concomitant increase in degradation products eluting in region I. M6P was also able to reduce lymphocyte heparanase
Fig. 5.1

Representative elution profiles on a Superose-6 FPLC column showing the effects of heparin, M6P and CS on degradation products released from $^{35}$SO$_4$-labeled ECM by $10^6$ neutrophils isolated from human peripheral blood. Treatments (24hr, 37°C) are: medium control ( ), cell control ( ), cells in the presence of 50 ng/ml PMA ( ) and cells in the presence of both 50 ng/ml PMA and the compound indicated ( ).

The positions of regions I-IV is indicated, the properties of these regions being described in Table 4.1.
Heparin

Mannose-6-phosphate

Castanospermine
Fig. 5.2

Representative elution profiles on a Superose-6 FPLC column showing the effects of heparin, M6P and CS on degradation products released from $^{35}$SO$_4$-labeled ECM by $10^6$ lymphocytes isolated from human peripheral blood. Treatments (24hr, 37°C) are: medium control (-----), cell control (-----), cells in the presence of 50 ng/ml PMA (----------) and cells in the presence of both 50 ng/ml PMA and the compound indicated (-----).

The positions of regions I-IV is indicated, the properties of these regions being described in Table 4.1
activity. In 4/5 experiments with human peripheral blood lymphocytes or ConA-activated T lymphocytes, in the presence of M6P a reduction of region III degradation products, with an attendant increase in region I products, was observed (Fig. 5.2b). In contrast, CS treatment of lymphocytes consistently resulted in no effect on ECM degradation (Fig. 5.2c).

As reported in chapter 4, the incubation of monocytes and the monocytic cell line U937 with $^{35}$SO$_4$-labeled ECM generally resulted in poor levels of ECM degradation. At the time of writing, only MCAF was found to be able to increase the activity of U937 sulfatase activity above nonstimulated cell levels, PMA usually inhibiting the low level of endogenous sulfatase activity (Fig. 4.4c-d). Unfortunately, the ability of MCAF to stimulate monocyte sulfatase activity was only discovered just prior to writing. Consequently, the effects of heparin, M6P and CS were not investigated further with this particular cell type.

The incubation of PMA-stimulated HUVECs with $^{35}$SO$_4$-labeled ECM resulted in the release of labeled degradation products eluting in all of the major regions (I-IV) at levels substantially above the medium control (Table 4.2 and Fig. 4.4e). This indicated the presence of protease, heparanase and sulfatase activity (Section 4.2.2). Heparin was again shown to inhibit the appearance of degradation products eluting in regions II, III and IV to below nonstimulated HUVEC levels (Fig. 5.3a). M6P did not effect the degradation of ECM by HUVECs (Fig. 5.3b). CS, however, consistently reduced HUVEC heparanase and sulfatase activity to non-stimulated cell levels (Fig. 5.3c). In 2/3 experiments, protease activity, represented by peaks in regions I and II, was also inhibited by CS.

The interaction of thrombin stimulated washed platelets with labeled ECM for 6 hr resulted in high levels of heparanase activity, as indicated by the appearance of most of the labeled degradation products in region III of the gel filtration elution profiles. Again, heparin inhibited the appearance of these degradation products, and caused an accumulation of degradation products eluting in region I of the chromatograms (Fig. 5.4a). Interestingly, a small peak at region IV also appeared with thrombin treated stimulated platelets in the presence of heparin (Fig. 5.4a). This peak was subsequently shown, using $^3$H-glucosamine-labeled ECMs, to represent labeled free sulfate (data not shown) implying the presence of some sulfatase activity in platelets. M6P did not have any effect on the degradation of the labeled ECM by
Fig. 5.3

Representative elution profiles on a Superose-6 FPLC column showing the effects of heparin, M6P and CS on degradation products released from $^{35}$SO$_4$-labeled ECM by $10^6$ HUVECs. Treatments (24hr, 37°C) are: medium control (——), cell control (---), cells in the presence of 50 ng/ml PMA (-----) and cells in the presence of both 50 ng/ml PMA and the compound indicated (----). The positions of regions I-IV is indicated, the properties of these regions being described in Table 4.1
Fig. 5.4

Representative elution profiles on a Superose-6 FPLC column showing the effects of heparin and M6P on degradation products released from $^{35}$SO$_4$-labeled ECM by $10^8$ platelets isolated from human peripheral blood. Treatments (6 hr, 37°C) are: medium control (———), platelet control (-----), platelets in the presence of 0.1 U/ml thrombin (———) and platelets in the presence of both 0.1 U/ml thrombin and the compound indicated (----). The positions of regions I-IV is indicated, the properties of these regions being described in Table 4.1.
thrombin-activated platelets (Fig. 5.4b). Since the degradative enzymes of platelets are present in pre-formed platelet granules and lysosomes, CS was not tested as an inhibitor of platelet degradation of the ECM.

5.3 Discussion

All of the anti-inflammatory compounds analysed in this study inhibited $^{35}$SO$_4$-labeled ECM degradation, but M6P and CS were shown to be cell-type specific in their effects. Heparin inhibited the heparanase activity of all cell types examined, confirming the results of previous studies using similar in vitro techniques (reviewed by (Vlodavsky, Eldor et al., 1992)) and consistent with the view that heparin is a non-cleavable substrate of most mammalian heparanases. Interestingly, heparin was also found to inhibit the sulfatase activity of HUVEC (Fig. 5.3a) and to inhibit the release of high mol. wt. (region II) ECM fragments by HUVEC (Fig. 5.3a). In the case of the former phenomenon, it is possible that the sulfate groups of heparin enable it to act as a competitive inhibitor of sulfatases. There are at least two possible explanations for the inhibition of the release of high mol. wt. material by heparin. First, the peak in region II may be composed not only of intact HS side chains, but also of HSPG partially degraded by heparanase. Secondly, previous studies indicate that heparin can directly inactivate human leukocyte proteinases such as elastase and cathepsin G (Redini et al., 1988) and interfere with the expression and activity of proteases such as the plasminogen activators (Au et al., 1991).

Unlike heparin, M6P selectively inhibited lymphocyte heparanase, with no effect on the heparanase activity of platelets and the other cells examined. In contrast, CS selectively inhibited HUVEC heparanase, sulfatase and protease activity, but inhibited only PMA-induced degradative enzyme expression, without affecting the constitutive expression of degradative enzymes by nonstimulated HUVECs. This result supports the current understanding that CS perturbs the formation of nascent N-linked oligosaccharide hydrolases and their targeting to lysosomes, but does not affect preformed lysosomal enzymes (Elbein, 1987; Elbein, 1991).

The cell type specific effects of CS and M6P on BM degradation provides important clues as to the mode of action of these compounds. CS is a potent inhibitor of glucosidase I and glucosidase II (Elbein, 1991) and should perturb
appropriate N-linked glycosylation of lysosomal enzymes irrespective of the cell type. However, some cells possess an alternative endomannosidase activity which can provide a bypass mechanism to circumvent these glycosidases (Karlsson et al., 1993). In fact, the concentrations of CS used in this study are far in excess of those required to inhibit glucosidase I and II activity (Saul, 1983). Presumably lymphocytes, and possibly neutrophils, but not HUVECs, possess this alternate trimming route. The inhibition of lymphocyte, but not HUVEC, degradation of ECM by M6P further suggests that heparanases in these two cell types may be expressed on the cell surface in different ways. While it appears that lymphocyte hydrolytic enzymes are expressed on the cell surface via MPR, it is possible that HUVEC hydrolytic enzymes contain a different complex type oligosaccharide structure and thus utilise an alternate hydrolytic enzyme-receptor system. In support of this observation, studies in this laboratory utilising antibodies against MPR, have shown that activated T cells, but not HUVECs, express significant amounts of cell surface MPR (E. Hindmarsh, C. Parish, personal communication).

It is useful to compare the results of the present study with those of previous studies examining the effects of sulfated polysaccharides, M6P and CS on animal models of inflammation. In this regard, perhaps the most informative of the in vivo experiments is the rat EAE model (Parish, Willenborg et al., 1990). EAE is a CD4+ T cell mediated disease in which the target antigen is myelin basic protein (MBP) (section 1.5.2.2). While some investigators suggest that entry of T cells into the CNS is antigen specific, there is more evidence to support the non-immunological mechanism proposed by Wekerle (Wekerle et al., 1986) who showed that activated T cells of any specificity emigrate through CNS endothelium into the parenchyma of the CNS as part of a surveillance mechanism. Activated T cells entering the CNS which encounter an antigen for which they have specificity, respond to it and initiate inflammatory events. In the case of EAE, this migration of activated cells, some of which can respond to MBP, results in inflammation and disease. Willenborg and co-workers studied the effects of heparin, M6P and CS on EAE (Parish, Willenborg et al., 1990). They found that all three compounds exhibited marked inhibitory effects on the symptoms of EAE, but there were variations in the histology taken from treated animals. For example, control rats showed diffuse inflammatory lesions in the spinal cord, whereas heparin- and M6P-treated rats had no lesions, or a dramatically reduced number of lesions compared to controls (Willenborg and Parish, 1988;
Willenborg, Parish et al., 1989; Parish, Willenborg et al., 1990). On the other hand, rats treated with CS had inflammatory lesions which varied from control rats only in distribution and quality. Specifically, while lesions in the control rats were diffuse, with mononuclear cells migrating away from the vessels and infiltrating extensively into the parenchyma of the spinal cord, perivascular inflammatory lesions in the CS treated rats were characteristically intense and compact, with inflammatory cells remaining closely marginated around the vessels (Willenborg, Parish et al., 1989). Electron microscopic studies revealed that most of these marginating cells were monocytes (C. Parish, personal communication) which were clearly able to cross the endothelium, but subsequently accumulated between the endothelium and a limiting structure which appeared to be the BM. Thus, CS appeared not to be affecting leukocyte-EC adhesion and transmigration events, but appeared to be inhibiting the subsequent passage of monocytes through the subendothelial BM.

The discovery of characteristic lesions in CS-, but not heparin- and M6P-treated rats suggests a subtle difference in the mode of action of these compounds and, in light of the results of the present study, an appropriate model can be proposed. Both heparin and M6P, which inhibit lymphocyte heparanase activity or cell-surface expression respectively, prevent BM degradation by lymphocytes and thus inhibit their extravasation into the CNS parenchyma. Thus, inhibition of an inflammatory response by MBP-specific T cells entering the CNS would be prevented by these two agents. Interestingly, studies in this laboratory using monoclonal antibodies to MPR, (E. Hindmarsh, C. Parish, personal communication) showed that activated T cells transiently expressed MPR following EC adhesion. Presumably such T cells use the MPR to concentrate heparanase activity at the cell surface, thereby facilitating their extravasation. On the other hand, CS, which was shown in this study to have no effect on lymphocyte ECM degradative activity, allows activated, MBP-specific T cells to enter the CNS. Once in the CNS they encounter the target antigen and initiate the events that would normally recruit further mononuclear cells, resulting in inflammation and disease.

Stimulation of endothelium by inflammatory agents induces the expression of adhesion molecules and results in the margination of leukocytes. The results presented in chapter 4 have shown that inflammatory stimuli may also induce the degradation of BM by EC. Indeed, EC may be required to prepare the BM for invasion by certain leukocyte types. CS, which inhibits EC
expression of hydrolytic enzymes, may frustrate this cooperative process, resulting in the accumulation of mononuclear cells at the subendothelial BM as observed in CS-treated rats with EAE (Parish, Willenborg et al., 1990). In addition, CS may inhibit chemokine-induced expression of degradative enzymes by monocytes, a point which requires further investigation.
5.4 Summary

Hydrolytic enzymes of lysosomal origin, secreted by activated leukocytes, have been implicated in the degradation of the subendothelial basement membrane (BM) (Vettel, Bar-Shavit et al., 1991; Vlodavsky, Eldor et al., 1992) and may facilitate the extravasation of leukocytes into foci of inflammation. It has been suggested previously that the anti-inflammatory effects of M6P and CS may be due to their ability to inhibit MPR-lysosomal interactions which are critical in the intracellular transport and cell-surface expression of lysosomal enzymes. Similarly, the anti-inflammatory effect of heparin may be due to the direct inhibition of leukocyte heparanase activity.

In this chapter, heparin, M6P and CS were examined for their ability to prevent in vitro degradation of a $^{35}$SO$_4$-labeled ECM by leukocytes, ECs and platelets. The products of labeled ECM degradation released into the culture supernatants were analysed by gel filtration chromatography. This assay provided both a qualitative and quantitative measurement of the different enzymic activities utilised by the cells to degrade the ECM and the ability of the three anti-inflammatory compounds to inhibit these enzymes.

All three anti-inflammatory compounds analysed in this study inhibited $^{35}$SO$_4$-labeled ECM degradation, but M6P and CS were shown to be cell-type specific in their effects. Heparin inhibited the heparanase activity of all cell types examined, confirming the results of previous studies using similar in vitro techniques. M6P selectively inhibited lymphocyte heparanase, with no effect on the heparanase activity of platelets and the other cells examined. CS selectively inhibited HUVEC heparanase and sulfatase activity, but inhibited only PMA-induced degradative enzyme expression, without affecting the constitutive expression of degradative enzymes by nonstimulated HUVECs. The finding that heparin was able to inhibit heparanase in each cell type tested, while the actions of CS and M6P were cell type specific, provided important clues as to the mode of action of these compounds and the characteristic inflammatory pathology associated with the use of these anti-inflammatory agents. In particular, the data support the view that leukocytes markedly differ in the mechanisms they use to degrade BM/ECM to enable extravasation, and that some degree of cooperation with EC is required in this process.
**Chapter 6**

**Venular and arterial endothelial cells differ in their expression of adhesion molecules and their ability to degrade the subendothelial basement membrane**

**6.1 Introduction**

It has been known for over a century that during inflammation, leukocytes adhere to the wall of microvascular endothelium prior to migrating into the tissue. This phenomenon was investigated in detail by Cohnheim (Cohnheim, 1882), whose graphic descriptions still form the basis of our understanding of the histopathology of inflammation today. These early studies clearly demonstrated that leukocyte adhesion and extravasation during acute inflammation takes place on the venular side of the micro-vasculature. Many more recent studies by light, electron and intravital microscopy (Allison et al., 1955; Florey, 1962; Tonnesen et al., 1982; Fiebig et al., 1991) have confirmed the original findings and led to the wide acceptance that leukocyte transmigration is spacially specific, occurring primarily in post capillary venules. The apparent propensity of leukocytes to adhere to postcapillary venules has been related primarily to a decrease in vessel wall shear forces (Harlan, 1985) and increased adhesive interactions between leukocytes and the activated venular EC surface, the molecular events of which have only recently been characterised (Springer, 1990; Hynes, 1992; Lasky, 1992).

Once arrested in the vasculature, leukocytes must also passage through the endothelium and penetrate the subendothelial basement membrane (BM), a significant barrier to leukocyte extravasation. A number of studies have demonstrated the importance of the expression of inducible hydrolytic enzymes by leukocytes in overcoming this connective tissue barrier (Savion, Vlodavsky et al., 1984; Bar-Ner, Kramer et al., 1985; Kramer et al., 1985; Vlodavsky, Eldor et al., 1992), but evidence is accumulating that ECs also contribute to the dissolution of this barrier (Stolpen et al., 1986) (chapters 4 and 5). Thus, the observed predilection of leukocytes to adhere to, and extravasate via, endothelium of the postcapillary venule, rather than to endothelium on the arterial side of the microvasculature, may also be due to significant differences of particular vascular beds in the expression of enzymes capable of degrading the vascular BM.
The aim of this study was to investigate the hypothesis that there are differences in the ability of venular versus arterial EC to: a) express adhesion molecules in response to pro-inflammatory cytokines and/or b) degrade the subendothelial BM in response to the same pro-inflammatory cytokines. If such differences do exist they may go some way towards explaining the selective entry of leukocytes into inflammatory sites via venular endothelium. In order to test this hypothesis, EC derived from human umbilical vein and human umbilical artery were tested for their expression of E-selectin, VCAM-1 and ICAM-1, and for their ability to degrade a subendothelial extracellular matrix (ECM) both before and following exposure to pro-inflammatory cytokines.

6.2 Results

6.2.1 Expression of cytokine induced adhesion molecules by venular and arterial EC
In order to determine whether there are differences between arterial and venular EC in their expression of adhesion molecules, human EC isolated from umbilical vein (HUVEC) and umbilical artery were examined for E-selectin, VCAM-1 and ICAM-1 expression in the presence and absence of the pro-inflammatory cytokines TNF and IL-1. Fig. 6.1 shows that pretreatment of HUVEC (passage 2) for 18 hr with IL-1 and TNF resulted in the induced expression of the adhesion molecules E-selectin and VCAM-1, and an increased expression of ICAM-1. Arterial EC (passage 2) showed a similar pattern of adhesion molecule induction by IL-1 and TNF, the only notable difference being a consistently (5/5 experiments) higher constitutive expression of VCAM-1 (Fig. 6.2). For both HUVEC and umbilical artery EC, the cytokine-induced expression of adhesion molecules diminished with increasing passage number. However Table 6.1 shows that the diminution of adhesion molecule expression with passage number in culture was much more marked with the arterial EC than with the venular EC. In fact, following cytokine treatment, passage 7 arterial EC expressed the three adhesion molecules at only marginal levels above non-treated controls.

6.2.2 Comparison of the ability of venular and arterial EC to degrade the ECM
In the next series of experiments, arterial and venular EC isolated from human umbilical cord were examined for their ability to degrade EC-derived, $^{35}$SO$_4$-labeled ECM in tissue culture dishes. As already described in chapter
Fig. 6.1

Expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 on passage 2 HUVEC following 18 hr culture either in the presence or absence of TNF (2.5 ng/ml) and IL-1 (0.2 ng/ml). Adhesion molecule expression was assessed by immunofluorescent flow cytometry with HUVEC treatments being: control cells, no mAbs (--), cytokine treated cells, no mAbs (-----), control cells + mAbs (---) and cytokine treated cells + mAbs (-----). Non-mAb controls are shown in the top figure only.
Fig. 6.2

Expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 on passage 2 human umbilical artery EC following 18 hr culture either in the presence or absence of TNF (2.5 ng/ml) and IL-1 (0.2 ng/ml). Adhesion molecule expression was assessed by immunofluorescent flow cytometry with arterial EC treatments being: control cells, no mAbs (--), cytokine treated cells, no mAbs (-----), control cells + mAbs (-----) and cytokine treated cells + mAbs (-----). Non-mAb controls are shown in the top figure only.
### Table 6.1
Effect of EC passage number on the cytokine-induced expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 on venular and arterial EC

<table>
<thead>
<tr>
<th>EC type</th>
<th>Cell-surface antigen</th>
<th>Passage 2</th>
<th>Passage 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venular</td>
<td>E-selectin</td>
<td>1400&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1320</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>4258</td>
<td>984</td>
</tr>
<tr>
<td></td>
<td>ICAM-1</td>
<td>2304</td>
<td>997</td>
</tr>
<tr>
<td>Arterial</td>
<td>E-selectin</td>
<td>970</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>1900</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>ICAM-1</td>
<td>2360</td>
<td>120</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cytokine concentrations: TNF (2.5 ng/ml) and IL-1 (0.2 ng/ml). Results of one representative experiment are presented.

<sup>b</sup> EC derived from human umbilical vein and human umbilical artery.

<sup>c</sup> Median fluorescence intensity of cytokine treated cells expressed as percentage of non-treated cells.
4, the products of labeled ECM degradation released into the culture supernatants were analysed by FPLC gel filtration chromatography. The resultant elution profiles could be subdivided into four major regions representing four mol. wt. families of degradation products (Table 4.1), each region also providing evidence for the types of enzymes utilised by the cells in the degradation of the ECM.

The incubation of labeled ECM with nonstimulated arterial EC resulted in the release of similar amounts of radioactivity as with nonstimulated HUVECs (Fig. 6.3). Gel filtration analysis of the degradation products, however, revealed that, unlike HUVECs, which released labeled products in all four regions (I-IV) of their elution profiles (Fig. 6.4a), arterial EC released mainly degradation products detected in regions I and IV of the elution profiles (Fig. 6.4b). Since degradation products in regions II and III are generally indicative of heparanase activity, these data indicated that, unlike venular EC, arterial EC lack heparanase activity. Moreover, in contrast to HUVECs, treatment of arterial EC with PMA did not result in an overall increase in the release of labeled products from the ECM (Fig. 6.3) and there was only a marginal change in the nature of the degradation products released, i.e., slightly less material in region I and more in region IV of the FPLC elution profile (Fig. 6.4b).

The low mol. wt. degradation products eluting in region IV were shown previously for HUVECs to represent the activity of a sulfatase enzyme (chapter 4, Fig. 4.10). Similarly, incubation of PMA-stimulated arterial EC with $^{3}$H-glucosamine-labeled ECM demonstrated that no $^{3}$H-labeled glucosamine was associated with the very low mol. wt. products in region IV, confirming again the presence of sulfatase enzymic activity in these EC (Fig. 6.5). However, the arterial EC did release some $^{3}$H-labeled degradation products above medium control levels in regions I and II-III, the latter possibly representing desulfated heparan sulfate chains.

The cytokine response of arterial EC also differed markedly from venular EC (Fig. 6.6). Thus TNF + IL-1, like PMA, did not increase ECM degradation by the arterial EC (Fig. 6.6c) whereas HUVEC mediated degradation was modestly augmented, particularly the release of intact proteoglycans detected in region I of the elution profile (Fig. 6.6a). In contrast, IFN$\gamma$ selectively stimulated arterial EC sulfatase with little or no effect on the release of other ECM degradation products (Fig. 6.6d), a result totally
Fig. 6.3

Ability of HUVECs and EC isolated from human umbilical artery to release radioactivity from $^{35}$SO$_4$-labeled ECM. In each case, $10^6$ EC were incubated with ECM (24 hr, 37°C) in the presence or absence of PMA (50 ng/ml). Data represents the mean +/- SE of at least 3 experiments.
Fig. 6.4

Comparison of the degradation of $^{35}$SO$_4$-labeled ECM by venular and arterial EC. $10^6$ EC isolated from (a) human umbilical vein (HUVEC) or (b) human umbilical artery, were incubated with the sulfate-labeled ECM (24 hr, 37°C), the degradation products being separated by gel filtration chromatography on a Superose-6 FPLC column. Treatments were: medium control (············), nonstimulated EC (–––––) and EC + 50 ng/ml PMA (----------). The position of regions I-IV is indicated, the properties of these regions being described in Table 4.1
HUVECs

Arterial EC

Fraction No.

CPM

Tritiated-labeled ECM was incubated (24 hr, 37°C) with medium (-----) or $10^6$ PMA (50 ng/ml) stimulated EC (-----). Sulfate labeled ECM was also incubated with PMA (50 ng/ml) stimulated EC (-----). The incubation medium was collected and fractionated by gel filtration on a Superose-6 FPLC column. The sulfate cpm were 4-fold lower than indicated on the graph. The position of regions I-IV is indicated, the properties of these regions being described in table 4.1.

Fig. 6.5
Fig. 6. 6
Representative elution profiles on a Superose-6 FPLC column of degradation products released from $^{35}$SO$_4$-labeled ECM by HUVECs and EC isolated from human umbilical artery. $^{35}$SO$_4$-labeled ECMs were incubated (24 hr, 37°C) with medium control (———), $10^6$ nonactivated (———) or cytokine-stimulated (--------) cells. Cytokine concentrations used were TNF + IL-1 (2.5 and 0.2 ng/ml), and IFN$\gamma$ (150 U/ml). The position of regions I-IV is indicated, the properties of these regions being described in Table 4.1
different from the effect of IFNγ on HUVEC (Fig. 6.6b). In fact, the only effect of IFNγ on HUVECs was to inhibit their endogenous sulfatase activity.

6.3 Discussion

The results of this study show that the cytokine induced expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 did not differ significantly between early passage arterial and venular EC. However, this study also demonstrated a number of striking differences between venular and arterial EC. First, arterial EC expressed significant constitutive levels of VCAM-1 whereas, in agreement with earlier reports (Osborn, Hession et al., 1989; Rice and Bevilacqua, 1989), venular EC expressed low constitutive levels of VCAM-1. Second, TNF and IL-1 were virtually unable to induce adhesion molecule expression on late passage arterial EC whereas similar passage venular EC were only slightly reduced in their cytokine responsiveness. Third, analysis of ECM degradation products revealed substantial heparanase activity in venular EC, an enzyme activity lacking in arterial EC under a number of activation conditions. Fourth, there were marked differences in the ability of different stimuli to induce ECM degradation by the two types of EC. Thus, PMA substantially enhanced ECM degradation by venular EC but had no effect on arterial EC degradation. Furthermore, TNF and IL-1 induced an increase in the release of high mol. wt. proteoglycans from the ECM by venular EC but had virtually no effect on the release of degradation products by arterial EC despite these same cytokines inducing adhesion molecule expression on both EC types. Conversely, IFNγ selectively upregulated arterial EC sulfatase expression whilst inhibiting endogenous venular EC sulfatase activity.

It is clear from the findings summarised above that there are substantial differences between venular and arterial EC, but since both EC types were shown to express adhesion molecules in response to pro-inflammatory cytokines, the mechanism for the predilection of leukocytes for postcapillary venules during inflammation cannot be explained entirely on the basis of adhesion molecules. However, there are several other factors which may favour the adherence of leukocytes to postcapillary venules, rather than to arterial or arteriolar endothelium.
Firstly, despite the possibility that both arterioles and postcapillary venules express adhesion molecules in response to cytokines, shear forces resulting from the flowing blood moving parallel to the EC surface normally limit the initial interaction between circulating leukocytes and the endothelium. For example, integrins cannot mediate T cell binding to ICAM-1 under physiological shear forces (Lawrence and Springer, 1991). The postcapillary venule is the site of the first major decrease in vessel wall blood shear forces (Mayrovitz et al., 1977; Wilkinson and Lackie, 1979) and should therefore be the first possible site for leukocyte-endothelium interactions. Furthermore, blood shear forces are reduced by vessel dilation and increased vascular permeability (Pober and Cotran, 1991), conditions which are typical of an inflammatory site. TNF has been shown to cause EC contraction, EC cytoskeletal and junctional reorganisation and BM protein degradation in vitro (Stolpen, Guinan et al., 1986), - all alterations which can lead to increased fluid permeability and consequently a diminished shear force in vivo. In the context of the present study, it is conceivable that venular EC are more susceptible to such effects of TNF and IL-1, and that the relatively high levels of $^{35}$SO$_4$-labeled ECM degradation by venular EC observed in this study are a measure of the BM rearrangement that accompanies such phenomena.

Another factor which may influence the adherence of leukocytes to postcapillary venules, rather than to arterial or arteriolar endothelium is vessel-site specific differences in cytokine (and other) receptor expression. For example, it has been shown that vascular histamine receptors are predominantly distributed in post capillary venules, in comparison with arterioles (Heltianu et al., 1982). The EC-specific effects of TNF plus IL-1, and IFN$\gamma$ on ECM degradation shown in the present study also support this suggestion. In addition, arterial EC may differ from post-capillary venule EC in their ability to activate integrin mediated adhesion by leukocytes. For example, recent studies have suggested that following the initial, transient adhesion of leukocytes to endothelium, activation of the leukocyte by cell-cell contact mediated signals are required to trigger secondary adhesion molecules that mediate strong adhesion (Butcher, 1991; Shimizu et al., 1992). Finally, the present study has not compared arterial and venular EC for P-selectin expression. P-selectin, which is rapidly redistributed to the EC cell surface upon stimulation of agonists such as thrombin and histamine, is a receptor for neutrophils and monocytes, but not for lymphocytes (Bevilacqua and Nelson, 1993). This study cannot discount the possibility
that arterial EC do not express P-selectin, particularly in the light of their reported deficiency in histamine receptors (Heltianu, Simionescu et al., 1982).

It is important to note that expression of cytokine-induced adhesion molecules on EC is a relatively late event in the inflammatory response. As the first step in a typical DTH response, T-cells adhere to normal endothelium and subsequently transmigrate into the tissues, possibly in a random rather than antigen-specific fashion (Wekerle, Linstead et al., 1986; Mackay and Imhof, 1993). Thus, the experiments described here do not explore the initial phase of inflammation where the extravasation of T cells during such "surveillance" activity usually does not involve interaction with cytokine-induced adhesion molecules on the endothelium. It is conceivable that venular, rather than arterial, EC favour the initial extravasation of T cells into tissues and that this results in the subsequent entry of leukocytes into inflammatory sites being localised to the venular side of the vasculature.
6.4 Summary

During inflammation, EC play a central role in the expression of adhesion molecules and in the preparation of subendothelial BM for invasion by leukocytes. Previous studies have clearly demonstrated that the majority of adhering and extravasating leukocytes in inflammatory responses are found in postcapillary venules, and it is possible that this is due to differences in the EC from the different vascular beds. In order to test this hypothesis, venular and arterial EC were examined for their expression of adhesion molecules, and their ability to degrade or reorganise the subendothelial BM in response to pro-inflammatory cytokines.

In this study, the cytokine induced expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1 was shown to be similar between early passage venular and arterial EC. However, a number of striking differences between venular and arterial EC were demonstrated. First, arterial EC expressed significant constitutive levels of VCAM-1 whereas venular EC expressed low constitutive levels of VCAM-1. Second, TNF and IL-1 were virtually unable to induce adhesion molecule expression on late passage arterial EC whereas similar passage venular EC were only slightly reduced in their cytokine responsiveness. Third, analysis of ECM degradation products revealed substantial heparanase activity in venular EC, an enzyme activity lacking in arterial EC under a number of activation conditions. Fourth, there were marked differences in the ability of different stimuli to induce ECM degradation by the two types of EC. Thus, PMA substantially enhanced ECM degradation by venular EC but had no effect on arterial EC degradation. Furthermore, TNF and IL-1 induced an increase in the release of high mol. wt. proteoglycans from the ECM by venular EC but had virtually no effect on the release of degradation products by arterial EC despite these same cytokines inducing adhesion molecule expression on both EC types. Conversely, IFNγ selectively upregulated arterial EC sulfatase expression whilst inhibiting endogenous venular EC sulfatase activity.

These data provide evidence for EC heterogeneity between different vascular beds. Further, collectively, the adhesion molecule and ECM degradation data support the notion that margination and subsequent extravasation of leukocytes probably depends on a number of contributing processes, and not entirely on increased adhesive interactions mediated by adhesion molecules on endothelium.
Chapter 7

General discussion

A number of groups have demonstrated the potent anti-inflammatory activity of the SPS, heparin, and have suggested that this activity is due to inhibition of the endoglycosidase, heparanase. Following evidence that heparanase and a number of other BM-degrading enzymes were of lysosomal origin (Kaiser, 1980; Nakajima et al., 1983), and that lysosomal enzymes can be expressed on the cell surface via MPR (von Figura and Voss, 1979; Fischer, 1980), (reviewed by Kornfeld, 1987; Kornfeld, 1990), Parish and co-workers put forward the hypothesis that leukocyte extravasation is dependent upon the cell surface expression, and not merely the secretion, of lysosomal enzymes. They further hypothesised that M6P, a potent inhibitor of the lysosomal enzyme-MPR interaction, would displace lysosomal enzymes from the cell surface, thus preventing leukocyte extravasation. Willenborg and co-workers subsequently showed that the phosphosugar, M6P, and castanospermine (CS), an inhibitor of N-linked oligosaccharide processing, are both effective inhibitors of EAE and adjuvant induced arthritis (Willenborg and Parish, 1989; Willenborg et al., 1989; Willenborg et al., 1992). The major aim of this thesis was to test the hypothesis, proposed by Parish et al (1990), that the anti-inflammatory effects of CS and M6P are due to their ability to displace lysosomal enzymes from the leukocyte cell surface, thus preventing degradation of the subendothelial BM and ultimately impeding the ability of leukocytes to transmigrate into tissues.

7.1 Inhibition of inflammation by SPS, M6P and CS

Chapter 3 describes an initial study conducted to confirm the previous findings that SPS, M6P, and CS are effective inhibitors of inflammation. Keeping in mind the working hypothesis that these compounds inhibit leukocyte transmigration, the models used in this study were chosen on the basis of their ability to provide data on the level of infiltration of leukocytes, so that comparisons between control and treated animals could be made. In general, these experiments confirmed previous findings with these compounds in that some anti-inflammatory effects were observed. For example, CS, M6P and fucoidan inhibited leukocyte infiltration into rejecting thyroid allografts, with approximately 50% MNC infiltration into grafts of the treated animals compared to around 75% MNC infiltration in the control mice. Clearly, the rejection response in this model was extremely vigorous. It is important, therefore, to note that while the effects
of the three compounds did not appear dramatic, even the potent immunosuppressant, CsA, was only able to partially inhibit MNC infiltration into the rejecting grafts.

Subtle differences were also observed between the action of M6P, fucoidan and CS on the influx of leukocytes into the peritoneal cavity following the administration of an inflammatory stimulus. For example, during early stages in the thioglycollate induced model of peritoneal inflammation, neutrophils were the preponderant infiltrating cell type. M6P could inhibit these exudates in the early stages, but could not inhibit leukocyte infiltration in the MNC dominated DTH model. Further, cytocentrifuge smears of the thioglycollate induced exudates revealed that M6P and fucoidan acted primarily by reducing the relative numbers of neutrophils, their effects being more pronounced early, and less pronounced at later time points when a higher proportion of cells in the exudate were MNC. Taken together, these observations raised the possibility that lymphocytes and macrophages have a different mode of extravasating compared to neutrophils. Some of the differences may concern the participation of lymphocytes in immune surveillance, wherein they move into tissues via specialised sites in the vasculature.

Collectively, the results of chapter 3 highlight the highly selective nature of the inhibition of inflammation by these compounds, particularly M6P and CS. The data also suggest that CS, M6P and the SPS fucoidan, while exhibiting subtle differences in their anti-inflammatory activity, probably inhibit inflammation at the level of leukocyte extravasation.

7.2 Comparison of BM degrading potential of the major cells involved in inflammation

The in vivo data of chapter 3 supported the proposal that M6P, SPS and CS inhibit inflammation, and showed that they could generally reduce the numbers of leukocytes in inflammatory foci. However, these studies were unable to provide direct evidence of the effect of these compounds on BM-degrading enzymes expressed by the extravasating leukocytes. The major aim of chapters 4 and 5 was to address this issue.

A number of separate studies have already revealed that leukocytes possess ECM degrading capabilities but a single study comparing the relative potential of each cell type to degrade the ECM has not previously been reported. Thus, before the anti-inflammatory compounds were tested for
their effects on BM degradation, a preliminary study was conducted to investigate the comparative potential of the major cells involved in inflammation, both resting and activated, to degrade the subendothelial BM. These experiments confirmed that leukocytes, ECs and platelets have the capacity to degrade the subendothelial BM \textit{in vitro}. Further, the results of this study provided evidence that both nonstimulated and PMA stimulated HUVEC and platelets possess the greatest potential for solubilizing the ECM, with levels of degradation 2-5 fold higher than that of the leukocytes tested. These results were significant because they indicated that in spite of the emphasis placed on leukocytes as the sole participants in the penetration of BM during inflammation, ECs themselves may play an important role. These results also suggest that while the novel inhibitors studied in this thesis were assumed to be acting primarily on extravasating leukocytes, their effects on the endothelium may also be relevant.

Leukocytes were also shown in chapter 4 to display great heterogeneity in the spectrum of degradative enzymes that they expressed. For example, lymphocytes and platelets expressed mainly heparanase activity, while activated neutrophils expressed predominantly protease activity. In contrast, monocytes and the monocytic cell line U937 expressed only sulfatase activity in significant amounts while HUVECs, whether stimulated or nonstimulated, expressed high levels of all three major enzymes. These \textit{in vitro} observations may partly explain the subtle differences observed in the anti-inflammatory effects of M6P, fucoidan and CS \textit{in vivo} described in chapter 3. Since there is evidence that ECs are involved in BM degradation, the heterogeneity of degradative enzyme expression by leukocytes could also suggest differences in the dependence of each leukocyte type on ECs during the extravasation process. Alternatively, even though the \textit{in vitro} experiments of chapter 4 indicated that leukocytes preferentially express different enzyme activities, it is possible that given the appropriate stimulus or conditions, all of the leukocyte cell types could express the entire repertoire of hydrolytic enzymes. For example, while neutrophils were shown in this study to express mainly protease activity, with very little heparanase activity, other studies showed that neutrophil cell lysates, and neutrophils under certain pH conditions, expressed active heparanase activity (Matzner et al., 1985; Matzner et al., 1992).
7.3 Detection of a novel sulfatase in ECs and monocytes

The comparatively high levels of released radioactivity resulting from the interaction of ECs with labeled ECM, shown in chapter 4, provides evidence for the proposal that ECs participate directly in the process of leukocyte extravasation by preparing their BM for invasion. As the cells forming the luminal vascular surface, it is logical that EC may play a pivotal role in the outcome of any inflammatory event. Thus, the finding that ECs degrade the ECM in response to inflammatory stimuli is, perhaps, not surprising. However, an important finding reported in this thesis was that ECs, as well as monocytes, express a novel sulfatase. The N-and O-sulfated residues of HSPGs confer a highly anionic character to the HS side chains of the BM, this anionic charge being important in controlling the entry and diffusion of macromolecules and in stabilising interactions between different components of the BM (Sewell, 1989; Yurchenco and Schittny, 1990). Sulfatase enzymic activity could, therefore, reduce the negative charge barrier of the BM and thus allow greater physical interaction between leukocytes and the ECM, further increasing the accessibility of BM components to degradation by hydrolytic enzymes. Indeed, it is conceivable that the action of a sulfatase may so alter BM characteristics as to render it penetrable by invading leukocytes without further need for degradation by heparanases and proteases. The fact that sulfatase activity is the predominant enzymic activity expressed by chemokine stimulated monocytes supports this view. Furthermore, the expression of a sulfatase by ECs supports the hypothesis that ECs play an important role in preparing their ECM for leukocyte extravasation. In further support of this hypothesis, the EC sulfatase was found to be upregulated by inflammatory cytokines, and also expressed by activated EC monolayers, which are probably more representative of their in vivo counterparts than EC suspensions.

7.4 The effects of heparin, M6P and CS on BM degradation in vitro

According to the hypothesis of Parish and co-workers, the cell-surface expression of hydrolytic enzymes is a critical factor in determining the ability of leukocytes to breach the BM and transmigrate into tissues. In their model they proposed that M6P, which is a potent inhibitor of the lysosomal enzyme-MPR interaction, displaces lysosomal enzymes from the cell surface, whereas CS prevents the correct formation of the M6P marker, thus preventing binding of lysosomal enzymes to the MPR. In contrast, there is evidence that heparin, a non-cleavable substrate for heparanases, inhibits the enzyme competitively. These compounds have been shown to
inhibit inflammation, and prolong allograft survival, but the only direct evidence for the cell-surface expression hypothesis of Parish et al was in the histology of CNS lesions of EAE rats, where CS appeared not to be affecting leukocyte-EC adhesion and transmigration events, but appeared to be inhibiting the subsequent passage of monocytes through the subendothelial BM.

The aim of the study presented in chapter 5 was to test the hypothesis that these inhibitors prevent degradation of the ECM by the cellular components of inflammation. Using 35SO4-labeled ECMs, the major cells involved in inflammation were tested for their ability to degrade ECM in the presence of heparin, M6P and CS. In general support of the Parish hypothesis, all of the anti-inflammatory compounds analysed in this study inhibited 35SO4-labeled ECM degradation.

Heparin inhibited the heparanase activity of all cell types examined, but M6P and CS were shown to be cell-type specific in their effects. The cell specific effects of M6P and CS in inhibiting BM degradation might support the general finding (chapter 4) that different cells express different profiles of degradative enzymes. However, some of the results of this chapter are in apparent contradiction with the in vivo results of chapter 3. For example, M6P was shown to be effective at inhibiting the infiltration of neutrophils into inflammatory sites. This in vivo action, however, did not translate to its effects in vitro, where M6P was unable to inhibit BM degradation by neutrophils. Moreover, M6P was able to inhibit lymphocyte heparanase in vitro, but was unable to inhibit inflammation in the DTH model described in chapter 3, although it should be noted that M6P appears to very effectively inhibit MNC mediated inflammation in EAE and adjuvant induced arthritis (Willenberg and Parish, 1989; Willenberg, Parish et al., 1992). It is possible that the mesentery vascular bed of the peritoneal cavity is not fully representative of normal tissue vasculature. Conceivably, lymphocytes participate in immune surveillance of the peritoneum and may move into the peritoneal cavity via specialised sites in the vascular bed of the mesentery. This type of transmigration may not require BM degradation and hence the cell-surface expression of lysosomal enzymes. However, during the acute early phase of inflammation, neutrophil extravasation into the peritoneal cavity may require the expression of degradative enzymes. Under these conditions, neutrophils would therefore be more sensitive to compounds which inhibit the cell-surface expression of degradative enzymes.
Interestingly, heparin was found to inhibit not only heparanase, but also the sulfatase, and to a lesser extent, the protease activity of EC. The results presented in this thesis suggest that sulfatase activity may play an important role in leukocyte extravasation, either as an initiating step in the BM degradative enzyme cascade, or as an alternative means of rearranging the BM in preparation for invasion by leukocytes. In the past it has been assumed that the anti-inflammatory effects of heparin were mainly due to its competitive inhibition of leukocyte heparanase. The findings of this thesis suggest that EC sulfatase inhibition cannot be ruled out as a possible mode of action of heparin in inhibiting inflammation. The effect of heparin on the cytokine-induced sulfatase activity of monocytes is yet to be tested.

7.5 Comparison of venular and arterial EC
The study presented in chapter 6 addressed the proposition that the predilection of leukocytes for the postcapillary venule as the site for margination and extravasation in inflammatory sites may be due to vascular bed heterogeneity. Differences between arterial and venular ECs which might influence leukocyte extravasation were proposed to be in the expression of adhesion molecules, and the capacity to express BM-degrading enzymes, particularly following exposure to pro-inflammatory cytokines.

Significant differences between arterial and venular ECs were demonstrated both in their expression of adhesion molecules and in their capacity to degrade the BM. For example, arterial ECs expressed significantly higher constitutive levels of VCAM-1 than venular ECs and TNF and IL-1, which were able to induce adhesion molecule expression on late passage venular ECs were unable to induce such expression in late passage arterial ECs. While the latter observation may be purely an in vitro phenomenon, nevertheless it suggests that the two cell types are inherently different in their response to certain stimuli and this may have some physiological significance in an inflammatory environment.

Further differences between the two cell types concerned their ability to degrade the BM. Analysis of ECM degradation products revealed substantial heparanase activity in venular ECs, an enzyme activity lacking in arterial EC under a number of activation conditions. Moreover, there were marked differences in the ability of different stimuli to induce ECM degradation by the two types of EC. Thus, PMA substantially enhanced ECM degradation by venular ECs but had no effect on arterial EC
degradation. Furthermore, TNFα and IL-1 induced an increase in the release of high mol. wt. proteoglycans from the ECM by venular EC but had virtually no effect on the release of degradation products by arterial ECs despite these same cytokines inducing adhesion molecule expression on both EC types. Conversely, IFNγ selectively upregulated arterial ECs sulfatase expression whilst inhibiting endogenous venular EC sulfatase activity.

These data provided evidence for EC heterogeneity between different vascular beds and support the notion that margination and subsequent extravasation of leukocytes probably depends on a number of contributing processes, and not entirely on increased adhesive interactions mediated by adhesion molecules on endothelium. For example, blood shear forces can still be regarded as having a fundamentally important influence on whether or not margination, the first step of extravasation, takes place. Nevertheless, taken together, the results of chapter 5 demonstrating the effects of heparin and CS on venular ECs, and the results of chapter 6, which highlight marked differences between venular and arterial ECs, strongly suggest that the endothelium may be an important target for anti-inflammatory strategies.

7.6 Future work

Studies presented in this thesis provide evidence that different cell types can preferentially express different hydrolytic enzymes and that the induced expression of these enzymes may vary depending upon the stimulus. Thus, an important unanswered question is the nature of the cytokines or other factors which control the expression of these enzymes during inflammation. It seems likely that the chemokines may emerge as important activating factors for leukocyte, and perhaps EC, degradative activity. MCAF, for example, was shown to selectively induce monocyte sulfatase activity, and one of the first questions to be answered is, for example, whether M6P, SPS or CS can inhibit MCAF-induced sulfatase expression. IL-8 was shown to have a relatively modest effect on the expression of hydrolytic enzymes by neutrophils, but there are many other chemokines that have not been examined in this system which may prove to be more potent in their induction of hydrolytic enzyme expression during chemotaxis. For example, a recently identified chemotactic cytokine, CP-10, was found to be an extremely potent chemoattractant for neutrophils, with maximal activity at 10^{-13}M (Lackmann et al., 1992). As the differential
effects of cytokines are further characterised, more insights may be gained as to the mode of action of M6P, SPS or CS in inhibiting inflammation.

Another area worthy of further study is the role of cell-cell contact in "triggering" the expression of degradative enzymes by leukocytes and endothelium. For example, as well as expressing selectins responsible for the initial capture and subsequent rolling of leukocytes along the endothelium, ECs participate in contact phenomena via CD31 and the selectins which result in the activation of integrins on the leukocyte surface. It is quite feasible that such contact phenomena may also upregulate the expression of hydrolytic enzymes by leukocytes, which could facilitate the release of adhesive interactions and enable the transmigration of leukocytes through the subendothelial BM. Alternatively, leukocyte-EC adhesive events may trigger the expression of hydrolytic or sulfatase enzymes by ECs.

Finally, while many of the proteases expressed by leukocyte and tumour cells have been well characterised, the heparanases and sulfatases have been far less studied. Further study of the sulfatase and heparanase enzymes, should be a fruitful area of research, but will require the development of better assays for the enzymes prior to their isolation and characterisation. It will be particularly interesting to examine the effects of sulfatase activity on the integrity of BM.

In conclusion, leukocytes, endothelium and the subendothelial BM are intimately involved in the inflammatory response, and any insights that can be gained into these cell-cell and cell-ECM interactions will be of considerable importance in providing an understanding of a number of clinical disorders including autoimmune pathologies. Clearly, a great deal of work remains to be done in defining the factors that regulate leukocyte extravasation through endothelium and the subendothelial BM. In particular, clarification of the mechanisms used by leukocytes and ECs to degrade the subendothelial BM, which represents a major barrier to leukocyte extravasation, may lead to exploitation of this vulnerable point of inflammation in the development of more effective and specific anti-inflammatory drugs.
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