THE EFFECTS OF MYELOID GROWTH FACTORS ON PHAGOCYTE-ENDOTHELIUM INTERACTIONS

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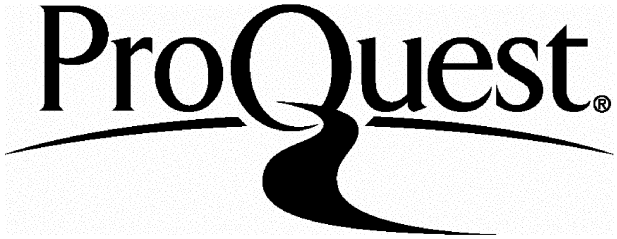

to

my husband and children

and to

my grandfather

ABSTRACT

The myeloid growth factors, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were initially defined by their effects on the proliferation and differentiation of haemopoietic progenitor cells, but are now recognised to play an important part in enhancing the functions of mature effector cells. This thesis explores and defines the effect of these growth factors on the adhesive properties of phagocytic cells, with particular emphasis on the interaction of neutrophils with endothelium, and the role of the different leucocyte adhesion molecules. GM-CSF and G-CSF produced upregulation of phagocyte surface CD11b/CD18 both *in vitro* and *in vivo*, but induced shedding of the L-selectin receptor on neutrophils only *in vitro*. *In vitro*, GM-CSF enhanced the adhesion of neutrophils to cultured human umbilical vein endothelial cells, while G-CSF was without effect. GM-CSF also demonstrated greater proadhesive effects *in vivo*: Systemic administration of GM-CSF led to a rapid neutropenia, with a much slower recovery of peripheral cell counts than was seen with G-CSF. Histological analysis using a non-human primate (*cynomolgus*) model showed that GM-CSF increased neutrophil adhesion to pulmonary vascular endothelium *in vivo*. *In vitro*, GM-CSF had differential effects on the transendothelial migration of neutrophils, depending on the conditions of endothelial activation. Neutrophils stimulated with GM-CSF showed enhanced migration across unstimulated endothelium, but impaired migration across cytokine activated endothelium.

Monoclonal antibodies to CD11b and to CD18 did not inhibit GM-CSF induced neutrophil margination *in vivo*; and a patient with a congenital deficiency of the CD11/CD18 receptors (leucocyte adhesion deficiency) demonstrated a normal margination response to GM-CSF, confirming that the CD11/CD18 receptor plays a minimal role in GM-CSF induced adhesion *in vivo*. In spite of this, the same antibodies blocked GM-CSF induced neutrophil adhesion and transendothelial migration *in vitro*. Neutrophil migration *in vitro* was not blocked by monoclonal antibodies to L-selectin, and migrated cells expressed lower levels of L-selectin than nonmigrated cells.

Finally, the possible direct effect of these growth factors on endothelial cells was studied. *In vitro*, GM-CSF and G-CSF had no effect on the proliferation of, or the

expression of procoagulant or fibrinolytic activites by, cultured human endothelial cells. However, systemic administration of GM-CSF produced a rise in plasma tissue plasminogen activator activity *in vivo*, an effect which may relate to the enhanced neutrophil adhesion to endothelium. These studies emphasize the role of the myeloid growth factors, in particular GM-CSF, as inflammatory mediators which regulate the immune responses of phagocytic cells, including the cellular adhesive interactions which underly phagocyte recruitment into inflammatory areas.

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CHAPTER ONE

INTRODUCTION

1.1. HAEMOPOIETIC GROWTH FACTORS

Overview of haemopoietic growth factors

The human haemopoietic growth factors (HGFs) can be divided into erythropoietin, the colony-stimulating factors (CSFs), and the interleukins (Table 1). The CSFs were defined by their effect on the clonal proliferation of haemopoietic progenitor cells in semi-solid culture *in vitro* (Metcalf, 1986a), while, in general, the interleukins (IL) were defined by their *in vitro* effects on T or B cell function/proliferation in liquid systems (Paul, 1989). Interleukin-3 (IL-3) is best regarded as a CSF, as reflected in its other name, multi-CSF (Sonoda et al, 1988a). Apart from the interleukins, other multifunctional cytokines such as transforming growth factor-beta (TGF-β) and tumour necrosis factor-alpha (TNF-α) have also been shown to be active in haemopoiesis (Haworth, 1989). Although the CSFs were initially defined as affecting specific lineages, it has become clear that lineage specificity is an exception, perhaps applicable only to erythropoietin which acts on the erythroid pathway (and possibly on megakaryocytes), and M-CSF, acting on the macrophage pathway (Browne et al, 1986, Becker et al, 1987). Most of the other HGFs display multiple cross lineage effects. An example is G-CSF, which, apart from acting on cells of the neutrophil lineage, also synergises with other CSFs to act on early stem cells and megakaryocytes (Ikebuchi et al, 1988, Takaue et al, 1990). The cellular sources of these HGFs are a wide range of cell types, including endothelial cells, monocyte-macrophages, T cells, fibroblasts, osteoblasts, mesangial cells, polymorphonuclear leucocytes, mesothelial cells and thymic epithelial cells (Metcalf, 1986).

Recently, a number of HGF receptors have been defined, and a haemopoietic receptor superfamily has been characterised (Bazan, 1990). This family of receptors includes the receptors for erythropoietin, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF as well as those for prolactin and growth hormone. Several members of this family, including IL-2, IL-6, GM-CSF, and IL-3, share a common theme in which the low affinity form of the receptor exists as an α subunit, while the high affinity receptor requires the coexpression of both α and β subunits (Nicola & Metcalf, 1991). It has been postulated that ligand binding of the α chain induces the association with the β subunit, hence converting the receptor from a low to a high affinity form.

TABLE 1.1. Haemopoietic growth factors

FACTOR	MAJOR TARGET CELL LINEAGE/ACTION
Erythropoietin	Erythroid progenitors
IL-3 (multi-CSF)	Granulocytes, monocytes, megakaryocytes, erythrocytes, early stem cells
GM-CSF	Granulocytes, monocytes, megakaryocytes, erythrocytes
G-CSF	Neutrophilic granulocytes
M-CSF (CSF-1)	Monocytes
Steel factor (c-kit ligand)	Synergises with multiple factors to stimulate proliferation and differentiation in many lineages, pre-B cells
IL-1	Fibroblasts, stem cells
IL-2	T cells, activated B cells
IL-4	B and T cells, myeloid co-factor
IL-5	B cells, eosinophils
IL-6	B cells, megakaryocytes, granulocytes, monocytes, early stem cells
IL-7	Pre B and T cells, megakaryocytes
IL-9	Helper T cells and erythroid progenitors
IL-11	B cells, megakaryocytes, mast cells

Several receptors, such as GM-CSF, IL-3 and IL-5, appear to share, and hence to compete for, a common β chain (Kitamura et al, 1991). Other growth factor receptors, such as the receptors for 'Steel factor' (stem cell factor), and that for M-CSF belong to the receptor tyrosine kinase family (Ulrich & Schlesinger, 1990).

In vitro actions of CSFs on primitive cells

IL-3 is a multilineage growth factor which supports the *in vitro* growth and differentiation of multilineage colonies (, CFU-GEMM), granulocyte-macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), megakaryocyte (CFU-Meg), eosinophil (CFU-Eo), basophil (CFU-Baso), and, in the presence of erythropoietin, both early and late (BFU-E and CFU-E) erythroid stem cells (Sieff, 1987, Sonoda et al 1988a, b, Emerson et al, 1988). GM-CSF acts not only on CFU-GM and CFU-Eo, but also CFU-GEMM, BFU-E and CFU-Meg, thus displaying some overlap in activity with IL-3 (Metcalf et al, 1986b, Emerson et al, 1988, Aglietta et al, 1991). As mentioned above, G-CSF acts on committed neutrophil precursors (Metcalf & Nicola, 1983, Metcalf, 1989, Souza et al, 1986), but can synergise with several other factors, including IL-3, IL-6, L-1, and stem cell factor to mediate effects on more primitive progenitor cells (Ikebuchi et al 1988, Ogawa, 1989, McNiece et al,1991). M-CSF (also called CSF-1) acts on progenitor and precursor cells of the macrophage lineage (Becker et al, 1987). A recent important development has been the molecular cloning of the c-kit ligand, also termed mast cell growth factor and stem cell factor (Zsebo et al, 1990). The c-kit ligand has relatively little effect *in vitro* when present on its own, but synergises with several growth factors, including IL-3, M-CSF, GM-CSF and G-CSF, to stimulate early haemopoietic progenitors, and with IL-7 to stimulate pre-B cell production (McNiece et al 1991).

An important feature of the action of the CSFs is that many display synergistic, or additive interactions with one another, as seen in studies on erythroid stem cells (BFU-E), high proliferative potential colony-forming cells (HPP-CFC) or CFU-blast (Robinson & Quesenberry, 1990, Leary et al, 1988). The relative concentrations of synergistic factors present may also dictate which cell lineage is stimulated, or whether stem cells receive a proliferation or a differentiation signal (Dexter, 1992, and references therein). In a similar fashion, multifunctional cytokines such as IL-1 and IL-6 can act on progenitor cells, to render them more sensitive to the later acting factors (Bagby, 1989, Leary et al, 1988), and IL-4 enhances the effects of G-CSF or erythropoietin on progenitor cells and also has effects on megakaryocytic precursors (Peschel et al, 1987).

Actions of CSFs on mature cell function

Just as cytokines initially named for their effects on differentiated cells are now recognised to act also on more primitive cells at various stages in the differentiation pathway, the actions of the CSFs on mature cell function have been recently characterised. *In vitro*, GM-CSF increases neutrophil survival, and stimulates phagocytosis, degranulation, antimicrobial cytotoxicity and antibody dependent cellular cytotoxicity (ADCC) (Vadas et al, 1983, Gasson et al, 1984, Lopez et al, 1986, Fleishman et al, 1986) of neutrophils. GM-CSF also augments neutrophil superoxide production in response to physiological chemoattractants such as the bacterial peptide formyl-methionyl-leucyl-phenylalanine (FMLP), complement component C5a, and leukotriene B_4 (Weisbart et al, 1987), and enhances neutrophil production of leukotriene B_4 and platelet activating factor (PAF) (Wirthmueller et al, 1989, DiPersio et al, 1990). GM-CSF modulates neutrophil surface adhesion receptors (Arnaout et al, 1986, Devereux et al, 1989, Yong et al, 1992, Griffin et al, 1991), enhances homotypic cell adhesion (Arnaout et al, 1986), and, in some systems, is chemotactic for neutrophils (Wang et al, 1987). GM-CSF is also able to induce the expression of a growing list of genes, including *c-fos*, and the genes for IL-1, G-CSF, CSF-1 and TNF-α (Colotta et al, 1987, Lindemann et al, 1989, Wing et al, 1989). In addition to its actions on neutrophils, GM-CSF enhances eosinophil survival and functions such as cytotoxicity and leukotriene release (Silberstein et al, 1986), increases basophil histamine release (Lopez et al, 1986) and augments antigen presentation by mononuclear phagocytes (Morrisey et al, 1987). G-CSF acts mainly on neutrophils, enhancing phagocytosis and ADCC , causing priming of the respiratory burst, and upregulation of adhesion receptors, as well as acting as a chemoattractant (Avalos et al,1990, Yuo et al, 1990, Kitagawa et al, 1987, Wang et al, 1988). In exerting their actions on mature cells, these growth factors display synergism with one another, and with other inflammatory cytokines such as TNF-α and IL-8 (Khwaja et al, 1992a, Yuo et al, 1991), in a manner analogous to their actions on haemopoietic progenitor cells. M-CSF supports macrophage survival, and functions such as migration, superoxide production, anti-tumour and antimicrobial cytotoxic activities, cytokine release from monocyte and macrophages (Wing et al, 1985, Warren & Ralph, 1986, Wang et al, 1988, 1989, Ralph & Nakoinz, 1987, Karbassi et al, 1987, Rettenmier & Sherr, 1989). IL-3 enhances monocyte cytotoxicity, primes monocyte superoxide release in response to FMLP, and augments survival and functions of eosinophils (Cannistra et al, 1988, Rothenberg et al, 1988, Yuo et al, 1992).

These effects suggest that, quite apart from their role in haemopoiesis, the HGFs also function as proinflammatory mediators, which, together with other cytokines such as TNF (Klebanoff et al, 1986) and interleukin-8 (IL-8) (Baggiolini et al, 1989, Yuo et al, 1991) modulate the immune and inflammatory responses of phagocytic cells. The work in this thesis is concerned with the actions of these growth factors on the adhesion-dependent inflammatory responses of phagocytic cells, with particular emphasis on the interaction with vascular endothelium.

In vivo effects of CSFs in humans

Phase 1 clinical studies with rhGM-CSF demonstrated a dose dependent increase in circulating neutrophils, monocytes, eosinophils, and occasionally, lymphocytes (Lieschke et al, 1989b, Phillips et al, 1989). This was accompanied by an increase in bone marrow cellularity (Liesche et al 1989b, Aglietta et al, 1989), and in the number of circulating CFU-GM (Socinski et al, 1988). Leucocyte numbers returned to normal within a few days of stopping GM-CSF therapy. An increase in reticulocyte numbers was only seen in one study (Lieschke et al, 1989b) and no increase in platelet numbers was observed. RhG-CSF produces a selective increase, mainly in neutrophil numbers, although there has been one report of an increase in monocyte counts (Cohen et al 1987, Gabrilove & Jakubowski, 1989, Lindemann et al, 1989). Like GM-CSF, G-CSF also causes the release of early neutrophil precursors from the marrow (Durhesen et al, 1988). These "peripheral blood stem cells" may have potential in bone marrow reconstitution after high dose chemotherapy. With prolonged administration, G-CSF causes haemopoietic expansion in new marrow spaces as well as in the spleen (Gabrilove et al 1989, Morstyn et al, 1988). Purified urinary M-CSF has been shown to produce a mild neutrophilia in some studies, but similar effects have not been seen with the recombinant product (Komiyama et al, 1988, Nemunatitis et al, 1991b). In one trial of urinary CSF-1 in patients with lymphoma undergoing high dose chemotherapy and ABMT, there was possibly a beneficial effect on platelet recovery when the dose of mononuclear cells reinfused was limiting (Khwaja et al, 1992b). In one phase I study of IL-3 reported (Ganser et al, 1990b), there was a much slower rise in peripheral leucocyte numbers (as compared with G- or GM-CSF), and peak levels were modest compared to GM-CSF or G-CSF. All leucocyte subtypes were elevated, including basophils, and platelet numbers were also increased. Bone marrow cellularity was increased.

Several effects of the CSFs on mature cell function have also been demonstrated *in vivo*. Neutrophils from patients treated with G-CSF show an enhanced oxidative burst in response to FMLP, upregulation of CD11b/CD18 surface receptors (Ohsaka et al, 1989, Yuo et al, 1989), and appear to function normally in terms of *in vivo*

migration to sites of inflammation (Welte et al, 1987). GM-CSF also enhances neutrophil functions *in vivo*, causing priming of the respiratory burst, secondary granule release, upregulation of CD11b/CD18 (Socinski et al, 1989, Devereux et al, 1989, 1990, Sullivan et al, 1989, Jaswon et al, 1990,) but has been shown to inhibit the movement of neutrophils into skin windows (Peters et al, 1988, Addison et al, 1989). In contrast, M-CSF increases monocyte movement into skin windows, without any concomitant rise in peripheral monocyte numbers (Khwaja et al, 1991). Both GM-CSF and G-CSF produce an immediate but transient fall in circulating phagocyte numbers (Devereux et al, 1989, Lindemann et al, 1989, Morstyn et al, 1988), and radiolabelling studies suggest that the neutrophils are transiently sequestered in the lungs (Devereux et al, 1989). These margination responses to GM-CSF and G-CSF *in vivo* may be related to the increased expression of adhesion receptors on circulating cells. Thrombocytopenia has been reported in one study using rhM-CSF (Nemunaitis et al, 1991b), and in another using hu-CSF-1 (Khwaja et al, 1991). This effect may relate to the induction of Fc receptors on monocytes (Magee et al, 1987), and increased immune-mediated clearance.

Clinical applications and potential of the CSFs

The clinical potential of the CSFs can be broadly divided into four main areas. Firstly, early trials with G-CSF and GM-CSF were undertaken to explore their potential to accelerate bone marrow recovery following myeloablative chemotherapy, with or without bone marrow transplantation (BMT). In a randomized, double-blind placebo-controlled phase III study of patients with lung cancer receiving chemotherapy, G-CSF administration led to significant reductions in the duration of neutropenia, incidence of fever, antibiotic requirements and length of hospitalisation (Crawford et al, 1991). On the strength of this study, G-CSF obtained approval by the Food and Drug Administration (FDA) in the USA in early 1991. G-CSF has since been licensed for use in the UK, and has been shown to accelerate haemopoietic recovery in patients receiving high dose chemotherapy and bone marrow transplantation (Sheridan et al, 1989, Gabrilove et al, 1989). GM-CSF has also been shown to accelerate myeloid recovery after high dose chemotherapy and autologous marrow transplants (Brandt et al, 1988), and, in a recent, randomized, double-blinded, placebo-controlled Phase III trial, was shown to reduce infection rates, days of antibiotic administration and hospitalisation in patients with lymphoid malignancies undergoing ABMT (Nemunaitis et al, 1991a). On the basis of this trial, GM-CSF has also received FDA approval for use in BMT.

Secondly, the clinical potential of the CSFs to stimulate neutrophil production in patients with bone marrow failure has also been explored. Patients with aplastic anaemia (unresponsive to conventional chemotherapy), congenital neutropenia,

cyclic neutropenia, graft failure after BMT, and acquired immunodeficiency syndrome (AIDS), were investigated. In aplastic anaemia, improvements in haematological function tended to be seen in patients with higher pretreatment counts and greater residual myelopoiesis (Antin et al, 1988, Nissen et al, 1988, Vadhan-Raj et al, 1988, Ganser et al, 1990, Kojima et al, 1991). Similarly, in graft failure following BMT, sustained granulocyte recovery was seen in patients who had higher residual bone marrow cellularity prior to treatment (Nemunaitis et al, 1990). In a randomized phase III trial, G-CSF has been shown to be effective in inducing mature granulocyte production in patients with congenital neutropenia, cyclic neutropenia, and chronic idiopathic neutropenia (Bonilla et al, 1989, Hammond et al, 1989). These conditions appear to be rather less responsive to GM-CSF (Vadhan-Raj et al, 1990, Welte et al, 1990). Both GM-CSF and G-CSF have been used with some success in patients with AIDS, both in overcoming the primary bone marrow dysfunction, as well as in counteracting the myelosuppressive effect of azidothymidine (AZT) and ganciclovir (Groopman et al, 1987, Miles et al, 1991). Both GM-CSF and G-CSF have been used in patients with the myelodysplastic syndrome, and, in the majority of cases, are able to produce increases in neutrophil counts (Vadhan-Raj et al, 1987, Thompson et al, 1989, Antin et al, 1988, Ganser et al, 1989, Yoshida et al, 1991, Verhoff et al, 1991), and in some cases, a reduction in infection rates. In a phase I/II trial of IL-3 in secondary bone marrow failure, there were encouraging increases in leucocyte and platelet numbers (Ganser et al, 1990b), but no stimulation of thrombopoiesis was demonstrated in similar trials in patients with aplastic anaemia, or myelodysplasia (Ganser et al, 1989, 1990a). The risk of accelerating blast transformation in these patients remains unclear.

Thirdly, the effects of GM-CSF and G-CSF to increase circulating progenitors has led to an interest in the potential use of these growth factors in the harvest of peripheral blood stem cells for autotransplantation (Haas et al, 1990). Finally, the ability of the CSFs to enhance the functions of mature effector cells raises the possibility of their use to augment host defence mechanisms in patients with infections and/or malignancies. Studies in patients with HIV infection have demonstrated that GM-CSF and G-CSF are able to repair phagocyte defects in antimicrobial function (Baldwin et al, 1988, Riolides et al, 1990a,b). Similarly, G-CSF was able to partially correct the defect in the neutrophil respiratory burst (Yuo et al, 1987), and GM-CSF was found to improve antimicrobial capacity in patients with MDS, and to correct defective neutrophil function in patients after BMT (Zimmerli et al, 1989). A recent study of rhM-CSF in patients with invasive fungal infections following BMT has yielded encouraging results (Nemunaitis et al, 1991b). The possible use of rhM-CSF to enhance tumoricidal activity of monocytes/macrophages is being

assessed in a current trial in patients with metastatic melanoma (Bajorin et al, 1990).

Side effects of CSFs

Bone pain has been widely reported in trials of G- and GM-CSF, and appears to be most prominent in bones containing significant amounts of haemopoietic tissue, such as the sternum, lumbar spine and pelvis (Gabrilove et al, 1988, Lieschke et al, 1989, LIndemann et al, 1989, Barlogie et al, 1990). General lethargy, weakness, anorexia and fever are also commonly reported. In general, GM-CSF is more toxic than G-CSF, and dose-limiting toxicities have been reported for GM-CSF. At high doses of 20 - 64 μg/kg/day, features of the capillary leak syndrome have been observed with GM-CSF, including peripheral and pulmonary oedema, pericardial and pulmonary effusions and hypotension (Antman et al, 1988, Brandt et al, 1988, Steward et al, 1989, Lieschke et al, 1990a, Haas et al, 1990). Several groups have also reported venous thrombosis around central venous catheters (Antman et al, 1988, Nissen et al, 1988, Jost et al, 1990). Side effects of IL-3 include fever, chills, headaches, bone pains and facial flushing; the last may relate to histamine release from circulating basophils (Ganser et al, 1990a,b).

A specific syndrome of pulmonary dysfunction has been documented by Lieschke et al (1989a), related to the initial dose of GM-CSF. The clinical features include flushing, tachycardia, hypotension, musculoskeletal pain, dyspnoea, nausea and vomiting, rigors and syncope. These were associated with a fall in oxygen saturation and diffusion capacity, with a corresponding increase in alveolar-arterial oxygen gradients. These effects were more common after the first dose of GM-CSF, with intravenous rather than subcutaneous administration, and appeared to be dose related.

The observed clinical toxicity of these growth factors may be related to effects on mature cell function and thus provide insight into the ways in which the CSFs act to regulate the inflammatory responses of differentiated cells. Many of the side effects mentioned above may be related to monocyte production of TNF-α and IL-1 (Cannistra et al, 1990, Wing et al, 1989) in response to stimulation by GM-CSF. Increased expression of adhesion receptors on circulating phagocytes, resulting in a transient enhanced interaction with vascular endothelium in the lungs, may be responsible for the pulmonary dysfunction observed by Lieschke et al (1989a). The enhanced interaction of phagocytes with endothelium could affect endothelial cell function and integrity, and might account for the incidence of central venous thromboses, and the clinical features of a capillary leak syndrome noted at high doses

of GM-CSF. The lesser toxicity of G-CSF, as compared to GM-CSF, may be because its actions are mainly confined to neutrophils, and because it is, in general, a less effective activating agent than GM-CSF. The differences between these two growth factors in their range of actions, and proadhesive effects on neutrophils, and the implications for their different biological roles, will be discussed in later chapters.

Finally, decreased neutrophil migration into skin windows seen following GM-CSF administration (Peters et al, 1987, Addison et al, 1989) suggests that the presence of the growth factor in the circulation may impair the ability of circulating phagocytes to respond to appropriate signals from inflammatory areas. These observations raise the possibility that GM-CSF therapy might exacerbate deep seated infections, and hence systemically administered GM-CSF may not be appropriate treatment of severe infections in patients with normal neutrophil counts.
The specific effect of GM-CSF on the directed movement of neutrophils across endothelium is addressed in Chapter 8. The transendothelial movement of cells in response to signals from inflammatory sites is preceded by the initial attachment of circulating cells to vascular endothelium, and requires changes in surface adhesive properties, either of leucocytes, or endothelium, or both. Hence, the way in which growth factors alter the surface adhesive properties of phagocytes, and their adhesion to endothelium, forms the subject of the *in vitro* and *in vivo* studies presented in Chapters 3-5.

The adhesive responses of phagocytic cells are subserved by various cell surface adheison receptors. The way in which growth factors and other soluble mediators of inflammation modulate phagocyte-endothelial interactions is closely related to their effects on the surface expression and function of different adhesion receptors. The second part of this introduction will summarise what is known about the different leucocyte adhesion molecules which mediate the inflammatory and immmune response of these cells.

1.2. LEUCOCYTE CELLULAR ADHESION MOLECULES

Overview of leucocyte adhesion molecules

Leucocytes express adhesion promoting receptors which mediate cell-cell and cell-matrix interactions. These adhesive interactions are crucial to the regulation of haemopoiesis and thymocyte maturation, the direction and control of leucocyte traffic and migration through tissues, and in the development of immune and non-immune inflammatory responses. Several families of adhesion receptors have been identified

(Springer, 1990). The leucocyte integrin family (Table 1.2) (Arnaout , 1990a, Larson & Springer, 1990) comprises 3 αβ heterodimeric membrane glycoproteins which share a common β subunit, reactive with CD18 MoAbs. The α subunits of each of the 3 members, lymphocyte function associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1) and p150,95 are reactive with CD11a, b and c MoAbs respectively. Expression of the leucocyte integrins (also called β2 integrins) is restricted to leucocytes and haemopoietic precursor cells. CD11a is expressed on virtually all cells of the immune system except for some tissue macrophages (Krensky et al, 1983), while CD11b is found on granulocytes, monocytes, macrophages, large granular lymphocytes and some immature CD5+ve B cells (Miller et al, 1986, de la Hera et al, 1988). CD11c has a similar distribution to CD11b, but is also expressed on some cytotoxic T cells and activated lymphocytes, including cells from patients with hairy cell leukaemia (Keizer et al, 1987, Schwarting et al, 1985). Leucocyte integrin expression is linked to haemopoietic maturation. CD11a is upregulated during the differentiation of haemopoietic stem cells, while CD11b appears later, on committed granulocyte and monocyte precursors (Campana et al, 1986, Miller et al, 1986). CD11c expression is linked to monocytic differentiation, and high levels are found on tissue macrophages. Lymphocyte activation leads to increased surface expression of CD11a, together with other 'activation' markers such as CD2 and LFA-3 (Springer et al, 1987), while stimulation of neutrophils and monocytes leads to rapid upregulation of CD11b on cell surfaces (see below). These adhesion molecules play a critical part in the immune and inflammatory responses of leucocytes. The leucocyte integrin family is, in turn, part of the **Integrin Superfamily** (Hynes, 1987, 1992) members of which are evolutionally, structurally and functionally related. Each subfamily is identified by the β chain which is common to its members, and so the CD11/CD18 receptors, which share the β2 chain, are now more commonly termed the β2 integrins. Another Integrin subfamily found on leucocytes is the β1 integrin family (Hemler et al, 1987), also called the VLA group because the "very late antigens" VLA-1 and VLA-2 were originally found to appear late in T-cell activation. Members of this family function mainly as extracellular matrix adhesion receptors and are found both on haemopoietic and non-haemopoietic cells. They play a part in diverse cellular functions including tissue organisation, lymphocyte recirculation and T cell immune responses. A third integrin subfamily, the cytoadhesins or β3 integrin family (Ginsberg et al, 1988), are extracellular matrix protein receptors present on platelets and endothelial cells. At least five other β chains have been identified, some of which can associate with more than one α chain, and vice versa (Hynes, 1992).

A second family of adhesion receptors present on leucocytes is the **Immunoglobulin superfamily** (Williams & Barclay, 1988), members of which include CD2, lymphocyte function associated antigen-3 (LFA-3), and intercellular adhesion molecule-1 (ICAM-1), which participate in T cell adhesive interactions, and the antigen-specific receptors of T and B cells, CD4, CD8 and the MHC Class I and II molecules. Another member of this family is intercellular adhesion molecule-2 (ICAM-2, Staunton et al 1989) , which, together with ICAM-1 (Rothlein et al, 1986a), are endothelial cell surface ligands for the β2 integrin CD11a/CD18 (see later section). ICAM-1 is expressed on monocytes, tissue macrophages, memory T cells and germinal centre B cells, and its upregulation during cell activation suggests an inportant role in cellular immune responses, particularly in antigen presentation (Dustin et al, 1986, Dougherty et al, 1988, Altmann et al, 1989). Recently, a third ligand for CD11a, termed intercellular adhesion molecule-3 (ICAM-3) has been identified (Fougerolles & Springer, 1992); this is well expressed on all leucocytes, but can be further induced on lymphocytes following cell activation. A recently characterised family of adhesion receptors is the **selectin family,** a group of three integral membrane glycoproteins which share a common lectin domain. Leucocyte adhesion molecule-1 (LAM-1) also termed lectin adhesion molecule-1 (LECAM-1), or L-selectin is the human homologue of the murine homing receptor , MEL-14 (Tedder et al, 1989, Camerini et al, 1989), and is expressed on leucocytes, while endothelial leucocyte adhesion moleucle-1 (ELAM-1) or E-selectin, and granule membrane protein (GMP-140) or P-selectin are expressed on stimulated endothelial cells and activated platelets (Bevilacqua et al, 1989, Johnston et al, 1989).

Table 1.2. Families of Adhesion Receptors

	Functions known
Integrin Superfamily	
VLA antigens	
VLA-1	
VLA-2	Receptors for extracellular matrix proteins and cell surface receptors
VLA-3	VLA-4 is involved in adhesion to endothelium, and lymphocyte homing
VLA-4	
VLA-5	
VLA-6	
Leucocyte Integrins	
CD11a/18 (LFA-1)	adhesion strengthening in many immune and inflammatory leucocyte functions, leucocyte adhesion to endothelium and extravasation
CD11b/18 (Mac-1)	
CD11c/18 (p150,95)	
Cytoadhesions	
gpIIb/IIIa	mediates adhesive functions of platelets
vitronectin receptor	
Immunoglobulin Superfamily	
ICAM-1	leucocyte adhesion to endothelium, immune responses such as antigen presentation
ICAM-2	lymphocyte adhesion to endothelium
LFA-2	immune responses of lymphocytes
CD3	
VCAM-1	adhesion of lymphocyte and monocytes to endothelium may also mediate organ specific homing of lymphocytes
Selectins	
LECAM-1 (L-selectin)	lymphocyte homing to peripheral lymph nodes, neutrophil extravasation and interaction with endothelium
ELAM-1 (E-selectin)	inducible endothelial receptor for neutrophils
GMP-140 (P-selectin)	inducible receptor on platelets and endothelial cells, mediating cell-cell binding

Functional aspects of the β2 integrins and the neutrophil L-selectin receptor

CD11a is the major β2 integrin receptor involved in the adhesive and immune functions of lymphocytes (Springer et al,1987), mediating a broad range of lymphocyte functions, including T cell cytotoxicity, homotypic aggregation, helper T cell functions, T cell proliferative responses, B cell activation and differentiation into immunoglobulin-secreting cells, and lymphocyte adherence to endothelium. In addition, CD11a also participates in several important adhesive functions of phagocytes, such as monocyte aggregation, ADCC by neutrophils and monocytes, antigen presentation by monocytes and plays a part in non-stimulated neutrophil and monocyte adhesion to endothelium (Mentzer et al, 1986, 1987). CD11b, on the other hand, mediates the adhesive functions of myeloid cells, functioning both as the C3bi receptor (complement receptor 3) (Beller et al, 1982), and also in general as an adhesion-promoting molecule, mediating neutrophil aggregation and phagocytosis, neutrophil and monocyte chemotaxis and adhesion to artificial substrates and to endothelial and epithelial monolayers (Anderson et al, 1986, Wallis et al, 1985, Harlan et al, 1985, Brown et al, 1988). Different functional epitopes on CD11b subserve these different adhesive interactions (Dana et al, 1986). Apart from C3bi, several other ligands for CD11b have since been identified and CD11b has been implicated in a number of macrophage-microorganism interactions including the binding to E. Coli, Histoplasma Capsulatum and gp63 on Leishmania (Wright & Jong 1986, Bullock & Wright, 1987, Russell & Wright, 1988). Work using epitope-specific monoclonal antibodies suggests that there are at least two distinct binding sites on Mac-1, one that recognizes peptides containing the RGD sequence, and one that recognises lipopolysaccharide, and related carbohydrates (Wright et al, 1989). It is the peptide binding site that mediates granulocyte binding to endothelial cells, although the actual endothelial ligand/s involved have not been identified. Factor X and fibrinogen bind to an activated form of CD11b (Altieri et al, 1988a, Altieri & Edgington, 1988b, Wright et al, 1988).

CD11c contributes to the adhesive functions of neutrophils and monocytes, and seems particularly important in monocyte chemokinesis and chemotaxis, adherence to both plastic and endothelium, and phagocytosis of latex particles (Keizer et al, 1987b). CD11c also mediates some adhesive functions of lymphocytes, for example, it contributes to conjugate formation of some cytotoxic T cell clones with their targets (Keizer et al, 1987a) CD11c binds C3bi, this receptor activity is different from that of CD11b, and is designated CR4 (Myones et al, 1988).

Cytokines which activate neutrophils, such as GM-CSF, G-CSF , and TNF-α (Devereux et al, 1989, Socinski et al, 1989 , Yuo et al, 1989, Klebanoff et al, 1988) cause rapid upregulation of phagocyte expression of CD11b/11c, by translocation of the antigens from cytoplasmic stores to the surface membrane (Miller et al, 1987). GM-CSF and TNF-α increase neutrophil adhesion to endothelial cells in vitro (Devereux et al, 1989, Gamble et al, 1985, Yong et al, 1992), an effect which may be related to the increase in surface expression of these β2 integrins.

The function of the murine lymphocyte receptor, MEL-14, in organ specific homing has been well characterised in the murine system, where MoAB to MEL-14 almost completely abolishes lymphocyte adherence to peripheral lymph nodes (Gallatin et al, 1983). The recent identification of the human homologue, L-selectin, and the demonstration that it is identical to the leucocyte antigen recognised by Leu-8 MoAb has led to the recognition of its role in mediating neutrophil-endothelial interactions (see below). However, the role of L-selectin in mediating, more generally, the adhesion dependent responses of phagocytic cells is still largely unknown.
Stimulation of human neutrophils and monocytes with chemotactic factors, such as FMLP, or cytokines such as GM-CSF, results in rapid shedding of surface L-selectin (Griffin et al 1990). This rapid downregulation of L-selectin occurs coordinately with the upregulation of CD11b/CD18, and has led to the suggestion that these two receptors mediate distinct, but complementary adhesive events in the process of neutrophil extravasation.

1.3. ROLE OF ADHESION RECEPTORS IN PHAGOCYTE-ENDOTHELIUM INTERACTIONS

β2 integrin receptors

The recruitment of phagocytic cells at inflammatory sites requires the initial adherence of circulating cells to endothelium. In vitro, unstimulated neutrophils and monocytes demonstrate a baseline level of spontaneous adherence to cultured human endothelial monolayers. This unstimulated adherence is only partly mediated by the β2 integrins, mainly CD11a, with a lesser and variable contribution from CD11b, and , in the case of monocytes, from CD11c as well (Arnaout et al, 1988, Smith et al, 1989, Wallis et al, 1985).

Phagocyte adherence is greatly enhanced by inflammatory mediators. Some of these act directly on the leucocytes (lipid mediators, chemotactic peptides and cytokines),

others act directly to increase the adhesivity of endothelial cells (cytokines, lipid mediators) and there are yet others which act on both types of cells (Tonnesen, 1989). One of this last group (thrombin) acts by causing the secondary release by endothelial cells of platelet activating factor (PAF) which directly increases adhesivity of neutrophils (Zimmerman et al, 1985, 1990). In contrast to unstimulated adherence, the enhanced adherence of stimulated neutrophils is almost entirely dependent on the CD11/CD18 receptors, mainly CD11b, with a variable contribution from CD11a (Harlan et al, 1985, Zimmerman & McIntyre, 1988, Lo et al, 1989). Under conditions of endothelial activation, however, neutrophil adhesion is only partly dependent on the β2 integrins this time with CD11a playing a major part, but all three members have a cooperative effect (Pohlman et al, 1986, Dobrina et al 1989, Lo et al, 1989). The relative role of CD18 in the binding of neutrophils to activated endothelium also depends upon the stimulus, and the time course of stimulation (Smith et al, 1990)

Transendothelial migration of neutrophils occurs in response to cytokine stimulation of endothelial cells, or in response to chemoattractants, and appears to be heavily dependent on neutrophil CD18 receptors, and on ICAM-1 on endothelial cells (Smith et al, 1988, 1989, Luscinkas et al, 1991, Furie et al, 1991) .

L-selectin

L-selectin is expressed on all leucocytes and early evidence for the role of this receptor in mediating neutrophil-endothelial interactions comes from *in vivo* studies in the murine system, where MoAb to MEL-14 inhibits neutrophil extravasation into inflammatory sites (Lewinsohn et al, 1987, Jutila et al, 1989). *In vitro*, this receptor was also found to mediate the binding of human neutrophils to high endothelial venules (HEV) in frozen sections of rat lymph node in the Stamper-Woodruff assay (Lewinsohn et al 1987). *In vitro* studies of neutrophil adherence to cultured human endothelial monolayers demonstrate that L-selectin participates (together with the CD18 receptors) in the adherence of neutrophils to IL-1 activated endothelium, and that this dependence is increased when the adherence assay is conducted under dynamic conditions (Smith et al, 1991, Kishimoto et al, 1991, Hallman et al, 1991, Spertini et al, 1991). Recent studies suggest that the selectin receptors mediate neutrophil rolling on endothelium, which might be an early adhesive interaction in inflammation (Ley et al, 1991, Von Andrian et al, 1991, Lawrence & Springer, 1991). The endothelial cell ligand for L-selectin has recently been identified (Imai et al, 1991) and cloned (Lasky et al, 1992). In contrast to the CD18 receptors, L-selectin does not appear to mediate the migration

of neutrophils across cytokine-activated endothelium in the in vitro system (Smith et al,1991, Kishimoto et al, 1991).

The specific contribution of the different leucocyte adhesion receptors in mediating the adhesive response of phagocytic cells to G- and GM-CSF is directly addressed in Chapters 6 and 7. The relative contribution of the different neutrophil adhesion receptors in the various stages of neutrophil extravasation into inflamed tissues is discussed in Chapter 8.

Role of endothelial adhesion receptors and signalling molecules

The vascular endothelium is now recognised to play an active part in the control of leucocyte-endothelium interactions, by the coordinated expression of surface adhesion-promoting receptors, as well as by the elaboration of signalling molecules. ICAM-1 and ICAM-2 are expressed on unstimulated endothelium and both are ligands for CD11a, but only ICAM-1 binds CD11b (Rothlein et al, 1986a, Staunton et al, 1989, Diamond et al, 1990). ICAM-1 is upregulated on the endothelial cell surface by cytokine stimulation, and functions in the enhanced binding, and transmigration of neutrophils to, and across activated endothelium (Smith et al, 1990,). Cytokine stimulated endothelium also synthesizes E-selectin (Bevilacqua et al, 1989), which not only participates in the enhanced binding of neutrophils, but also functions in neutrophil activation (Lo et al, 1991). Another selectin receptor, P-selectin, is rapidly upregulated on endothelial cells upon stimulation with thrombin, leukotriene C4, histamine and phorbol esters, and mediates the transiently enhanced adhesion of neutrophils (Geng et al, 1990). There is evidence that under these conditions, platelet-activating factor (PAF) also plays a role, both as an adhesion-promoting ligand for neutrophil adherence (Zimmerman et al, 1990), as well as a signalling molecule to activate and prime neutrophils (see below, Lorant et al, 1991). Monocyte adherence to activated endothelium involves the above endothelial receptors, and also the cytokine-inducible vascular cell adhesion molecule-1 (VCAM-1), which is not involved in neutrophil adhesion (Carlos & Harlan, 199o).

Activated endothelial cells also synthesize signalling molecules, such as PAF and interleukin-8 (IL-8), which act as chemoattractants, as well as directly activating neutrophils, causing the upregulation of CD11/CD18, and increasing neutrophil adhesion and transendothelial migration (Lorant et al, 1991, Baggiolini et al, 1989, Huber et al, 1991, Yuo et al, 1991, Kuijpers et al, 1992). GM-CSF, which is also produced by cytokine-stimulated endothelial cells (Fibbe et al, 1989, Zsebo et al 1988), is another important neutrophil activator, and one of the main questions addressed in this thesis is the way in which GM-CSF functions as an inflammatory

cytokine, to regulate the adhesive interactions of neutrophils with vascular endothelium, *in vivo* and *in vitro.*

Finally, there is the possibility that G- and GM-CSF may have direct effects on vascular endothelium, and one group has reported that G- and GM-CSF induce endothelial cell proliferation and function (Bussolino et al, 1989), a finding which is of relevance to the clinical application of this growth factor. In Chapters 9 and 10 are presented the *in vitro*, and *in vivo* studies which address this question.

CHAPTER TWO

MATERIALS AND METHODS

2.1 GENERAL MATERIALS

2.1.1. Plastics

All plastics used were of tissue culture grade polystyrene unless stated otherwise.

Sterile disposable plastics	***Source***
30 mL universal containers	
250 mL specimen containers	Sterilin Ltd.,
7 mL bijou bottles	Feltham, UK.
50 mL polypropylene tubes	Falcon ware, Becton
25 cm^2, 75 cm^2 and 175 cm^2 tissue culture flasks	Dickinson, UK Ltd.,
96 well flat-bottomed tissue culture plates	Cowley, Oxford.
1.2 mL cryotubes (Nunc)	Gibco, Paisley, Scotland.
12 and 24 well flat-bottomed tissue culture plates	
6.5 mm 'Transwell-COL' (collagen-coated	
3.0 μ pore filter cell culture inserts)	Costar (UK) Ltd.
15 cm petri dishes	High Wycombe, UK.
0.22 μ cellulose acetate filters	
3 μ pore nitrocellulose membranes	Sartorius,
Collodion bags (for use with filter apparatus)	Belmont, Surrey.
0.22 μ Nalgene cellulose nitrate membrane	Nalge, obtained from
filter units (250 mL capacity)	Merck Ltd., Magna Park,
	Lutterworth,
	Leicestershire.

Syringes (1-50 mL capacity)	Sabre International Products Ltd., Reading, Berkshire, UK.
Quill filling tubes	Avon Medical, Redditch, Worcestershire.
5, 10 and 25 mL pipettes	Philip Harris, London.
Intravenous cannulae (14 gauge)	H.G. Wallace, Colchester Essex.
Nonsterile Disposable Plastics	
96 well round bottomed plastic flexible polyvinyl plates (300 µL/well)	Falcon ware, obtained from Becton Dickinson.
Pipette tips (Gilson)	Anachem, Luton, U.K.
96 well flat bottomed polystyrene plates for ELISAs	Costar (UK) Ltd. (3590) High Wycombe, UK.

2.1.2. Glassware

Disposable glassware	***Source***
Sterile 1 mL and 2 mL pipettes Glass slides	British Drug House (BDH), Poole, UK.
Tissue culture chamber slides (Lab-Tek)	Miles Lab. Inc. Naperville, Illinois, USA.
Non disposable glassware	
Filter flask (for concentrating protein)	Sartorius (SM 16305) Belmont, Surrey.

2.1.3. Reagents

	Source
Formyl-methionyl-leucyl-phenlyalanine (FMLP)	
12-0-tetradecanoylphorbol 13-acetate (TPA)	
O-phenylene diamine (OPD)	
2,2'Azino-bis(3-ethylbenzthiazine-6-sulphonic acid (ABTS)	
Sodium-p-nitrophenyl phosphate (PNP)	
Ethylenediaminetetraacetic acid (EDTA)	
Histopaque	
Tween 20	
Bovine serum albumin	Sigma Chemicals,
Ralmount	Poole, Dorset,
Thrombin	UK.
Protein A Sepharose	
Lymphoprep	Nycomed, Birmingham, UK.
Hetastarch	
Acid citrate dextrose	
Hydrogen peroxide	
Glutaraldehyde	BDH,
Preservative free Heparin	Poole, Dorset, UK.
^{51}Chromium	Amersham Ltd.
^{125}I-GM-CSF	Buckinghamshire,
^{3}H-thymidine	UK.

2.1.4. Buffers

i) Phosphate buffered saline (PBS) solution was made from PBS tablets (Oxoid Ltd., Basingstoke, Hampshire, UK). PBS/Tween for use in ELISAs was made by adding 0.05% of Tween 20 to PBS solution.

ii) Phosphate buffer, pH 8 was made by titrating 0.1M NaH_2PO_4 with 0.1M Na_2HPO_4.

iii) Citrate/phosphate buffer was made using 0.1M citric acid, and titrating in the appropriate amount of 0.1M Na_2HPO_4 to reach the required pH.

2.1.5. Tissue culture media, serum and enzymes

	Source
Phosphate buffered saline (PBS) without calcium or magnesium	
RPMI	
Hank's Balanced Salt Solution (HBSS)	
Iscove's Modified Dulbecco's Medium (Iscove's MDM)	
Penicillin/Streptomycin	Gibco,
Trypsin/ethylenediaminetetraacetic acid (EDTA)	Paisley, Scotland.
Collagenase (*Clostridium histolyticum*, Type A)	Boehringer
Fibronectin	Mannheim,
	Lewes, Sussex.

Foetal calf serum (FCS) was obtained from Gibco Ltd. (Paisley, Scotland). It was first heat-inactivated at 56°C for 30 minutes and aliquots were stored at -20°C. Batches were tested for their ability to support optimal cell growth *in vitro*.

2.1.6. Cytokines

Endothelial cell growth supplement (ECGS) was obtained from Sigma Chemicals, UK. Interleukin-1 beta (IL-1 β) was obtained from Boehringer Manheim, Germany. Recombinant human GM-CSF (rhGM-CSF, 5 X 10^7 U/mg) was generously supplied by Hoechst , UK., and recombinant human G-CSF (rhG-CSF, 1 X 10^8 U/mg) was a gift from Chugai, Japan. . Human urinary colony-stimulating factor-1 (CSF-hu, 1.5 X 10^o U/mg) was supplied by Alpha Therapeutics, Osaka, Japan. Recombinant tumour necrosis factor (TNF-α) and gamma interferon (γ-IFN) were gifts from Immunex Corp., Seattle, Washington, USA, and Biogen, Hounslow, UK, respectively. All cytokines were screened for endotoxin contamination using *Limulus polyphemus* lysate (Sigma Chemicals).

2.1.7. Antibodies

Commercial antibodies	***Source***
Fluorescein-conjugated rabbit anti-mouse Ig (FITC-RAM)	Dako Ltd., High
Anti-von Willebrand Factor monoclonal antibody	Wycombe, UK.
Leu-8 (anti-L-selectin)	
Fluorescein-conjugated Leu-8 (Leu-8-FITC)	Becton Dickinson,
Fluorescein-conjugated mouse IgG2a (IgG2a-FITC)	UK
Fluorescein-conjugated Mo-1 (anti CD11b, MO-1-FITC)	Coulter
Fluorescein-conjugated anti-mouse Ig (MsIg-FITC)	Electronics,
	Luton, UK

The following antibodies were gifts:

Table 2.1 Monoclonal Antibodies.

NAME	SPECIFICITY	SUB-CLASS	REFERENCE	SOURCE
44	CD11B	IgG1	Malhotra et al, 1986	Dr. Nancy Hogg, ICRF, London.
MHM23	CD18	IgG1	Hildreth et al, 1983	Drs. A McMichael and F. Gotch, Oxford.
DREG 56	L-SELECTIN	IgG1	Kishimoto et al, 1990	Dr.T K Kishimoto, Boehringer Ingelheim, Ridgefield, CT, USA.

All antibodies were titrated out and used at optimal concentrations.

2.2 GENERAL METHODS

2.2.1. Isolation and culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cords by a modification of the method originally described by Jaffe et al (1973). Briefly, umbilical cords were obtained within 8 hours of delivery, and stored in HBSS at room temperature until used. Only sections of cord greater than 15 cm. long, and free from needle puncture marks or clamp marks were used. The ends of the cord were trimmed and the umbilical vein was cannulated at either end using 14 guage intravenous cannulae (Wallace). The cannulae were secured with sterile umbilical cord ties, and the vein was gently flushed through with HBSS at room temperature until the effluent ran clear. At this stage, the vein was filled with collagenase solution (0.1% in PBS), and both ends sealed using cannulae bungs. Following an incubation of 20 minutes at 37°C, the contents of the cord vein were flushed out with IMDM, and the cells pelleted by centrifugation at 700 rpm for 5 minutes. The pellet was resuspended in complete culture medium (Iscove's Modified Dulbecco's Medium with 20% FCS, ECGS 25 μg/ml and heparin 25 U/ml), and cells were grown to confluence in fibronectin (2 $\mu g/cm^2$)-coated Falcon tissue culture flasks. Fibronectin coating of tissue culture flasks was carried out by covering the bottom of each flask with fibronectin solution (50 μg/mL in HBSS) for 20 minutes at room temperature. When the endothelial cell cultures had formed confluent monolayers (5-10 days, depending on seeding density), the cells were passaged using trypsin (0.05%) EDTA (0.01%) in PBS. For experiments on neutrophil adhesion, cells at passages 1-4 were seeded in 96-well fibronectin-coated tissue culture plates (Nunc) at $2x10^3$ cells/well and grown to confluence (5-7 days) before being used in adherence assays.

Identification of cells in culture was done using immunofluorescent labelling of von Willebrand Factor protein (vWF) (Jaffe et al, 1973). Cells for staining were either cytospin preparations of freshly passaged cells on glass slides or cells grown in tissue culture wells mounted on glass slides (Lab-Tek, Miles Lab.). Slide preparations were fixed in 70% methanol at 4°C for 15 minutes and washed with PBS, before overlaying with anti-vWF MoAb (1:100 in PBS). After an overnight incubation in a humidified chamber at 37°C, the slides were washed three times with PBS, overlayed with FITC-RAM (1:200), and incubated for a further 30 minutes. The nuclei were counterstained with propidium iodide (0.5 mg% m/v in PBS) and the slides mounted in glycerol:PBS (3:1) and viewed under a fluorescent microscope (Figure 2.1).

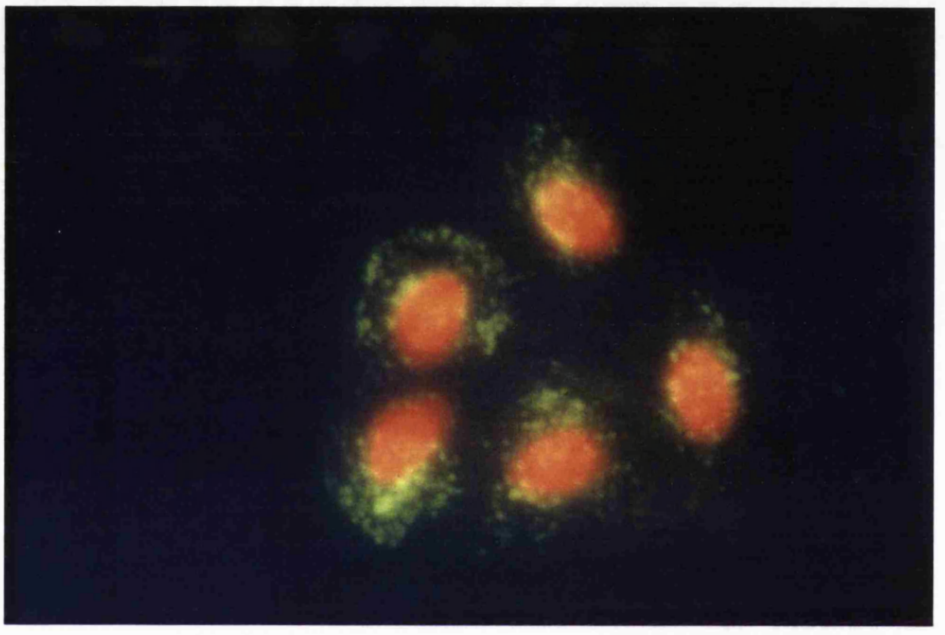


Figure 2.1. Immunofluorescent staining of HUVEC with anti-von Willebrand Factor antibody

2.2.2. Isolation of neutrophils from peripheral blood

For functional assays such as adhesion to endothelium, neutrophils were purified from heparinised citrated blood using starch sedimentation and double density Ficoll centrifugation (Histopague 1119, Sigma Diagnostics, Lymphoprep, Nycomed). Venous blood, obtained from healthy normal volunteers by easy venepuncture was taken into PFH (10 U/mL) and ACD (1:7) in polypropylene syringes. Hetastarch was added to a final concentration of 20%, and the red cells allowed to sediment at room temperature for 20-30 minutes. The leucocyte-rich fraction was then layered onto a double density gradient composed of Histopaque, SD 1119, on top of which had been layered Lymphoprep, SD 1077. Following centrifugation at 700g for 45 minutes, the neutrophils were collected from the interface between the Histopaque, and the Lymphoprep layers. The neutrophils were washed once in RPMI, and used immediately in functional assays. The neutrophils obtained by this method were >95% pure and >99% viable by trypan blue exclusion. This method for neutrophil purification was selected because it led to minimal cell activation, as assessed by changes in surface adhesion molecule expression.

2.2.3. Leucocyte Phenotyping

Quantitation of leucocyte surface antigen expression was done by indirect immunofluorescence, performed on whole blood samples in order to minimise activation of cells (Fearon & Collins, 1983). Aliquots of heparinised blood containing $2x10^5$ leucocytes were placed in microtitre wells and incubated with saturating concentrations of monoclonal antibodies or medium for 45 minutes at 4°C. Following three washes in RPMI, the cells were incubated with FITC-RAM for a further 45 minutes. This was followed by another three washes. All steps were done at 4°C. The red cells were then lysed and the white cells fixed using an automated system (Coulter Immunoprep Workstation, Jaswon et al, 1990) before analysing on an EPICS CD flow cytometer (Coulter Electronics, Luton, U.K.). The neutrophils and monocytes were selectively gated by virtue of their light scattering properties, and antigen density was measured as mean cell fluorescence (MCF) on a linear scale. In experiments studying the in vitro effect of growth factors or other cytokines on antigen expression, heparinised venous blood was preincubated with the relevant agent or medium as control for stated times at 37°C before being placed on ice for immunofluorescence.

2.2.4. Peripheral blood cell counts

Venous blood was taken into EDTA, and full blood counts were performed on an automated cell counter, the STAK-R (Coulter Electronics). All white cell differential counts were performed manually, under light microscopy.

2.2.5. Neutrophil adhesion experiments.

Freshly isolated neutrophils were suspended at 1 X 10^6 cells/ml in RPMI with 5% FCS. Confluent HUVECs in 96-well microtitre plates were washed twice with RPMI at 37°C , and 100 µL of neutrophil suspension (1 X 10^5 cells) was added to each well. GM-CSF, G-CSF, TPA or medium as control, were added to neutrophils at the start of an adhesion assay, and were present throughout. At least 6 wells were used for each data point. Following an incubation of 30 minutes at 37°C, unattached cells were removed by 3 washes of 300 µL exchanges of medium. At the end of the washing procedure, the plates were checked by microscopy to ensure that there was no disruption of the endothelial monolayers. The number of adherent neutrophils was then quantified by measuring the alkaline phosphatase activity of the adherent cells (Santini et al, 1987). Briefly 100 µL of 0.9% NaCl, followed by 100 µL of a solution containing 1 mg/ml of sodium-p-nitrophenyl phosphate (Sigma Chemicals) in diethanolamine buffer (BDH, 1M, pH 9.8) were added to each well. After incubation at 37°C for 30-45 minutes, the reaction was stopped by addition of 2N NaOH. 100 µL of the original neutrophil suspension, in doubling dilutions were incubated in the same plate with substrate. The optical density was read at 405 nm on a MR 700 Microplate Reader (Dynatech Laboratories). The adherent fraction was calculated from the standard curve of neutrophil numbers and expressed as a percentage of the total number of neutrophils added to each well.

2.2.6. Neutrophil aggregation experiments

Neutrophil aggregation was determined using an aggregometer (Biodata Corp., PA, USA). 300 µL of neutrophil suspension (5-10 X 10^6/mL in RPMI) was warmed for 10 minutes at 37°C before addition of agonist, and aggregation monitored continuously by measuring the light transmission through the suspension. A 1:1 mixture of cell suspension and medium was used as a blank, and percentage aggregation was measured assuming light transmission through the blank represented 100% aggregation.

2.2.7. Purification of monoclonal antibodies from ascites

Monoclonal antibodies (MoAbs) for use in functional blocking experiments were obtained as ascites, and purified over a Protein A column. Briefly, the column was washed with citric acid (0.1 M), followed by phosphate buffer (pH 8). The antibody, as ascites, was adjusted to pH 8, and then applied to the column, circulating overnight at about 5 mL/hour. The next day, the column was washed with phosphate buffer (pH 8), and the MoAb eluted with citrate buffer, at the appropriate pH for the isotype of antibody. Finally, the column was washed with citric acid, followed by phosphate buffer, pH 8. The concentration of the purified antibody was estimated by

measuring UV light absorbance at 280 nm (1 OD being approximately equal to 0.75 mg/mL).

2.2.7. Statistical methods

Statistical analyses were performed using Student's t-test, and the StatView programme on the Apple Macintosh.

CHAPTER THREE

EFFECT OF GM-CSF AND G-CSF ON PHAGOCYTE ADHESIVE PROPERTIES *IN VITRO*

INTRODUCTION

Among the reported effector functions of mature myeloid cells which are enhanced by GM-CSF and G-CSF are the adhesive responses of neutrophils and monocytes. Both growth factors modulate surface expression of adhesion molecules on neutrophils and monocytes. GM-CSF and G-CSF cause rapid and sustained upregulation of surface levels of the β2 integrin, CD11b/CD18 *in vitro* (Arnaout et al, 1986, Yuo et al, 1990) and *in vivo* (Devereux et al, 1989, Socinski et al, 1989, Ohsaka et al, 1989), and GM-CSF causes rapid shedding of L-selectin *in vitro* (Griffin et al, 1990). *In vitro*, GM-CSF increases neutrophil and monocyte adherence to endothelial monolayers (Devereux et al, 1989, Yong et al, 1992, Gamble et al, 1989), and G-CSF induces neutrophil adherence to nylon fibre (Yuo et al, 1989). Indirect evidence for GM-CSF mediated neutrophil-endothelial interactions *in vivo* comes from the observation that systemic administration of GM-CSF results in a rapid, transient leucopenia, which is due to a shift of circulating cells into the marginated pool, predominantly in the lungs (Devereux et al, 1989). Administration of G-CSF has also been reported to cause a transient leucopenia (Morstyn et al, 1988, Lindemann et al, 1989).

The exact relationship between alterations in adhesion molecule expression, and enhanced neutrophil adhesive functions remains unclear. Increased expression of CD11b/CD18 receptors in response to cell activation could account for the increased adherence of these cells. The following studies were carried out in order to define this relationship. This chapter documents the *in vitro* effects of GM-CSF and G-CSF on phagocyte surface expression of the CD11/CD18 receptor complex and L-selectin, and on the effects of these growth factors on the adhesion of normal neutrophils to plastic surfaces and to cultured human endothelial cells.

RESULTS

GM-CSF and G-CSF cause upregulation of CD11/CD18 receptors on peripheral blood neutrophils and monocytes.

Following 30 minutes incubation at 37°C, GM-CSF (10 ng/mL, 714 pM) increases neutrophil CD11b levels to 655 ± 255% (mean ± SD) of resting levels on control unincubated cells ($p < .0005$ when compared with cells incubated in medium alone, n=8), while G-CSF (100 ng/mL, 5.88 nM) produces a similar rise to 670 ± 368% of resting levels ($p <. 0005$, n=5). Cells incubated with medium alone for 30 minutes at 37°C demonstrated a rise in CD11b expression to 288 ± 90% of resting levels (n=11, Figure 3.1a). The maximal response achieved was equivalent for both growth factors, when used at optimum levels of 50-100 ng/mL (2.99-5.88 nM) for G-CSF, and 5-10 ng/mL (357-714 pM) for GM-CSF. The surface expression of CD11a and CD11c on neutrophils was also enhanced by GM-CSF (Figure 3.1b). The increase in CD11c expression (191 ± 37% of control, $p < .05$, n=3) was comparable to that seen for CD11b, but CD11a levels showed a more modest rise to 140 ± 52% of control ($p < .05$, n=3).

A similar upregulation of CD11/CD18 expression was also seen on peripheral blood monocytes in response to GM-CSF (10 ng/mL). Monocyte surface expression of CD11b and CD11c is increased by incubation with medium alone, to 184 ± 58% and 150 ± 33% of resting levels, respectively, however, GM-CSF is able to produce a further rise in levels. Monocyte CD11a, CD11b and CD11c levels rose to 169 ± 38% (N.S. by Student's t-test, n=3), 315 ± 94% ($p<.005$, n=7), and 213 ± 49% ($p < .005$, n=4) respectively, of resting levels found on unincubated cells (Figure 3.1d). G-CSF was also able to upregulate the CD11b receptor on monocytes to 306 ± 30% ($p < .05$, n=3) of resting levels (Figure 3.1c). M-CSF had no effect on the expression of CD11b on peripheral blood neutrophils or monocytes (Figures 3.1a, c).

These effects of growth factors on β2 integrin expression are dose dependent; GM-CSF has maximal effect at 1 ng/mL, while G-CSF is only effective at concentrations of 10 ng/mL or greater (Figure 3.2). Upregulation of these adhesion receptors occurs rapidly, with near maximal effects seen after 30 minutes incubation at 37°C, and is sustained for up to 2 hours (Figures 3.3a,b).

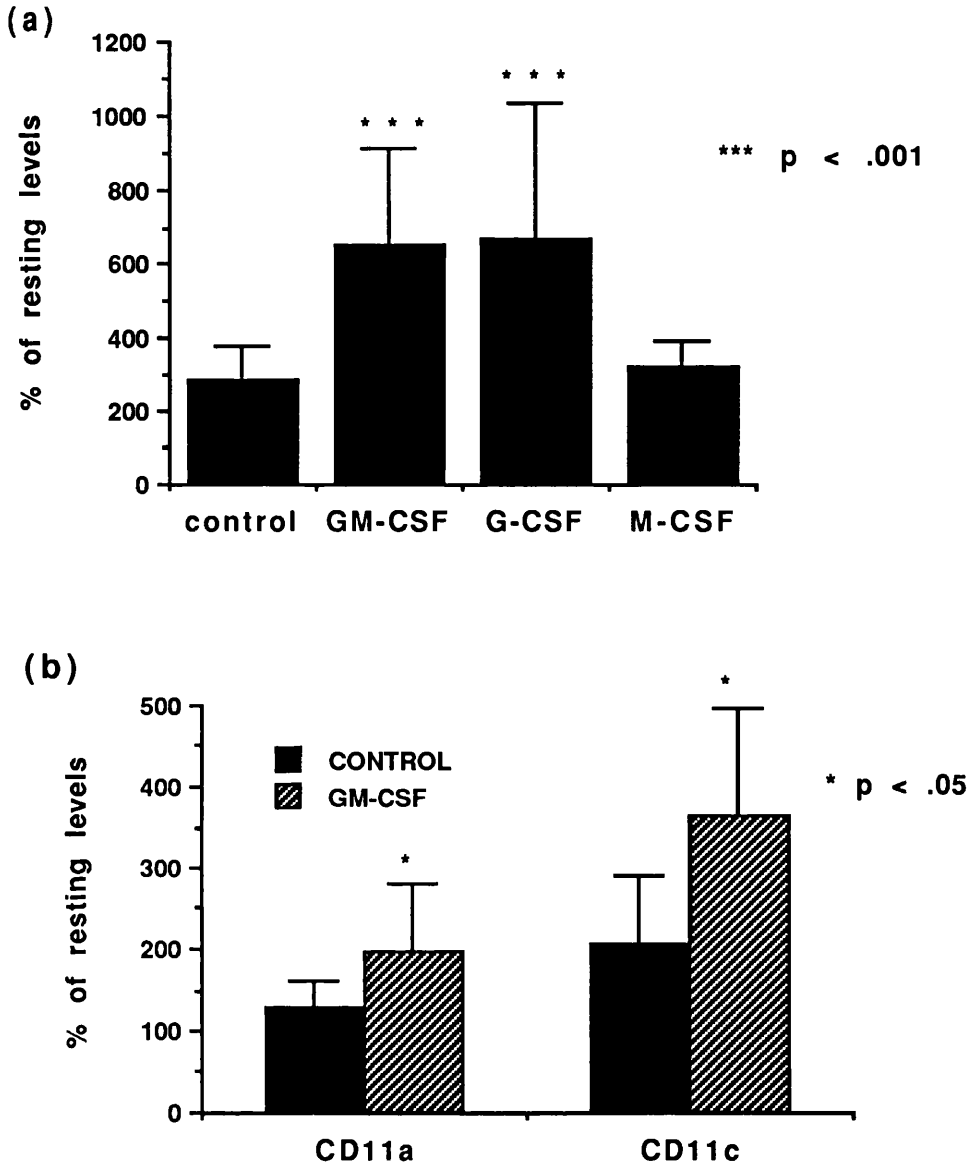


Figure 3.1. Effect of (a) growth factors (8 experiments with medium alone, 8 with GM-CSF, 5 with G-CSF and 3 with M-CSF) on neutrophil CD11b, and of (b) GM-CSF on neutrophil CD11a and CD11c (n=3). Data, expressed as percentage of the MCF of unincubated cells, is mean ± SD.

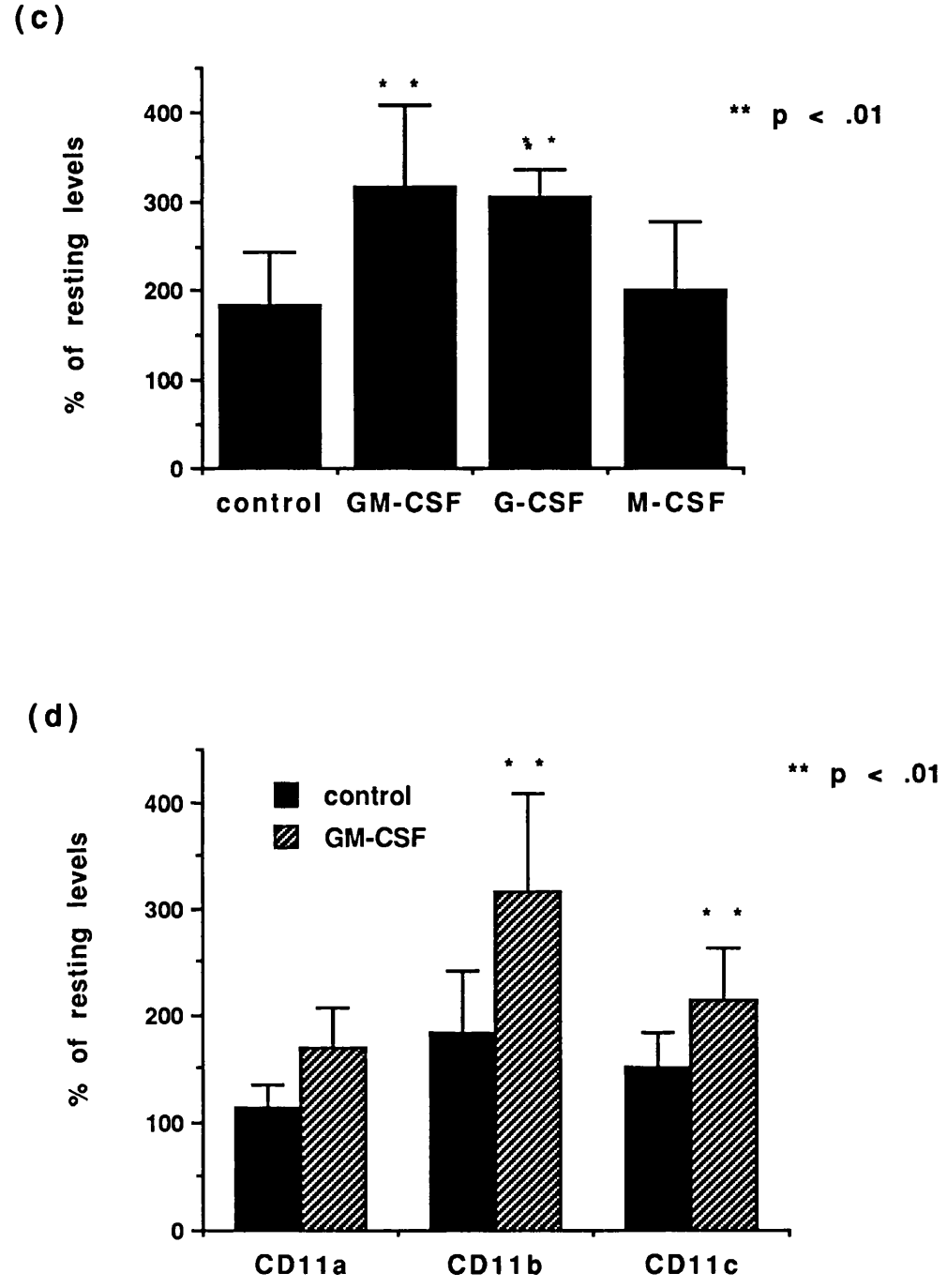


Figure 3.1 . Effect of (c) growth factors (7 experiments with medium and GM-CSF, and 3 experiments with G-CSF and M-CSF) on monocyte CD11b, and of (d) GM-CSF on monocyte CD11a (n=3), CD11b (n=7) and CD11c (n=4). Data is expressed as for (a) and (b).

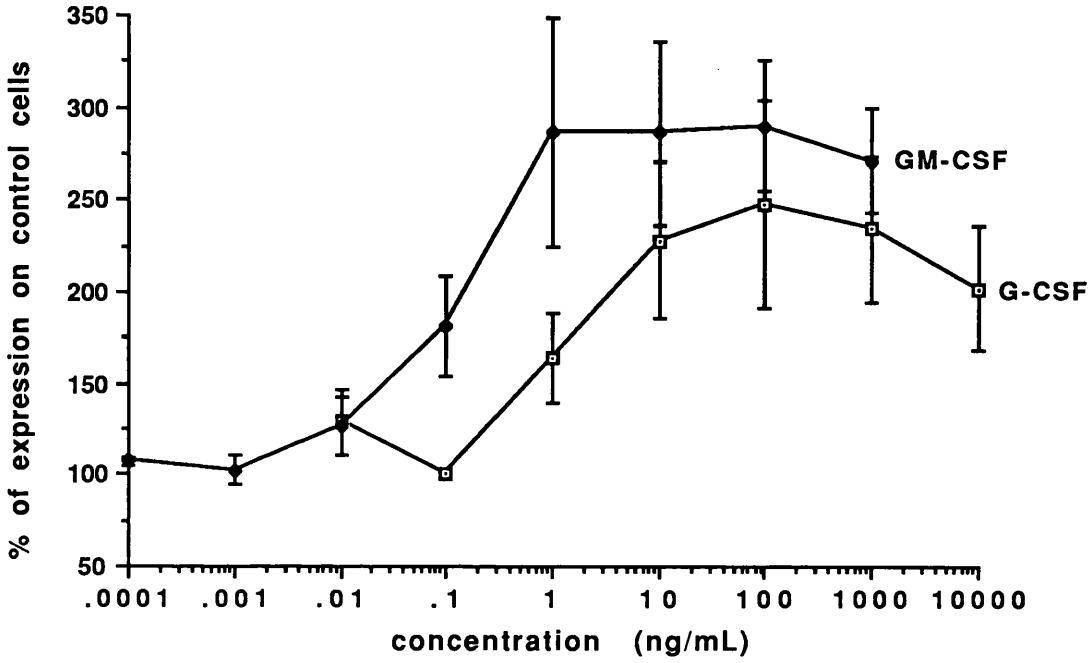


Figure 3.2. Dose dependent upregulation of neutrophil CD11b by GM-CSF and G-CSF. Data, expressed as percentage of MCF on control cells incubated with medium alone, is mean ± SD of 3 experiments with each growth factor.

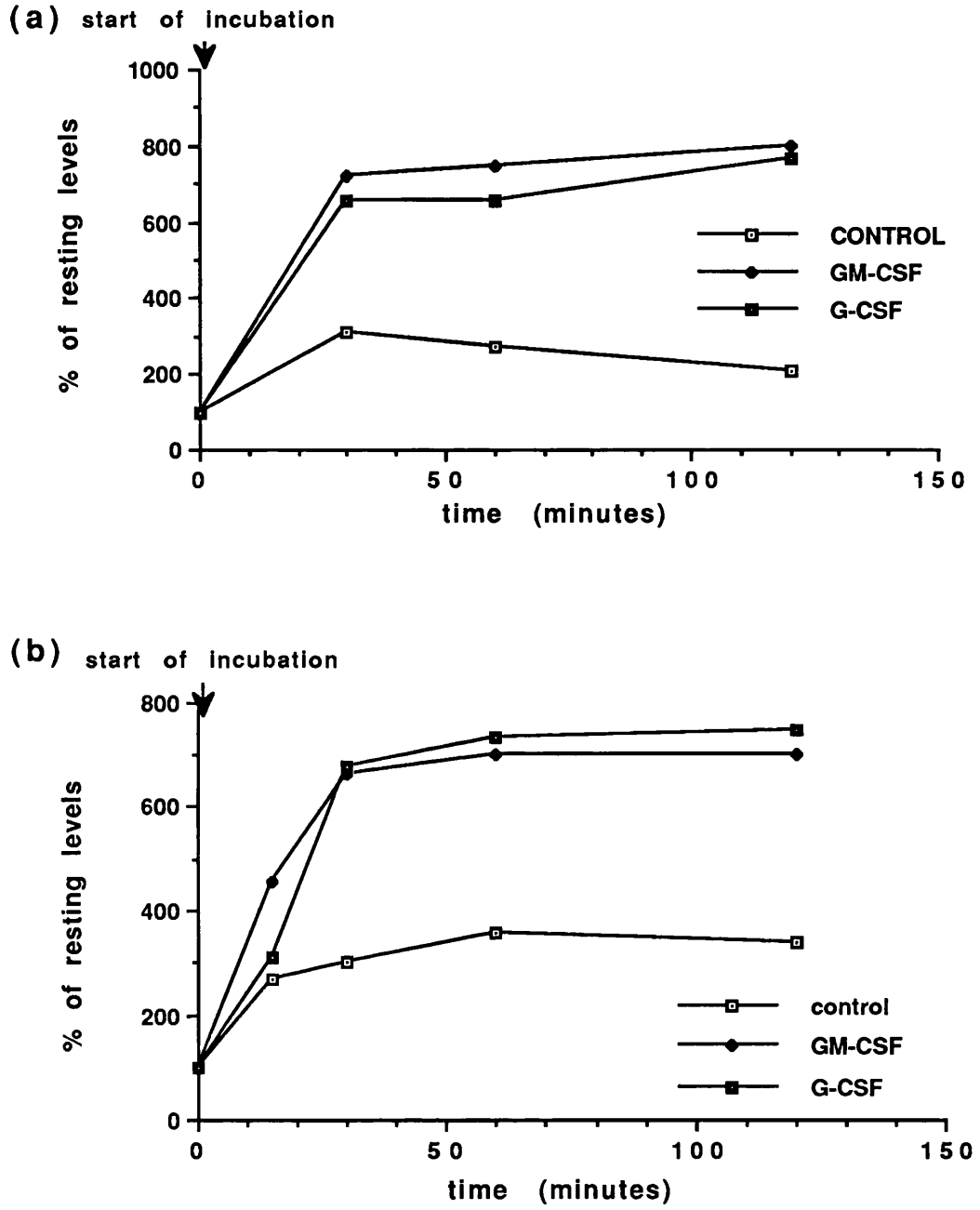


Figure 3.3. Time course of GM-CSF and G-CSF induced CD11b on (a) neutrophils, and (b) monocytes. Data, expressed as percentage of resting levels on unincubated cells, is given for 1 experiment in each which is representative of 2.

G- and GM-CSF downregulate neutrophil and monocyte L-selectin expression

In contrast to their effects on β2 integrin expression, both G- and GM-CSF cause rapid loss of surface L-selectin expression on neutrophils and monocytes (Figure 3.4a). While incubation in medium alone for 30 minutes at 37°C causes a fall in L-selectin levels to 81 ± 11% on neutrophils (n=9), and to 66 ± 16% on monocytes (n=3), G-CSF is able to cause a further fall to 52.7 ± 14% on neutrophils ($p < .005$, n=6) and to 48 ± 15% on monocytes ($p < .01$, n=3), and GM-CSF reduces levels to 26.9 ± 3% on neutrophils ($p < .0005$, n=5) and 37 ± 13% on monocytes ($p < .01$, n=3). Purified neutrophils (prepared as detailed in Chapter 2) also demonstrate loss of L-selectin in response to GM-CSF and G-CSF, of about the same magnitude as is seen in whole blood (Figure 3.3b). L-selectin levels are reduced to 34.7% and 24.2% of resting levels by G-CSF and GM-CSF respectively. Loss of L-selectin occurs in a dose dependent manner, with G-CSF showing maximal activity at concentrations of 100 ng/mL or greater while maximal effects with GM-CSF are achieved at 1-10 ng/mL (Figure 3.4c). This difference in potency between the 2 growth factors was noted earlier with respect to the upregulation of the integrin receptors. In contrast to the upregulation of the CD11/CD18 molecules, however, G-CSF is much less effective than GM-CSF is at downregulating L-selectin expression. Figure 3.4a shows that, at optimal concentrations for each growth factor, GM-CSF (10 ng/mL) produces a 73% fall in neutrophil L-selectin levels, while G-CSF (100 ng/mL) only causes a 47% fall. Growth factor mediated loss of this receptor occurs rapidly, is maximal by 60 minutes and is sustained for up to 4 hours (Figure 3.4d). M-CSF had no effect on L-selectin levels on neutrophils or monocytes (n=3, data not shown).

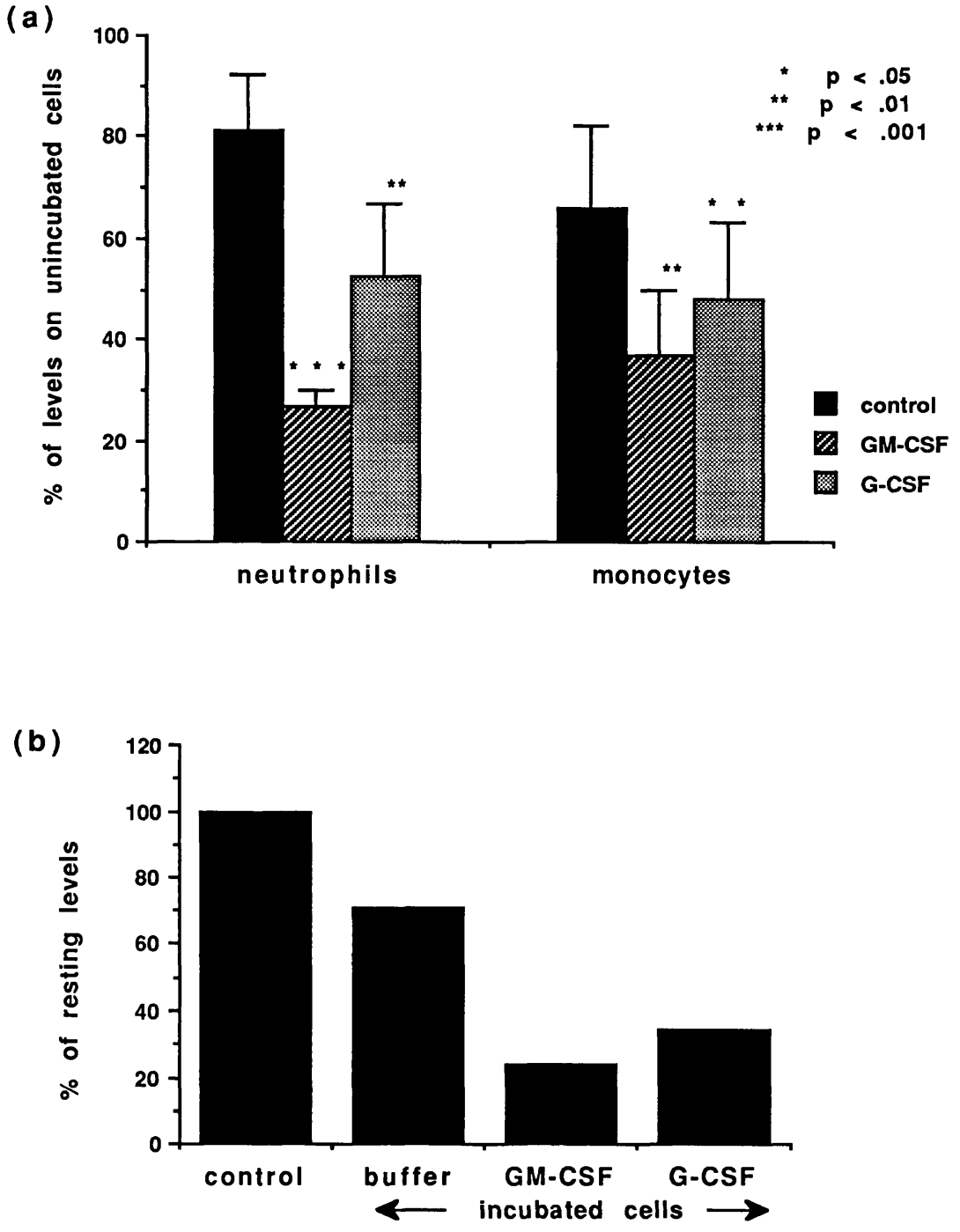


Figure 3.4 (a). GM-CSF and G-CSF downregulate L-selectin levels on neutrophils (n=6) and monocytes (n=3) in whole blood, and also on (b) purified neutrophils. Data, expressed as percentage of resting levels on unincubated cells, is mean ± SD in (a), and is the mean of duplicate samples in 1 experiment in (b).

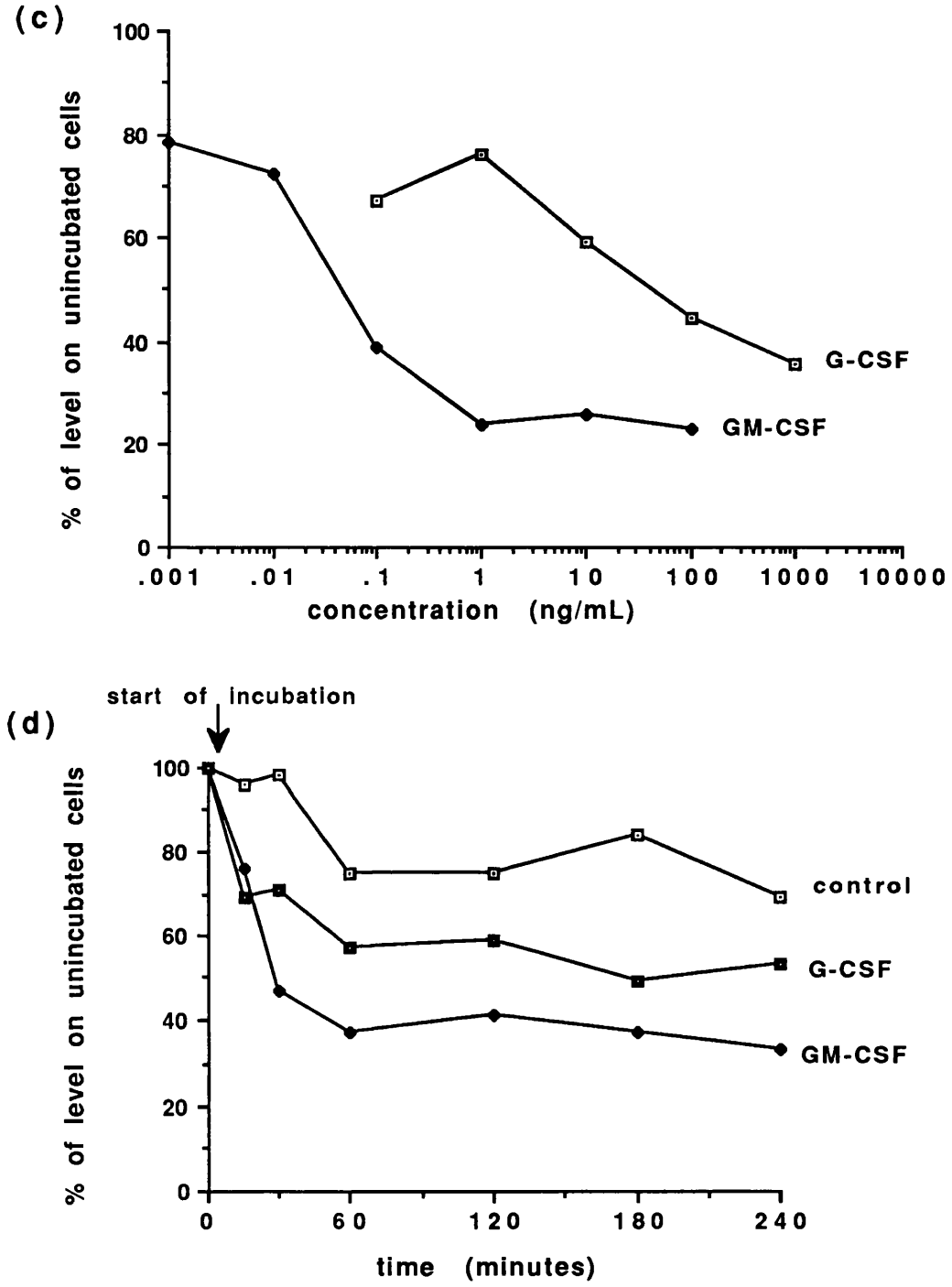


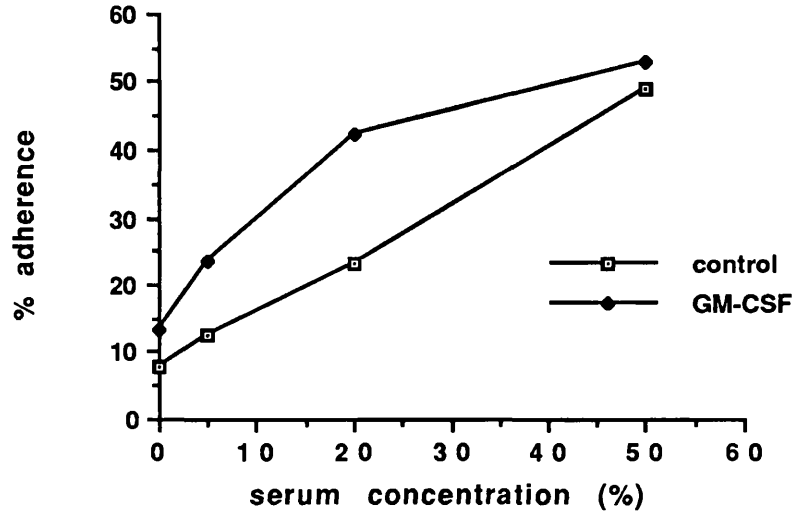
Figure 3.4 (c). Growth factor induced loss of neutrophil L-selectin is dose-dependent. (d) Time course of growth factor induced downregulation of neutrophil L-selectin levels. Data, expressed as percentage of MCF on unincubated cells, is given for 1 experiment in each case which is representative of 2.

GM-CSF enhances neutrophil adhesion to endothelial monolayers

In preliminary experiments, the adhesion of peripheral blood neutrophils to cultured human umbilical vein endothelial cells (HUVECs) was studied using different serum concentrations in the assay medium. Figure 3.5a shows that the percentage of adherent neutrophils increases with increasing serum concentrations. GM-CSF (100 ng/mL) enhances the adhesion of neutrophils to HUVECs under all serum conditions. However, it was found that this effect of GM-CSF was best demonstrated between serum concentrations of 5-20%. Higher serum concentations led to larger standard errors between replicate wells, and, in the absence of serum, the percentage of adherent cells in the control wells was often too low to be accurately quantified on the standard curve using the alkaline phosphatase assay. In the presence of 5% FCS, the adherence of unstimulated neutrophils to cultured human endothelial cells was 9.3±0.7% (mean ± SEM), and GM-CSF (100 ng/mL) increased this to 15.4±1.3% ($p < .0005$, n=10, Figure 3.5b). In 5 experiments using 20% FCS, GM-CSF increased neutrophil adhesion from 23.7±1.8% to 37.0±3.7% ($p < .005$). GM-CSF induces neutrophil adhesion in a dose dependent manner, with maximal effects seen at doses of 10 ng/mL or greater (n=5). G-CSF, on the other hand, in all of 5 individuals tested, had no significant effect on the adhesion of peripheral blood neutrophils to endothelial cells (Figure 3.5c).

It is important to note that this effect of GM-CSF on neutrophil adhesion was demonstrated under serum free conditions (in the absence of serum, GM-CSF increased neutrophil adhesion from 6.4±0.8% to 13±2.9%, $p<.01$, n=4, and Figure 3.5a), and was therefore not dependent upon any contaminating agonists in foetal calf serum. A serum concentration of 5% was selected for use in all subsequent experiments, because under these conditions, the levels of baseline adhesion were high enough to register reliably using the alkaline phosphatase assay, and the effect of GM-SCF was optimal.

(a)



(b)

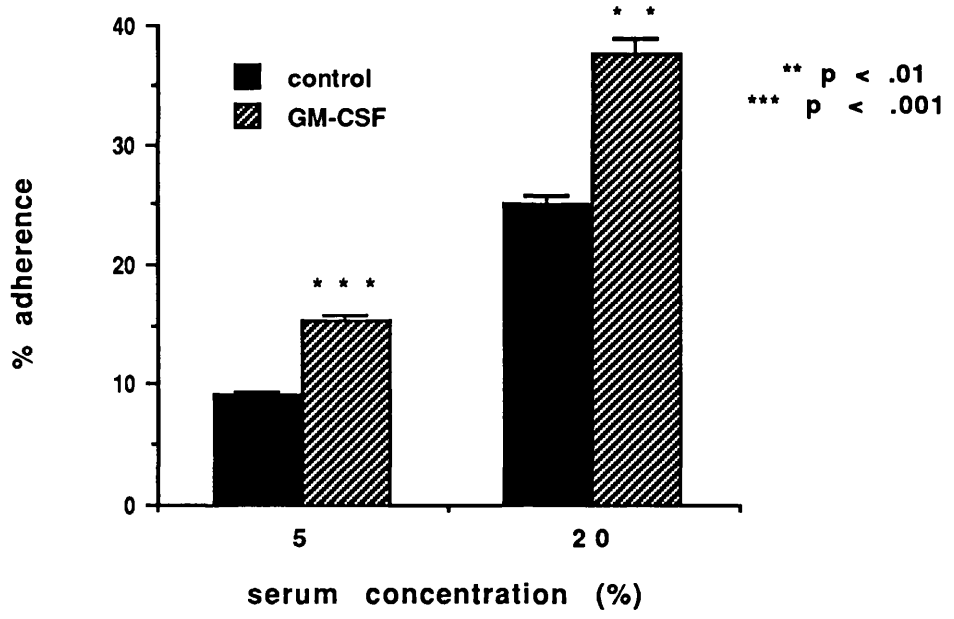
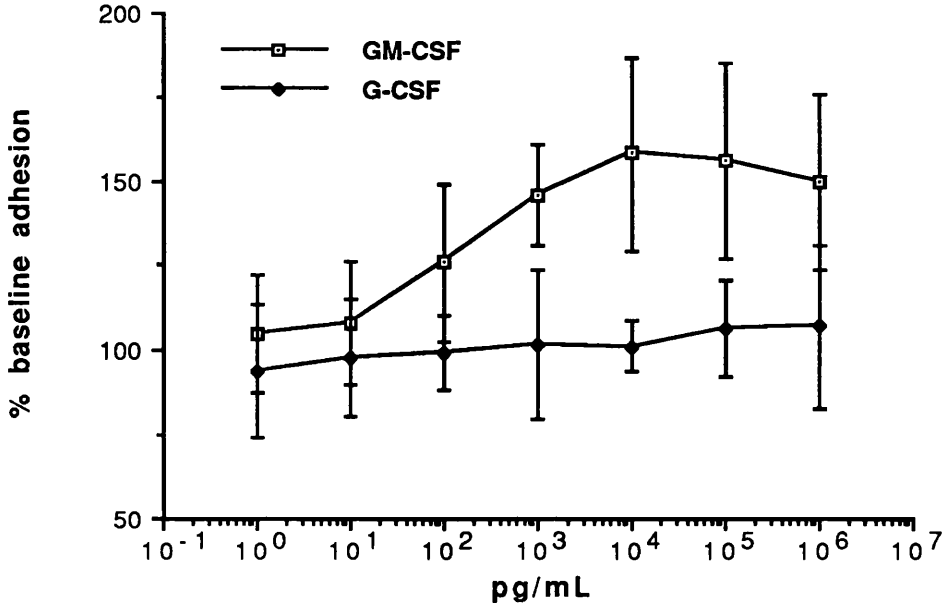


Figure 3.5. Effect of GM-CSF on neutrophil adhesion to HUVEC under different serum conditions. (a) Percentage neutrophil adhesion in 1 experiment, which is representative of 2 (b) Percentage adhesion in 5% (10 experiments) and 20% (5 experiments) serum, mean ± SD.

In order to examine the kinetics of GM-CSF enhanced adhesion to endothelium, separate plates of endothelial monolayers were set up, for each time point of an adhesion assay. Neutrophils were added to all plates simultaneously, together with GM-CSF, TPA, or medium as control, the plates were spun at 400 rpm for 1 minute in order to bring cells into direct contact with the endothelial monolayer, and then placed in an incubator. At each time point, nonadherent cells were removed from one plate, and the number of adherent cells quantitated as detailed above. Standard curves using doubling dilutions of known numbers of neutrophils were included in each plate. In order to control for the release of neutrophil alkaline phosphatase from cells stimulated by GM-CSF or TPA, separate standard curves were constructed for each condition. Aliquots of cell suspensions were incubated in microfuge tubes with GM-CSF, TPA or medium. At each time point, one set of tubes were centrifuged, and the supernatant removed, the cells were resuspended in saline and the counts adjusted before appropriate aliquots were placed in the microtitre wells for the standard curve. This was done for each time point. GM-CSF incubated cells demonstrated no loss of alkaline phosphatase activity, whereas TPA stimulated cells showed a loss of up to 50% (see Appendix 1). GM-CSF enhanced adherence is evident at 15 minutes, and sustained for at least 2 hours (Figure 3.5d). In contrast, TPA stimulated adhesion peaks by 30 minutes, following which there is a sharp decline approaching baseline levels. There is no significant change in the adherence of control cells over this time period (Figure 3.5d).

TPA was chosen as a neutrophil agonist in this series of experiments on the kinetics of neutrophil adhesion because it had been previously demonstrated (Lo et al, 1989) that TPA induced adhesion is transient, with a time course similar to the margination response seen *in vivo* following GM-CSF administration. It was therefore of interest to see if GM-CSF induced adhesion *in vitro* was also transient and reversible.

(c)



(d)

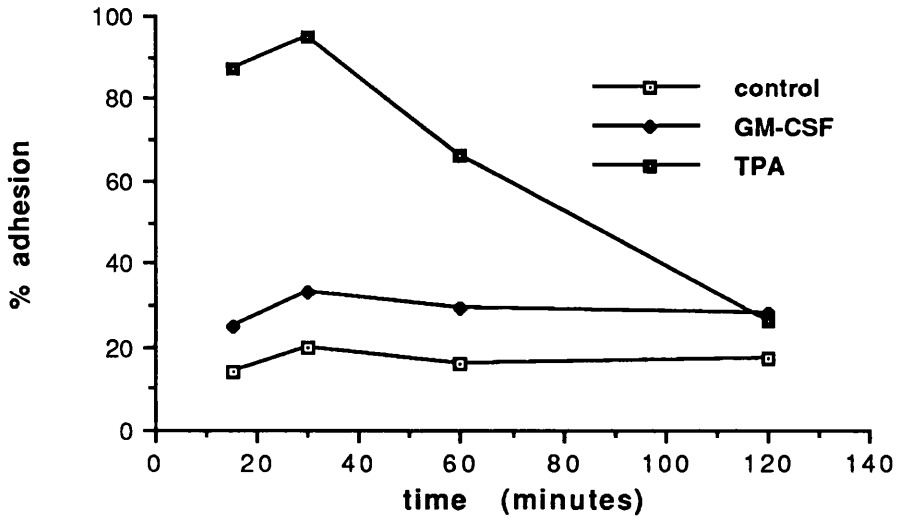
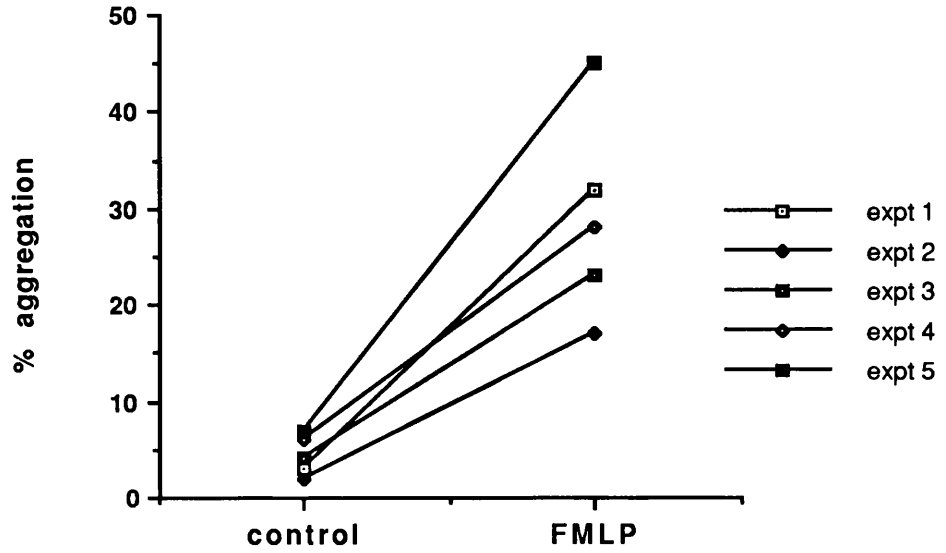


FIGURE 3.5 (c). GM-CSF enhances neutrophil adhesion to HUVEC in a dose dependent manner, while G-CSF is without effect. Data, expressed as percentage of baseline adherence (adherence of cells in the presence of medium alone), is mean ± SD of 3 experiments. (d) Different kinetics of GM-CSF and TPA induced neutrophil adhesion to HUVEC. Percentage of adherent neutrophils is given for 1 experiment, which is representative of 2.

Neutrophil aggregation is not enhanced by G- and GM-CSF

Neutrophil aggregation experiments were carried out in the absence of Cytochalasin B, and in serum-free medium, as it was found, in preliminary experiments, that considerable spontaneous aggregation occurred even at low serum concentrations. 5 experiments were performed using FMLP and GM-CSF. In all 5 experiments, FMLP (10^{-6} M for 3 minutes at 37°C) produced a marked aggregation response (Figure 3.6a). GM-CSF (100 ng/mL for 5 minutes at 37°C), on the other hand, had no direct effect on neutrophil aggregation in 3 out of the 5 experiments (Figure 3.6b). In 2 experiments (experiments 3 and 5) there was a small effect of GM-CSF, producing 10% and 20% aggregation respectively, while aggregation in the presence of buffer was 4% and 7% respectively (Figure 3.6b). Any aggregation response seen in the presence of GM-CSF was maximal by 5 minutes, while the aggregation response to FMLP was maximal by 2-3 minutes, following which the cells began to disaggregate (data not shown). G-CSF did not cause neutrophil aggregation above spontaneous levels (data not shown).

(a)



(b)

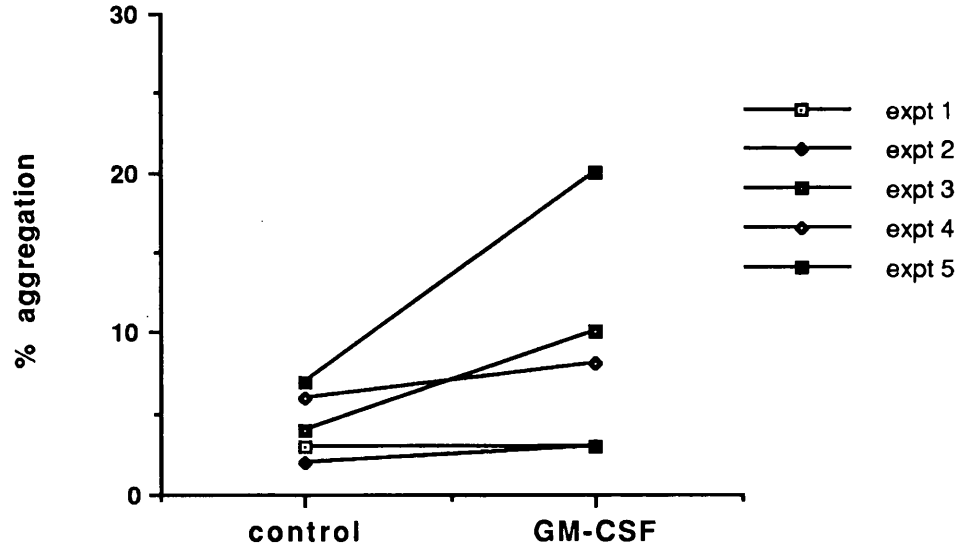


FIGURE 3.6. Effect of (a) FMLP and (b) GM-CSF on neutrophil aggregation. Data, expressed as percentage aggregation, is given for 5 individual experiments for each agonist.

DISCUSSION

Both GM-CSF and G-CSF modulate the surface expression of adhesion receptors on phagocytes *in vitro*. Stimulation by these growth factors at 37°C induces a rise in surface expression of the integrin receptors, CD11/CD18, but a fall in surface levels of L-selectin. Both these effects occur rapidly, are near maximal after 30 minutes, and are sustained for up to 4 hours. GM-CSF is more potent than G-CSF, being maximally effective at doses of 1 ng/mL (71 pM) or greater, while G-CSF requires concentrations of 100 ng/mL (5.9 nM) or greater for maximal response. Both these growth factors act on neutrophils and monocytes isolated from normal healthy donors, while M-CSF is without effect. Both GM-CSF and G-CSF are equally effective in upregulating phagocyte surface expression of the leucocyte integrins, in particular, CD11b and CD11c. In contrast, GM-CSF is more effective at downregulating L-selectin levels, producing up to a 75% fall, while G-CSF only achieves a reduction about 50% at maximally effective doses.

GM-CSF and G-CSF also differ in their ability to augment the adhesive functions of neutrophils *in vitro*. GM-CSF stimulation produces a significant and dose dependent enhancement of the adherence of peripheral blood neutrophils to cultured human endothelial cells *in vitro*, while G-CSF has no effect. GM-CSF is maximally effective at concentrations of 10 ng/mL (714 pM). G-CSF does not cause peripheral blood neutrophils to aggregate, while, in some individuals, GM-CSF has a small effect.

The upregulation of the CD11b/CD18 integrin receptors by G- and GM-CSF *in vitro* is in accord with previous reports (Arnaout et al, 1986, Devereux et al, 1989, Yuo et al, 1989). The downregulation of L-selectin in response to stimuli such as GM-CSF has also been previously reported for human neutrophils and monocytes (Griffin et al, 1990), with a similar magnitude of loss of this receptor as we have shown in these studies (65-75% loss). However, Griffin et al reported that G-CSF was without effect, while the data presented here show clearly that G-CSF also induces downregulation of L-selectin on neutrophils and monocytes, albeit to a lesser degree. The studies reported here were performed in whole blood, whereas those reported by Griffin were done on purified cells. However, similar results were obtained in these studies using purified neutrophils (Figure 3.4b), confirming that G-CSF does induce downregulation of surface L-selectin levels on phagocytes, but to a lesser extent than GM-CSF.

GM-CSF enhancement of neutrophil adhesion to cultured endothelium has been previously reported using radiolabelled neutrophils (Devereux et al, 1989). However, an earlier study failed to demonstrate any effect of GM-CSF on neutrophil adhesion to endothelium in vitro (Lopez et al, 1986). This difference in results may relate to the way in which neutrophils are isolated. Purification procedures themselves are known to cause cell activation (Fearon & Collins, 1983, Kuijpers et al, 1991), as evidenced both by a rise in CD11b/CD18 receptor as well as a fall in L-selectin receptor levels (Griffin et al, 1990). Furthermore, incubation at 37°C, in the absence of agonist, also produces a rise of CD11b, and a fall in L-selectin expression, as described above. Hence radiolabelled cells may already be partially activated and hence unable to demonstrate enhanced adhesion in the presence of a stimulus which is not maximally proadhesive, while still being able to respond to very proadhesive stimuli like phorbol esters. Using an adherence assay which avoids a radiolabelling step, the data reported here confirm that GM-CSF increases the binding of peripheral blood neutrophils to endothelial monolayers under static conditions *in vitro.* It is perhaps not surprising that Lopez et al failed to find any effect of GM-CSF at doses up to 2 ng/mL (100 pM), because the studies presented here show that maximal effects are only achieved at doses of 10 ng/mL or greater. GM-CSF enhanced adhesion occurs within15 minutes of stimulation, and is sustained at the same levels for up to 2 hours, in contrast with phobol ester stimulated adhesion, which also occurs rapidly, but which is transient, and declines after 30 minutes. The transient nature of phobol ester induced neutrophil adherence to endothelium has been commented on by others (Lo et al, 1989a). This difference between GM-CSF and TPA in their effects on neutrophil adhesion is supported by studies on leucocyte adhesion deficency neutrophils, as discussed in Chapter 8.

The lack of a reliable aggregation response to GM-CSF contrasts with a previous report by Arnaout et al (1986) who showed that at 2 ng/mL, rhGM-CSF was able to induce granulocyte aggregation, as measured by an increase in light transmission. This difference in results may relate to the fact that, in the studies reported by Arnaout et al, neutrophil aggregation was studied in the presence of Cytochalasin B.

The question arises as to whether, and to what extent the modulation of surface receptors can account for growth factor induced neutrophil adhesion, both homotypic and heterotypic. In particular, CD11b has been shown to be crucial in mediating the adhesive functions of neutrophils and monocytes. Agonists which upregulate the surface expression of these receptors on phagocytes also augment the adherence related functions of these cells, within the same dose range. A simple explanation would be that quantitative increases in cell surface receptor numbers leads to

enhanced cell adhesion. However, there is accumulating evidence that the functional activity of the β2 integrin receptors is not simply directly related to the quantity of cell surface protein expressed. There is a disparity between the kinetics of surface receptor upregulation, and the increased homotypic, and heterotypic adhesive responses of neutrophils, which has been noted in studies employing the chemotactic bacterial peptide, FMLP and phorbol esters as neutrophil agonists (Buyon et al, 1988, Phillips et al, 1988, Lo et al, 1989a). Chemotactic factor induced receptor upregulation, like that mediated by GM-CSF and G-CSF, occurs gradually over 30 minutes, and is sustained for up to 2 hours. Homotypic aggregation is maximal by 2 minutes, and disaggregation occurs by 5 minutes. Furthermore, the magnitude of the aggregation response of neutrophils does not correlate with the degree of surface molecule upregulation with different agonists (Buyon et al, 1988, Vedder & Harlan, 1988). Neutroplasts, which do not contain intracellular stores of integrin receptors are able to aggregate in response to FMLP, suggesting that upregulation of new surface receptors is not required. The effect, however, is blocked by anti-CD11b MoAbs. Precoating neutrophils with anti-CD11b MoAb and washing before activation inhibits the aggregation response without affecting the upregulation of new receptors (Buyon et al, 1988).

Similarly, phorbol ester induced neutrophil adhesion to endothelium is maximal at 15 minutes, but falls to baseline by 60 minutes, when CD11b expression remains high. In contrast, GM-CSF induced neutrophil adhesion to endothelium is sustained and would appear to parallel the increased surface expression of CD11b rather more closely. However, anti-CD11b MoAbs are able to abolish the increase in adherence in response to both agonists (Devereux et al, 1989, Lo et al, 1989, Yong et al, 1992), suggesting, once again, that quantitative changes in surface expression are not directly related to the enhanced adhesion.

Qualitative, rather than quantitative changes in surface integrin molecules may be important in functional activation, and newly upregulated molecules may not be competent to mediate adhesive responses. Cell activation can affect the binding activity of surface adhesion receptors without altering the amount of surface antigen expressed, and this has now been demonstrated for several of the integrin receptors. Firstly, cell activation can lead to exposure of new ligand binding sites, or 'activation epitopes', such as the binding of Factor X and fibrinogen by CD11b on monocytes and neutrophils activated by adenosine diphosphate (ADP), without any quantitative change in surface receptor levels (Altieri et al, 1988a,b). Cell activation can also lead to an increased avidity of surface receptors for their ligands, without causing any quantitative change in receptor levels. Functional interactions with other membrane receptors may be important. Binding of ELAM-1 by neutrophils leads to

enhanced adhesive activity of CD11b (Lo et al, 1991). Similar effects have been demonstrated for other integrin receptors. In lymphocytes, cell activation via TCR crosslinking leads to a transient increase in CD11a binding without any change in surface receptor density (Dustin & Springer, 1989). Similarly, the binding activity of the β1 integrins, or VLA receptors, is transiently increased upon lymphocyte activation (Shimizu et al, 1990). Therefore it is not clear to what extent, and how the upregulation of the CD11/CD18 receptor levels by G- and GM-CSF is directly related to the enhanced neutrophil-endothelial interactions demonstrated in these and other studies.

A further question relates to the relevance of the L-selectin molecule, which is downregulated by GM-CSF and G-CSF, in contrast to the integrin receptors. The fall in surface receptor density is rapid, and is sustained for up to 4 hours *in vitro*. A variety of other agonists are also able to downregulate L-selectin on neutrophils and monocytes *in vitro*. Stimulation with phorbol esters, chemotactic peptides (FMLP), leukotriene B4 and cytokines such as TNF leads to rapid downregulation of the MEL-14/L-selectin receptor on murine and human neutrophils (Kishimoto et al, 1989, Griffin et al, 1990). Therefore, levels of L-selectin appear to reflect the state of activation of these cells, activated cells express low levels, and vice versa.

A role for this receptor in mediating neutrophil adhesive functions *in vivo* was suggested initially by studies in the murine system, where neutrophils which are either activated *in vitro*, and hence have low levels of MEL-14, or have been treated with monoclonal antibody to MEL-14, *in vivo* or *in vitro*, are unable to migrate into inflammatory areas *in vivo* (Jutila et al, 1989). In addition, the loss of surface expression of this molecule on activated neutrophils appears to be associated with extravasation of cells *in vivo* (Kishimoto et al, 1989). Neutrophils which have localised in inflamed tissues have decreased levels of L-selectin. In another study, a soluble immunoglobulin chimaera containing the extracellular domain of MEL-14 was able to block neutrophil accumulation into the inflamed peritoneum *in vivo* (Watson et al, 1991). The downregulation of L-selectin on cell activation may provide a mechanism for the detachment of adherent cells which is required for leucocyte migration into inflammatory areas. The situation is likely to be far more complex, however, as the L-selectin molecule itself has recently been shown to exhibit a transient increase in affinity following cell activation, prior to the actual loss of receptors from the cell surface (Spertini et al, 1991). Neutrophils stimulated with G-CSF, GM-CSF or TNF for 5 minutes at 37°C showed increased binding of the fluoresceinated ligand, PPME, followed by a fall in binding, due to the subsequent shedding of the receptor, which occurs maximally by 30 minutes. Hence

activation induced changes in the binding activity of the L-selectin receptor might underlie the initial neutrophil-endothelial interactions which precede the migration of neutrophils into inflammatory tissues. The roles of the β2 integrins, and L-selectin, in mediating distinct types of neutrophil-endothelial interactions at different stages in the immune response is discussed further in Chapters 8 and 11.

CHAPTER FOUR

EFFECTS OF GM-CSF *IN VIVO*: A PRIMATE MODEL FOR GM-CSF INDUCED NEUTROPHIL ADHESION

INTRODUCTION

Systemic administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) in man results in an immediate transient fall in circulating neutrophils, monocytes and eosinophils (Devereux et al, 1987, Lindemann et al, 1989, Morstyn et al, 1988). Recovery of peripheral leucocyte numbers occurs within 2-3 hours, following which there is a steady increase in neutrophils, with appearance of immature forms. Radiolabelling of leucocytes has shown that this transient leucopenia is associated with the sequestration of cells, predominantly in the lungs, and that the recovery of peripheral counts coincides with the release of labelled cells back into the circulation (Devereux et al, 1989). The margination of phagocytes following GM-CSF administration may be important clinically, particularly as large vein thrombosis, generalised erythroderma and the capillary leak syndrome with fluid retention, pleural and pericardial effusions have been reported at high doses of the growth factor (Brandt et al, 1988, Lieschke et al,1989a, Haas et al, 1990).

In the investigation of the effects of GM-CSF on neutrophil-endothelial adhesion in vivo, a nonhuman primate model was used in order to afford histological analysis. GM-CSF displays some species specificity, and rhGM-CSF has no effect on murine haemopoietic cells (Wong et al, 1985), but is biologically active in nonhuman primate systems (*rhesus* and *cynomolgus* monkeys), both *in vitro*, to induce proliferation and differentiation of bone marrow cells and *in vivo*, producing a leucocytosis and activation of peripheral neutrophils (Donahue et al, 1986, Mayer et al, 1987). *Cynomolgus* was selected as an appropriate nonhuman primate model in which to study the *in vivo* effects of GM-CSF on neutrophil adhesion.

SPECIAL METHODS

Animals

Adult *Macaque fascicularis* monkeys were used in the study. Anaesthesia was induced with ketamine (Parke-Davis Medical, Eastleigh, UK) and maintained using propofol (ICI Pharmaceuticals, Wilmslow, UK). Vascular access was achieved using a cut-down technique in the groin, and the femoral vein and artery were cannulated separately. GM-CSF, at 2 μg/kg or 15 μg/kg, in 0.9% saline was administered by slow intravenous bolus injection over 5 minutes. Venous blood samples were drawn prior to starting the infusion, and at regular time intervals afterwards. Control animals received a bolus injection of saline in the same volume.

A technique of perfusion-fixation was employed in order to preserve tissues for histological examination. At 25 minutes after the injection of GM-CSF, flush-through of the circulation was commenced with rapid venous infusion of Hartmann's solution at 37°C accompanied by simultaneous drainage via the femoral artery. When the arterial outflow was very dilute the infusion fluid was changed to 4% glutaraldehyde in Hartmann's solution (sodium lactate intravenous infusion) at 37°C, and flush-through continued as rapidly as possible. Asystole occurred within 1-2 minutes of commencing glutaraldehyde perfusion, which was carried on for 20-30 minutes. Postmortems were performed on the animals and lungs and kidneys were removed and preserved in glutaraldehyde.

Peripheral blood counts

Blood counts were done on an automated S-Plus STKR (Coulter Electronics), and white blood differentials were performed manually on blood films stained with May-Grunwald-Giemsa.

Leucocyte phenotyping

Heparinised venous blood from animals was taken into microfuge tubes and placed immediately on ice. Cell surface antigen density (CD11b) was determined by indirect immunofluorescence performed in whole blood using MoAb 44, and samples were analysed on an EPICS CD flow cytometer (Coulter Electronics, Luton, U.K.) as detailed in chapter 2. In some experiments, heparinised blood samples from animals not receiving infusions were preincubated *in vitro* with GM-CSF 10 ng/mL or RPMI as control for 30 minutes at 37°C before being placed on ice, and examined for surface CD11b expression as detailed above.

Histological processing of samples

These procedures were performed by P M Rowles, Histopathology Department, University College and Middlesex School of Medicine. For light microscopy, representative cross-sectional areas of the fixed lung tissue were processed through to paraffin wax in a VIP tissue processor (Miles Scientific, UK). Sections of 15 mm thickness were cut from the blocks and stained with haematoxylin and eosin. Adjacent tissue samples were selected for examination by Scanning electron microscopy. These samples, about 4 mm in thickness, were dehydrated in ethyl alcohol, frozen in liquid nitrogen and fractured into several pieces. The fractured surfaces revealed the luminal surfaces of blood vessels, bronchioles, alveolar ducts and sacs. The fractured pieces of tissue were rehydrated, secondarily fixed in 1% osmium tetroxide and dehydrated through a graded series of ethyl alcohol. They were then transferred to Arklone (ICI Chemicals, UK) and critically point dried using carbon dioxide as the transitional fluid. Specimens were mounted onto aluminium stubs, coated with gold using a diode sputter coater (E500, Polaron Ltd.) and examined on an ISI DS130 scanning electron microscope at magnifications between 300 and 8000 times at an accelerating voltage of 20 kV.

Assessment of neutrophil adherence in vivo

This was done by two independent observers who were ignorant of the treatment group of each animal. For quantitation of pulmonary leucostasis, sections of lung were selected which contained only alveolar septae, with no bronchiolar tissue. One section was selected from each lung of each animal. Using an eyepiece graticule and a X40 objective, the number of neutrophils within the graticule grid of 10 microscopic fields, chosen at random from each of the lung sections was counted. 2 sections, 1 from each lung, were examined for each animal.

RESULTS

Cynomolgus neutrophils express the antigen which is recognised by MoAb 44, and which is upregulated by GM-CSF in vitro.

Preliminary studies were carried out to characterize the expression and regulation of CD11b on *cynomolgus* leucocytes, using MoAb 44. Figure 4.1 shows the results of indirect immunofluorescence staining of cells in heparinised whole blood. The cell population staining positive with MoAb 44 are the neutrophils (panel e) and the fluorescence intensity of these cells increases following incubation with GM-CSF (panels c and f). In whole blood samples (from 2 animals not receiving infusions), *in vitro* incubation with GM-CSF 10 ng/mL for 30 minutes at 37°C produced a rise in neutrophil surface CD11b expression to 184% and 140% of control (incubated with medium alone, Figure 4.2). Thus the surface antigen recognised by MoAb44 on *cynomolgus* neutrophils is upregulated by GM-CSF *in vitro* in the same way as it is on human neutrophils (see Chapter 3).

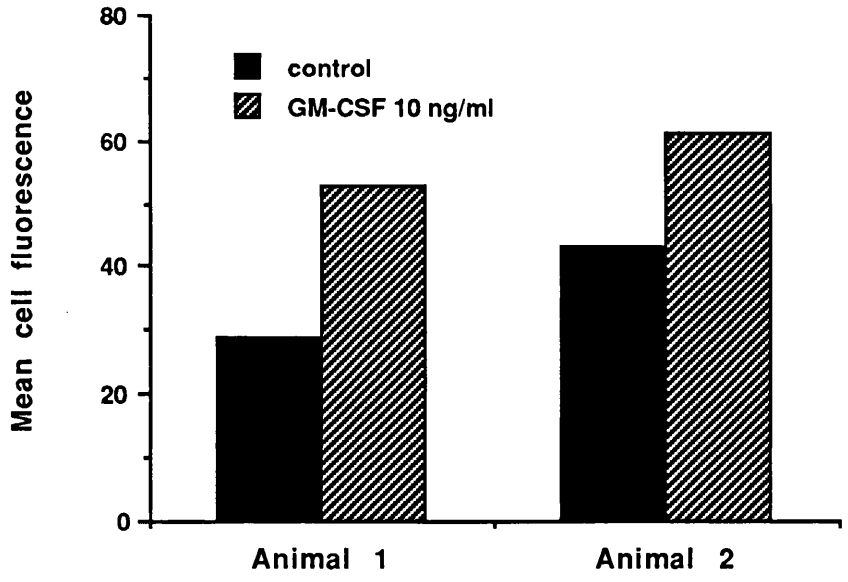


Figure 4.2. Effect of GM-CSF (100 ng/mL) on CD11b expression of *cynomolgus* neutrophils. MCF (mean of duplicates) of cells from 2 separate animals.

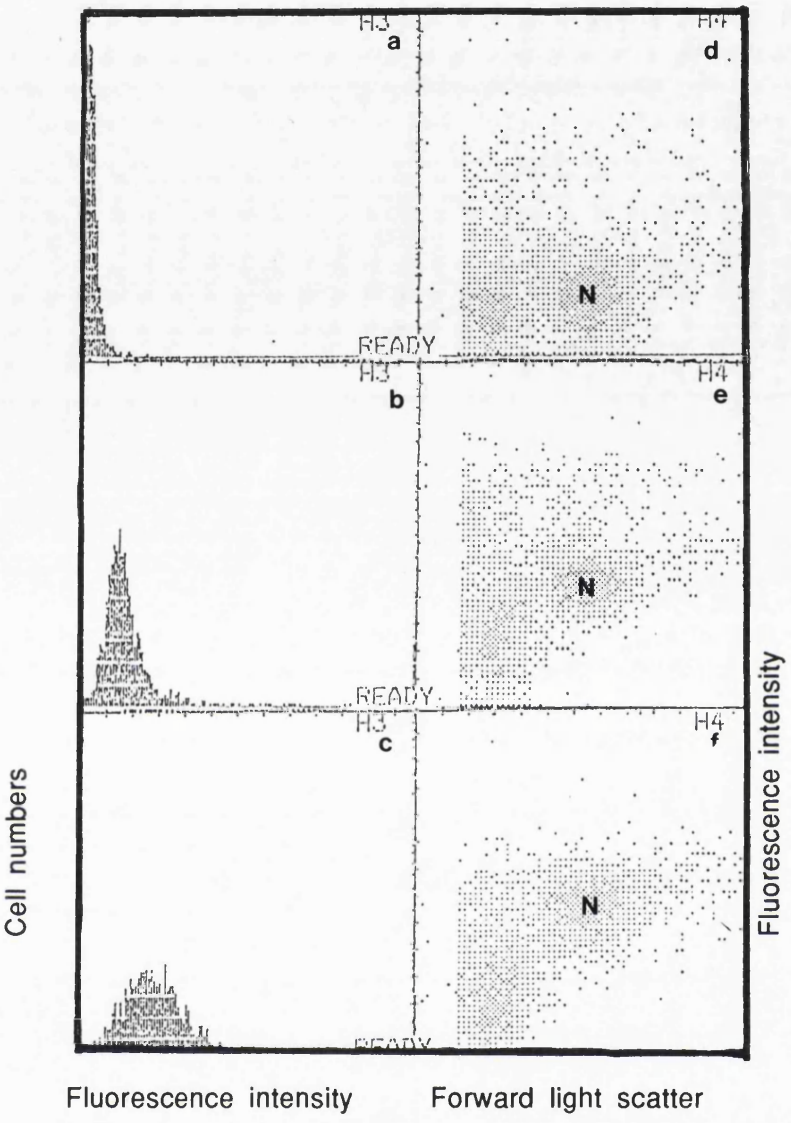


Figure 4.1. Surface CD11b expression on *cynomolgus* leucocytes. Immunofluorescence profiles showing, in panels (a)-(c), cell number against fluorescence intensity, and (d)-(f), fluorescence intensity against forward light scatter. The neutrophil cloud (**N**) stains positive, and staining increases following GM-CSF (10 ng/mL for 30 minutes).

Effect of in vivo GM-CSF on peripheral cell counts and CD11b expression in cynomolgus

Administration of an intravenous bolus of GM-CSF (2 or 15 μg/kg) to *cynomolgus* monkeys produces a rapid fall in circulating neutrophils and monocytes, but is without significant effect on the peripheral lymphocyte count (Figure 4.3a). There was no significant difference in effect between the two doses of GM-CSF used. The data (mean ± SD) is shown for 4 animals which received GM-CSF. Figure 4.3b shows the cell counts in a control animal which received an injection of saline, and is representative of 2 control animals.

Associated with the disappearance of phagocytes from the peripheral circulation is a rise in the surface expression of CD11b on circulating neutrophils (Figure 4.4). The surface levels of this adhesion molecule continue to rise even when the cells start returning into the peripheral circulation. Figure 4.5 shows the fluorescence profiles of whole blood samples drawn at various times from an animal receiving 15 μg/kg of GM-CSF, stained with MoAb 44. Fluorescence intensity of neutrophils starts to rise by 15 minutes (panel c) and is still elevated at 135 minutes (panel e)

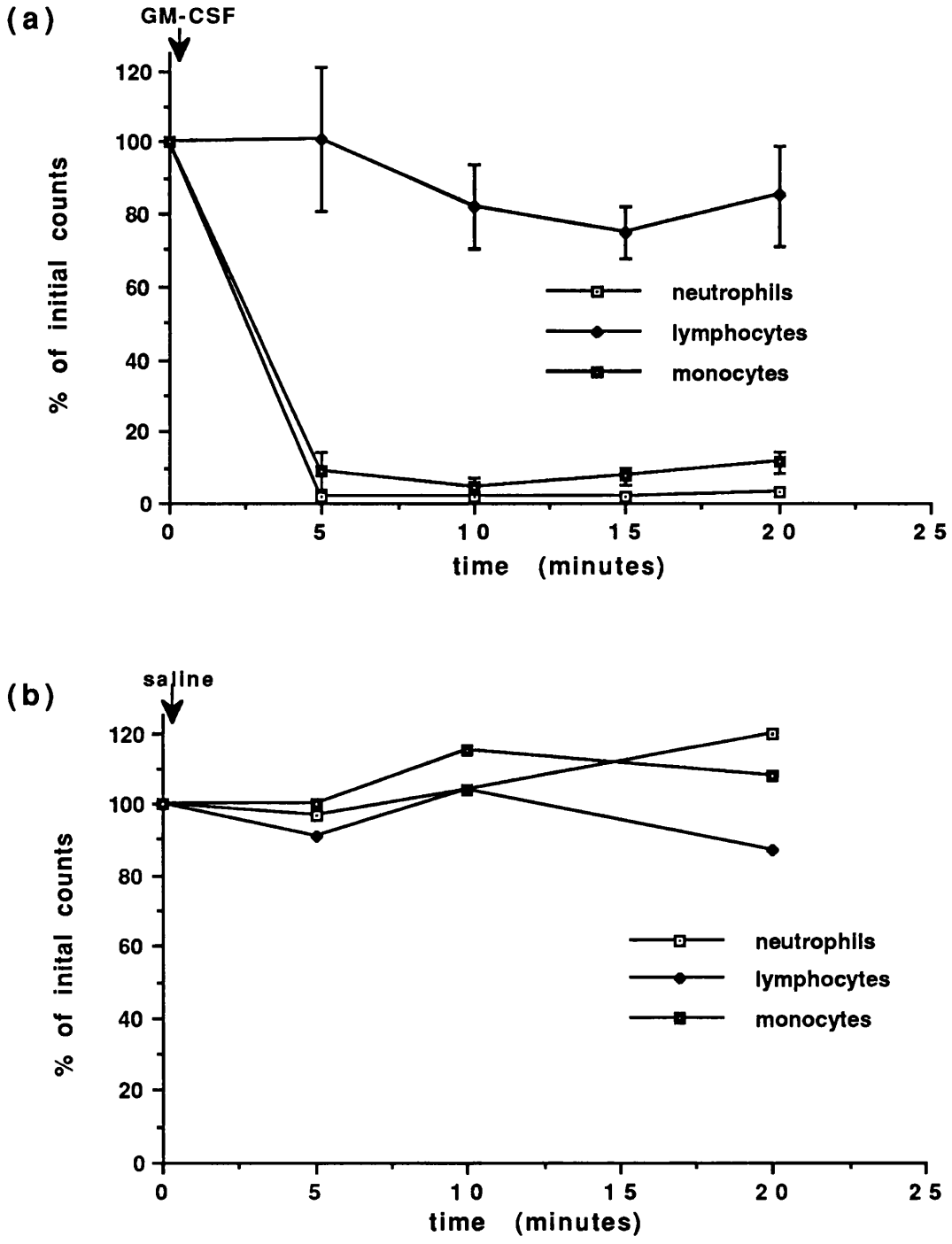


Figure 4.3. Leucocyte counts, expressed as percentage of initial counts, in (a) 4 animals receiving GM-CSF (2 or 15 μg/kg over 5 minutes), mean ± SD and (b) in 1 control animal, representative of 2 which received saline injections.

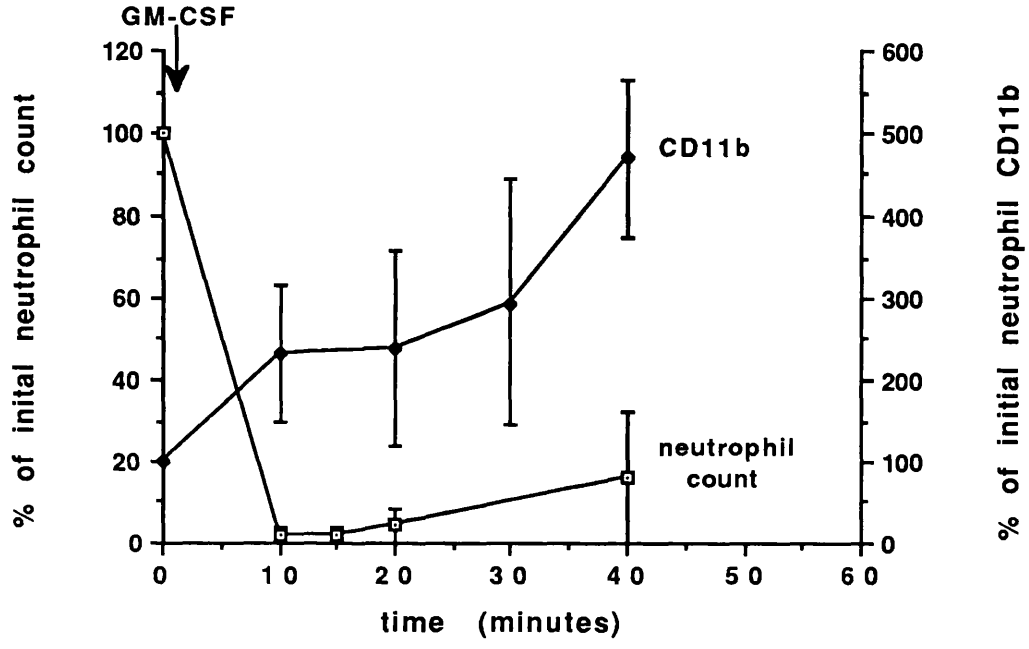


Figure 4.4. Concomitant changes in neutrophil counts and CD11b expression in 3 animals receiving GM-CSF (2 μg/kg). Mean ± SD percentage of initial levels.

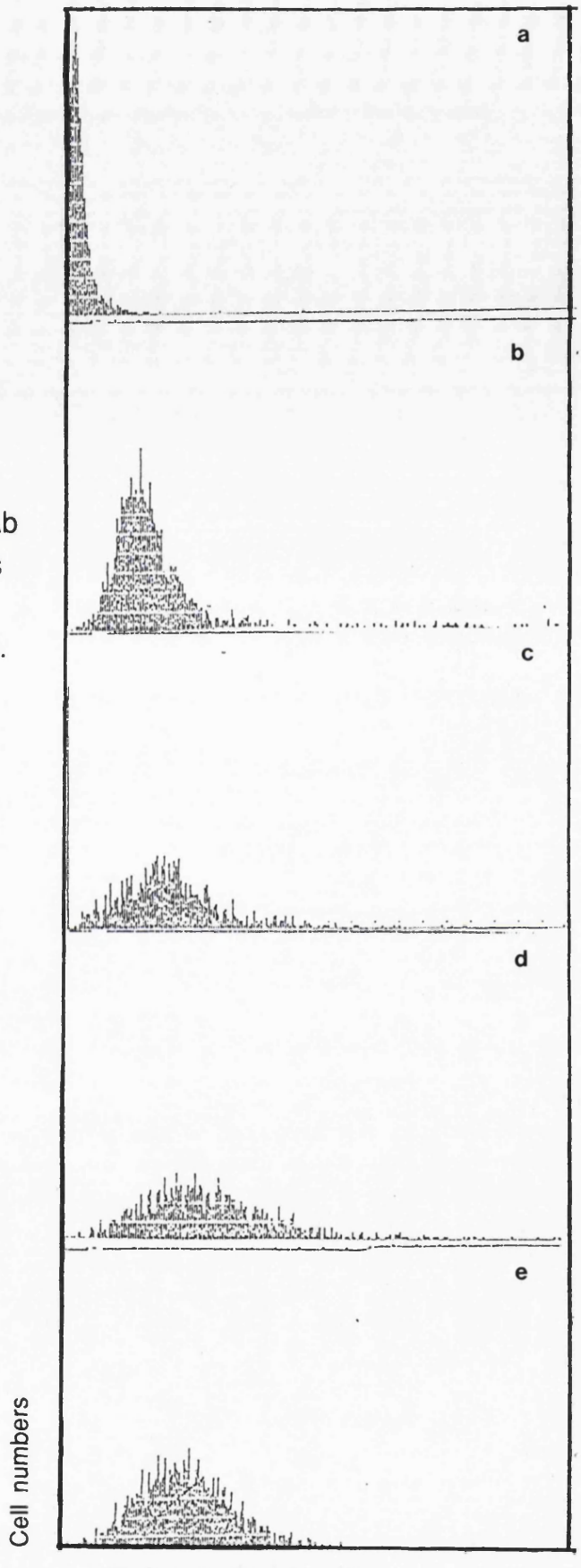


Figure 4.5.
Immunofluorescence profiles of whole blood samples stained with MoAb 44, at various time points following GM-CSF (15 μg/kg) administration. Cell numbers are plotted against fluorescence intensity;
a, negative control,
b, 0 minutes,
c, 15 minutes,
d, 75 minutes, and
e, 135 minutes

GM-CSF induces adherence of neutrophils to pulmonary vascular endothelium

Histological sections of animal lungs show a great increase in the number of neutrophils in the pulmonary microcirculation of animals receiving GM-CSF, particularly in the alveolar capillaries (Figure 4.6a). All neutrophils were seen to be contained within the vessels. In contrast, in lung sections from control animals, there are very few neutrophils to be seen in the pulmonary microvasculature (Figure 4.6b). The paucity of red cells in these sections suggests that the flush through was complete, and therefore the leucocytes seen remaining in the microcirculation are adherent to endothelium. Figure 4.7 shows the number of neutrophils counted in lung sections taken from each of 6 animals, 4 of which received GM-CSF, and the other 2 received control bolus injections of saline. Lung sections from animals receiving GM-CSF contained 36 ± 8, 17 ± 7, 21 ± 6, 15 ± 8 (mean ± SD, n=20) neutrophils, counted within a graticule grid, per microscopic field viewed under a X40 objective, as compared to the 2 control animals receiving saline injections whose lung sections contained 2.1 ± 1.6 and 3.1 ± 2.1 (mean ± SD, n=20) neutrophils, counted in the same way. 20 fields, 10 from a section of each lung, were counted for each animal by each observer. Scanning electron microscopy shows leucocytes within alveolar capillaries (Figure 4.6c), and also adherent to the endothelial surface in larger vessels (Figure 4.6d). The adherent cells show membrane ruffling but there is little morphological evidence of endothelial cell damage. No neutrophils were seen to be migrating out of the circulation into the alveolar spaces.

Effect of maternal administration of GM-CSF on foetal heart rate

One animal who received GM-CSF at 15 μg/kg was pregnant, and foetal heart rate was monitored by ultrasound throughout the procedure. Within 5 minutes of the bolus injection of GM-CSF into the mother, there was a marked foetal bradycardia which lasted 5-10 minutes (Figure 4.8). Maternal heart rate showed no change.

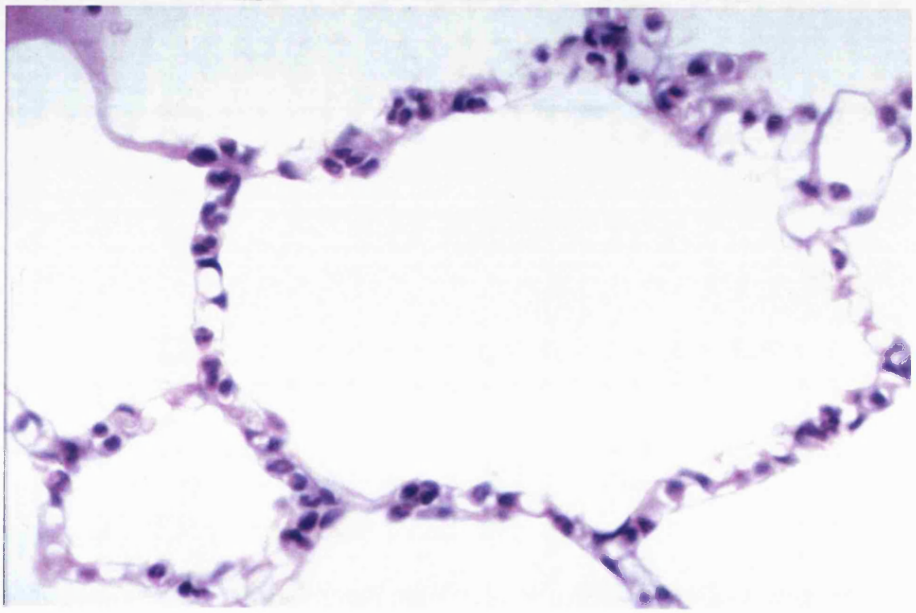


Figure 4.6 (a). Haematoxylin and eosin stained lung section (7 micron thick) taken from an animal receiving GM-CSF (2 μg/kg). Magnification X400

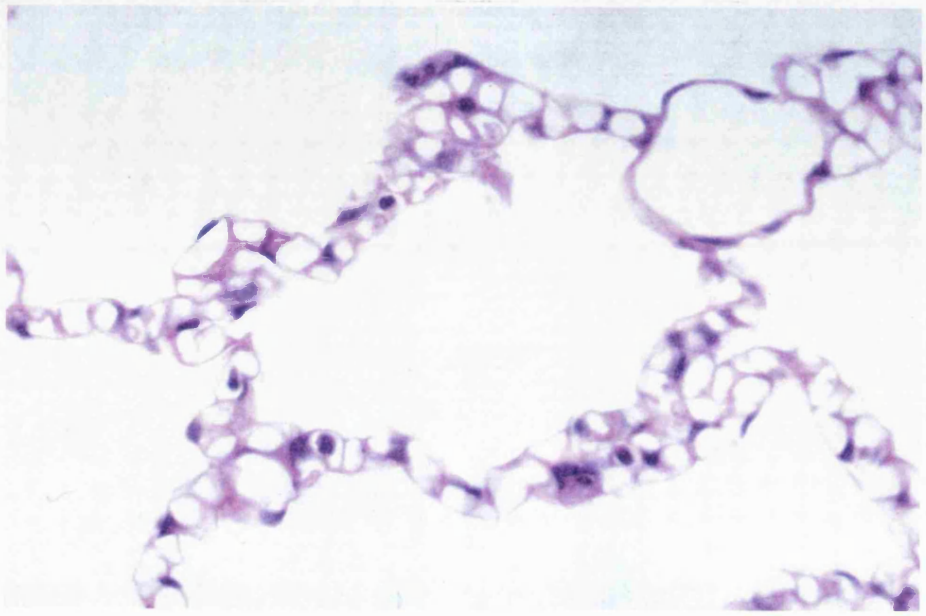
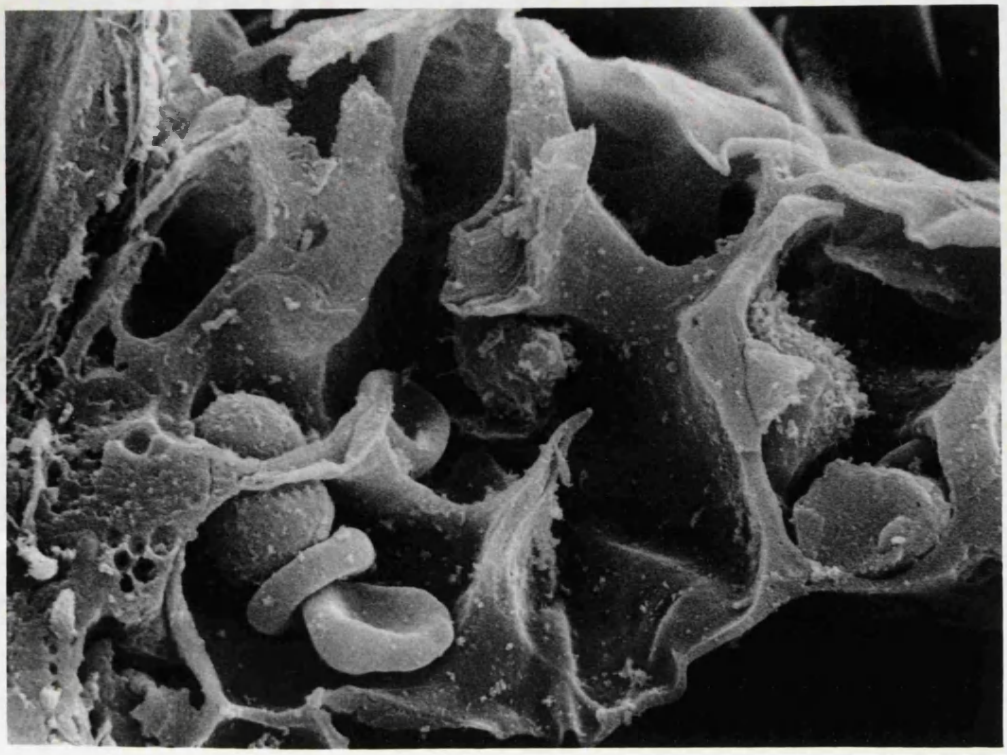


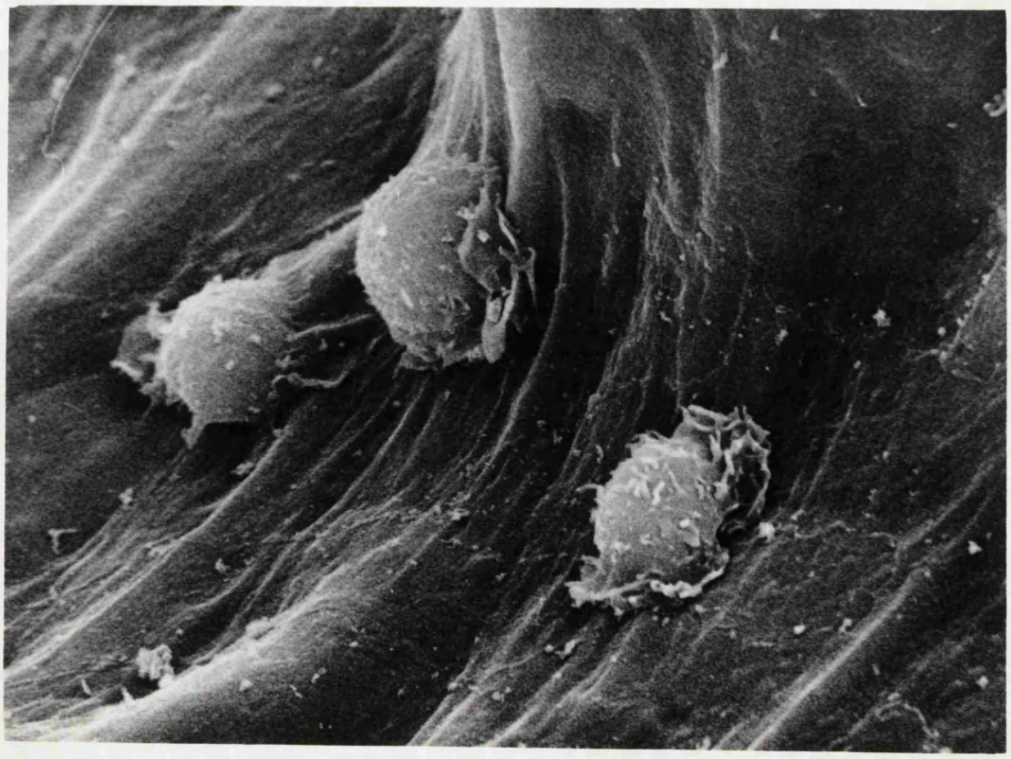
Figure 4.6 (b). Haematoxylin and eosin stained lung section (7 micron thick) taken from a control animal which received a saline injection. Magnification X400

Legends to figures on page 77

Figure 4.6(c) Scanning electron micrograph of cynomolgus lungs taken 30 minutes after administration of GM-CSF, showing leucocytes contained within alveolar capillaries.

Figure 4.6(d) Scanning electron micrograph of lung section from cynomolgus 30 minutes following GM-CSF (2 μg/kg) administration, showing leucocytes adherent to the endothelial surface of a large vessel.





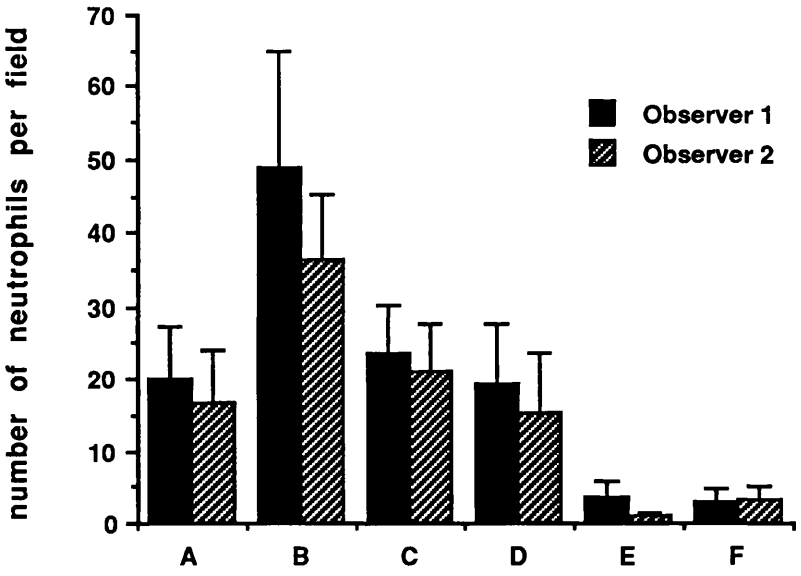


Figure 4.7. Effect of GM-CSF on neutrophil numbers in lung sections, counted by 2 independent observers. 20 microscopic fields, 10 from a section of each lung, were examined for each of 4 animals receiving GM-CSF at 2 μg/kg (B, C), or 15 μg/kg (A, D), and 2 control animals which received saline injections. Data is mean ± SD (n=20) of the number of neutrophils counted within the graticule grid for each animal by each observer.

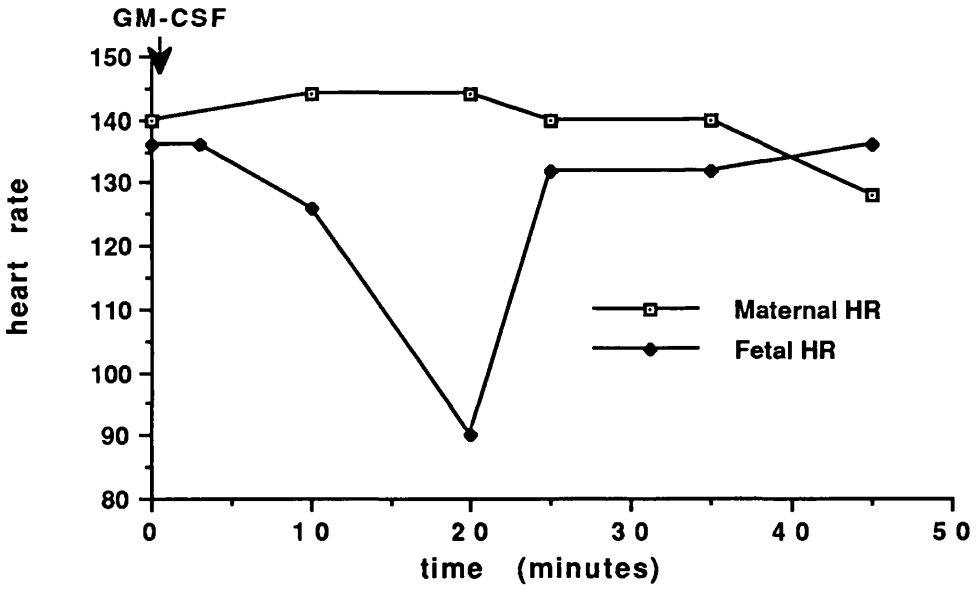


Figure 4.8. Effect of GM-CSF (15 μg/kg) on foetal and maternal heart rates in 1 animal.

DISCUSSION

In vivo administration of GM-CSF in *cynomolgus* produces a rapid transient leucopenia, associated with a rise in CD11b expression on circulating neutrophils. Expression of the CD11b receptor on *cynomolgus* neutrophils is also upregulated following incubation with GM-CSF *in vitro*. Although the degree of upregulation in the two animals tested in this way is modest when compared with the effects in man (see Chapter 3), this difference could relate to the delay caused by transporting the blood samples some 30 miles back to the laboratory before experiments could be performed. The similarity of these effects to those seen in man establish *cynomolgus* as a suitable primate model in which to study GM-CSF induced neutrophil adhesion *in vivo*.

These studies confirm that the transient neutropenia following GM-CSF administration is associated with increased numbers of neutrophils in the pulmonary microvasculature. These cells appear to be adherent to the endothelium and show evidence of activation (membrane ruffling), but there is no evidence of transendothelial migration of marginated cells, and no morphological evidence of damage to the pulmonary vascular endothelium , as seen on scanning electron microscopy.

The relationship between enhanced neutrophil adhesion to vascular endothelium in the lungs, and increased expression of CD11b on these cells remains unclear. The disparate kinetics between the two processes has been noted in humans (Socinski et al, 1989, Devereux et al, 1989), and is also seen in the studies presented here. There is evidence from *in vitro* studies that CD11b/CD18 at least partially mediates the adhesion of activated (by FMLP, phorbol esters) neutrophils to cultured human endothelium (Lo et al, 1989, Smith et al, 1990). These stimuli upregulate the surface expression of CD1b /CD18 in the same way as GM-CSF does. These *in vitro* studies, however, were done under static conditions, and there is accumulating evidence that the participation of the different adhesion receptors in neutrophil-endothelial interactions is dependent on the shear stresses involved (Lawrence et al, 1990). The CD11/CD18 receptor complex seems to be important at low flow rates of up to 0.5 dynes/cm^2, such as might exist in inflamed tissues. However, at the flow rates which exist in postcapillary venules (1.85 dynes/cm^2), neutrophil-endothelial interaction appears largely integrin independent, and other adhesion molecules such as L-selectin may be more important. The precise relevance of these different studies to the mechanisms underlying the margination of cells which

follows administration of GM-CSF *in vivo* remains to be clarified, but it is likely that the neutrophils which remain adherent to vascular endothelium in the alveolar capillaries as well as in larger vessels (Figure 4.6c, d) at the end of the flush through procedure employed in these studies do so under shear stress rates greater than 0.5 dynes/cm^2 (that is, at sheer stresses higher than those within which the CD11/CD18 receptors are thought to operate).

The question of the participation of the CD11b/CD18 complex in GM-CSF induced neutrophil-endothelial interactions *in vivo* is directly addressed by functional inhibition studies using MoAbs to CD11b and CD18 (Chapter 6).

Finally, administration of GM-CSF to a pregnant animal produced an immediate transient fetal bradycardia, without any effect on maternal heart rate. This suggests, either that the sequestration of cells in the lungs may have caused a temporary hypoxia, which although subclinical in the mother had clinical effects in the fetus, or that a similar margination of circulating cells in the placental vessels may have occurred. These effects indicate that this growth factor should not be administered in pregnancy. The clinical significance of the neutrophil-endothelial interactions induced by GM-CSF *in vivo* is not known, although one group has reported pulmonary dysfunction as manifested by dyspnoea, hypoxaemia, and decreased carbon monoxide diffusion capacity in 7% of patients receiving15 μg/kg of GM-CSF daily by subcutaneous injection (Lieschke et al, 1989a). The relation of these effects to the sudden increase in the number of neutrophils in the pulmonary microcirculation as demonstrated in our studies is unclear. The onset of leucopaenia preceded the fall in oxygen saturation. Furthermore, transient leucopenia occurred on subsequent days of GM-CSF administration, and larger numbers of cells left the circulation, but hypoxia did not occur. However, the fall in lung diffusion capacity for carbon monoxide paralleled the GM-CSF induced leucopenia, and also recurred with subsequent leucopenic episodes.

Apart from altering gas exchange, the enhanced adherence of neutrophils in pulmonary vessels could have effects on endothelial cell function and integrity. Adherence itself can prime neutrophils for increased oxidative response to cytokines, and activated neutrophils have been implicated in the vascular injury that occurs in pathological states such as ARDS and HUS (Tate & Repine, 1983, Forsyth et al, 1989). We have not found any morphological evidence of endothelial damage, at least at up to 2 hours after GM-CSF administration. However, other parameters of endothelial perturbation/damage need to be

explored, such as an alteration in haemostatic and fibrinolytic activities, which may be relevant to the reported side effects of central venous thrombosis and the capillary leak syndrome seen at high doses of this growth factor (Antman et al, 1988, Brandt et al, 1988). In Chapter 9 and 10, studies on the *in vitro* and *in vivo* effects of GM-CSF and G-CSF on endothelial cell proliferation and function are presented.

CHAPTER FIVE

EFFECTS OF GM-CSF, G-CSF AND M-CSF ON PHAGOCYTE MARGINATION AND ADHESION MOLECULE EXPRESSION : *IN VIVO* STUDIES IN MAN

INTRODUCTION

The preceding chapters have detailed the way in which the myeloid growth factors are able to alter the surface adhesive properties of neutrophils and monocytes. GM-CSF also enhances neutrophil adhesion to cultured endothelial monolayers *in vitro* and, *in vivo,* both G- and GM-CSF induce a transient leucopenia, which, in the case of GM-CSF, has been shown histologically to be related to an increased interaction of neutrophils with endothelium in the pulmonary circulation (Chapter 4). The molecular mechanisms which underly the enhanced neutrophil-endothelial interactions seen in response to GM-CSF *in vivo* remain to be clarified. The surface receptors which are known to mediate neutrophil adhesion are the leucocyte β2 integrin receptors, CD11/CD18 (Arnaout et al, 1990), and the L-selectin receptor (Jutila et al, 1989). Phagocyte activation *in vitro* causes upregulation of CD11b/CD18 receptor expression, but downregulation of surface L-selectin expression. When administered systemically, both G- and GM-CSF produce an *in vivo* rise in the levels of CD11b/CD18 on peripheral neutrophils (Devereux et al, 1989, Socinski et al, 1989, Morstyn et al, 1988).

The studies described below monitor the changes in surface receptor expression on cells which remain in the peripheral circulation following administration of G-CSF, GM-CSF, and M-CSF in relation to the time course of the disappearance and subsequent reappearance of cells into the peripheral circulation.

SUBJECTS

The myeloid growth factors, GM-CSF, G-CSF and M-CSF were administered to patients with Hodgkin's disease or non Hodgkin's lymphoma undergoing myeloablative chemotherapy, with or without autologous bone marrow transplantation (ABMT) as part of clinical trials in the department. All growth factors were administered intravenously. GM-CSF, at 30 μg/m^2 (0.6 μg/kg), G-CSF at 5 μg/kg or M-CSF, at doses ranging from 4-128 MU/m^2, were infused intravenously over 2 hours. Venous blood samples were drawn at various time points before and after the first dose of the growth factor for cell counts and antigen expression, which was determined using a whole blood technique, as detailed in Chapter 2.

RESULTS

Immediate effects of GM-CSF, G-CSF and M-CSF on peripheral cell counts

GM-CSF (30 μg/m^2 over 2 hours) produces a transient disappearance of neutrophils and monocytes from the peripheral circulation, but has little effect on lymphocyte numbers (Figure 5.1a, n=3). G-CSF (5 μg/kg over 2 hours, n=3) has a similar effect (Figure 5.1b). With G-CSF administration, recovery of peripheral counts occurs by 60 minutes. In contrast, at 120 minutes following GM-CSF infusion, circulating phagocyte numbers are still only 50% of preinfusion levels.

M-CSF, administered at doses ranging from 4-128 mU/m^2 over 2 hours had no significant effect on circulating neutrophils, as assessed by peripheral counts at the end of a 2 hour infusion, and at 2 hours after that (Figure 5.1c). The only significant effect on circulating monocytes, measured also at these time points, was seen at 2 hours after the infusion finished, in 5 patients who received a dose of 16-128 mU/m^2 (Figure 5.1d). In these patients, the monocyte count fell to 61 ± 17.3% of preinfusion levels ($p < .05$). In addition, in 3 patients, who received 4, 8, and 16 mU/m^2 respectively, neutrophil and monocyte counts were also monitored during the infusions, and were not seen to change (data not shown).

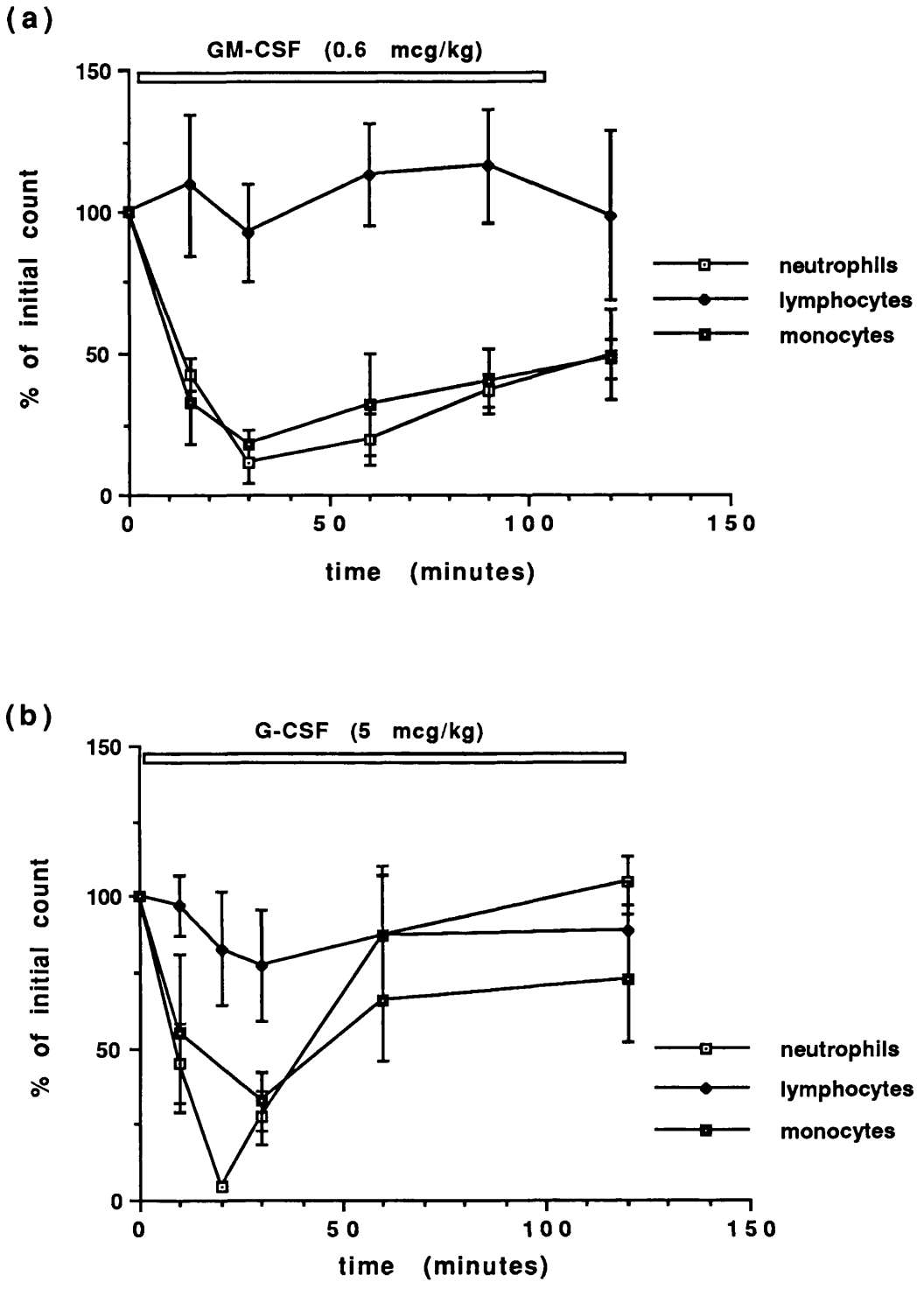


Figure 5.1. Effects of (a) GM-CSF (0.6 μg/kg over 2 hours), and (b) G-CSF (5 μg/kg over 2 hours) on peripheral leucocyte counts, expressed as a percentage of preinfusion counts. Data is given as mean ± SD of 3 patients in each.

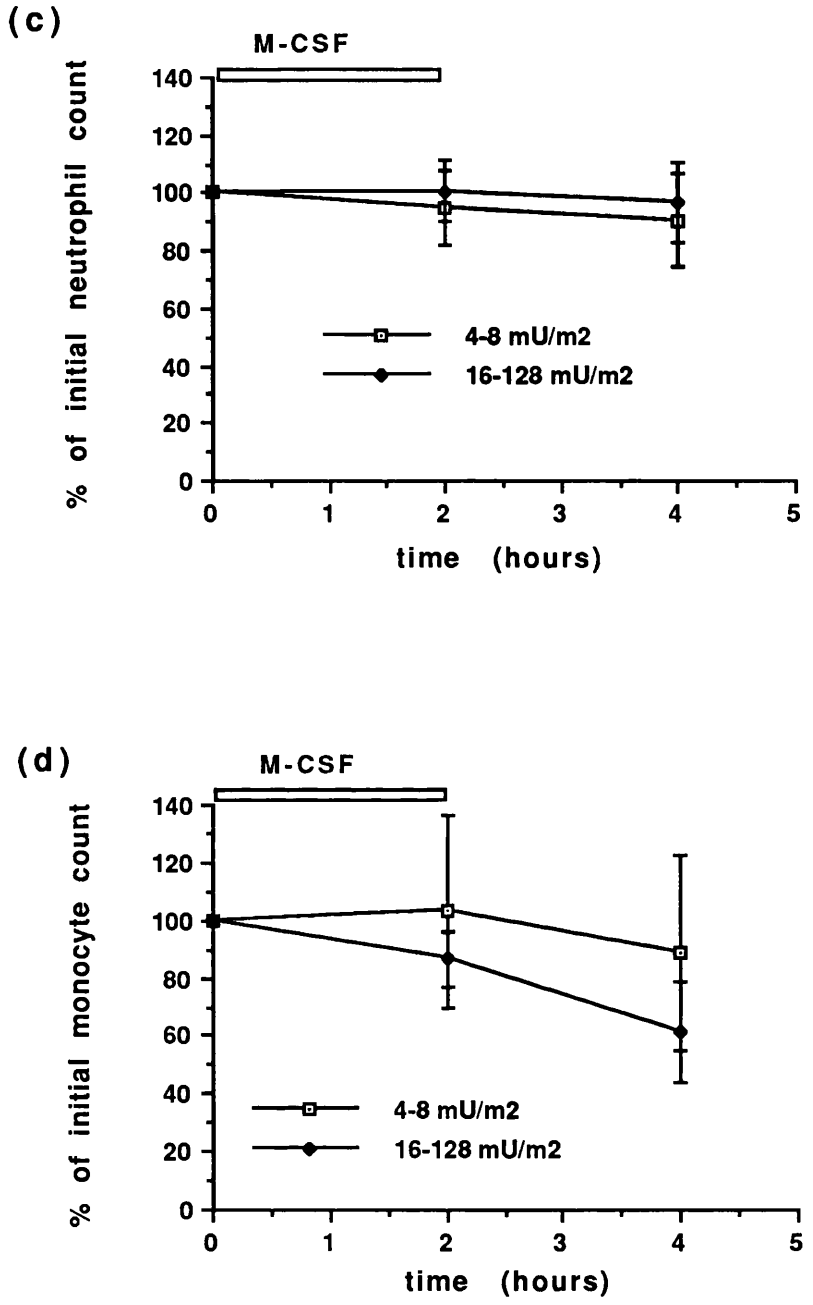


Figure 5.1. Effects of M-CSF, at two dose ranges, on peripheral (c) neutrophil (d) monocyte counts. Data, expressed as percentage of preinfusion counts, is the mean ± SD of 5 patients at each dose range.

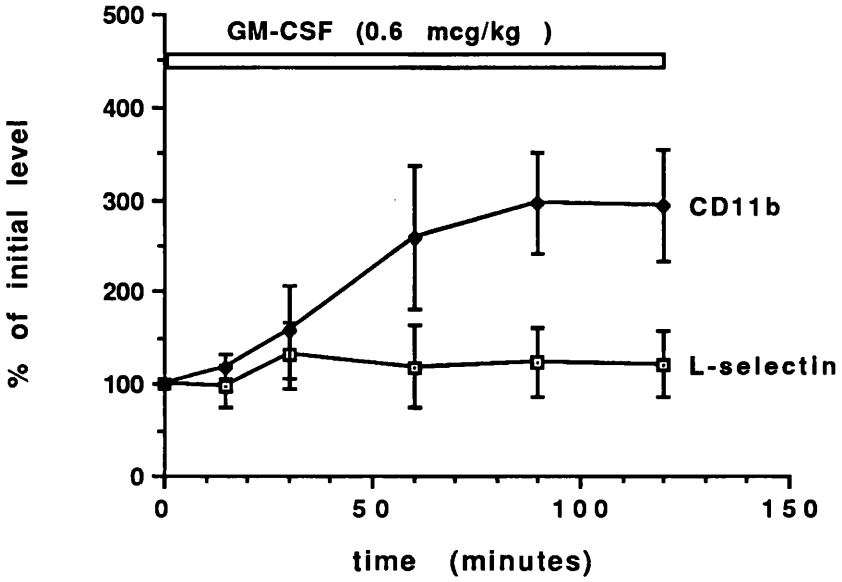
Effects of growth factors on phagocyte CD11b/CD18 in vivo

CD11b expression on peripheral cells was measured in a whole blood assay using MoAb 44. As has been previously reported, systemic administration of GM-CSF leads to an *in vivo* rise in the surface expression of CD11b on neutrophils and monocytes. In 3 patients receiving GM-CSF (30 $\mu g/m^2$) over 2 hours, CD11b expression on peripheral neutrophils rose rapidly over the first 60 minutes to reach a peak of 296 ± 45% of baseline by 90 minutes ($p < .001$, Figure 5.2a). Levels of CD11b remained high at the end of the infusion, despite the fact that 50% of the cells had demarginated by this time (Figure 5.1a). Figure 5.2b shows a similar effect on monocyte surface CD11b expression in the one patient in whom it was measured. In this patient, circulating monocytes continued to express high levels of CD11b at 60 minutes, when half the cells had demarginated. G-CSF also upregulates surface CD11b on circulating neutrophils (to 368 ± 115% of baseline by 60 minutes, $p < .01$, n=3, Figure 5.2c). Again, at 60 minutes, levels of the adhesion molecule remained elevated even though the peripheral neutrophil count had recovered completely. M-CSF at doses less than 16 mU/m^2 had no significant effect on CD11b expression in neutrophils or monocytes, but at doses of 16 mU/m^2 or more, there is an increase in neutrophil and monocyte CD11b expression (to 214 ± 102% and 221 ± 180% of preinfusion levels respectively, n=5, $p < .01$ for both) at the end of a 2 hour infusion (Figures 5.3a, b). 2 hours after the infusion finished, however, the levels of the adhesion molecule fell to within pre-infusion levels.

Effect of GM-CSF and G-CSF on phagocyte L-selectin in vivo

Levels of this adhesion molecule were determined by indirect immunofluorescence using Leu-8 MoAb in the whole blood assay. L-selectin expression on peripheral neutrophils (n=3) and monocytes (n=1) remained relatively unchanged folowing GM-CSF infusions (Figure 5.2a, b). In view of these rather unexpected results which contrast with the loss of L-selectin in response to GM-CSF *in vitro* , in two further GM-CSF infusions, a different antibody to L-selectin, Lam 1.1 MoAb (kindly supplied by Dr. T F Tedder, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA), was used to determined neutrophil L-selectin expression. In these experiments, GM-CSF was also without significant effect on L-selectin levels *in vivo*. However, it must be emphasized that it is only possible to measure antigen expression on those cells that are left in the peripheral circulation. When GM-CSF is given as a continuous infusion (250 $\mu g/m^2$) over 24 hours, similar changes in peripheral neutrophil count, and CD11b and L-selectin expression are seen (Figure 5.2d). Similarly, in 3 patients receiving G-CSF, neutrophil L-selectin expression on peripheral cells remained unchanged (Figure 5.2c).

(a)



(b)

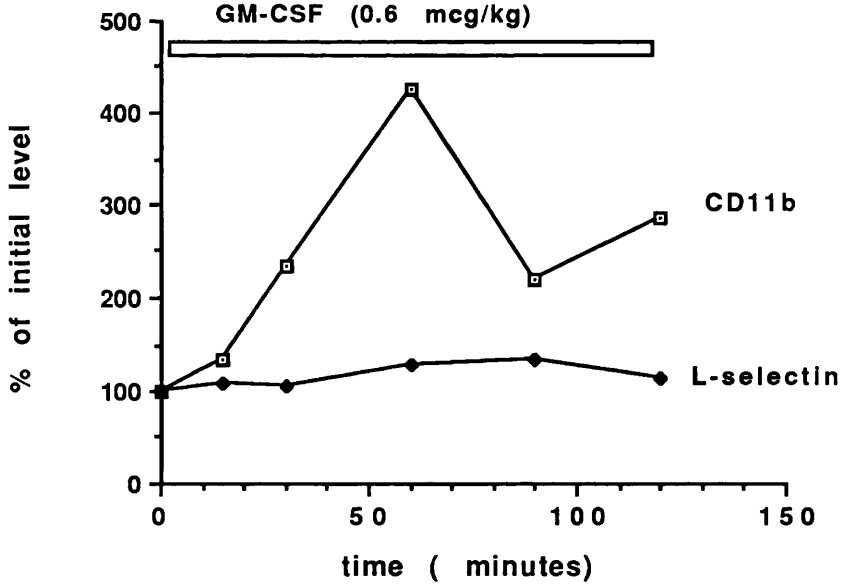


Figure 5.2. Effect of GM-CSF on CD11b and L-selectin levels on (a) peripheral neutrophils in 3 patients, and (b) peripheral monocytes in 1 patient, measured as MCF and expressed as percentage of preinfusion levels. Data in (a) is mean ± SD, n=3.

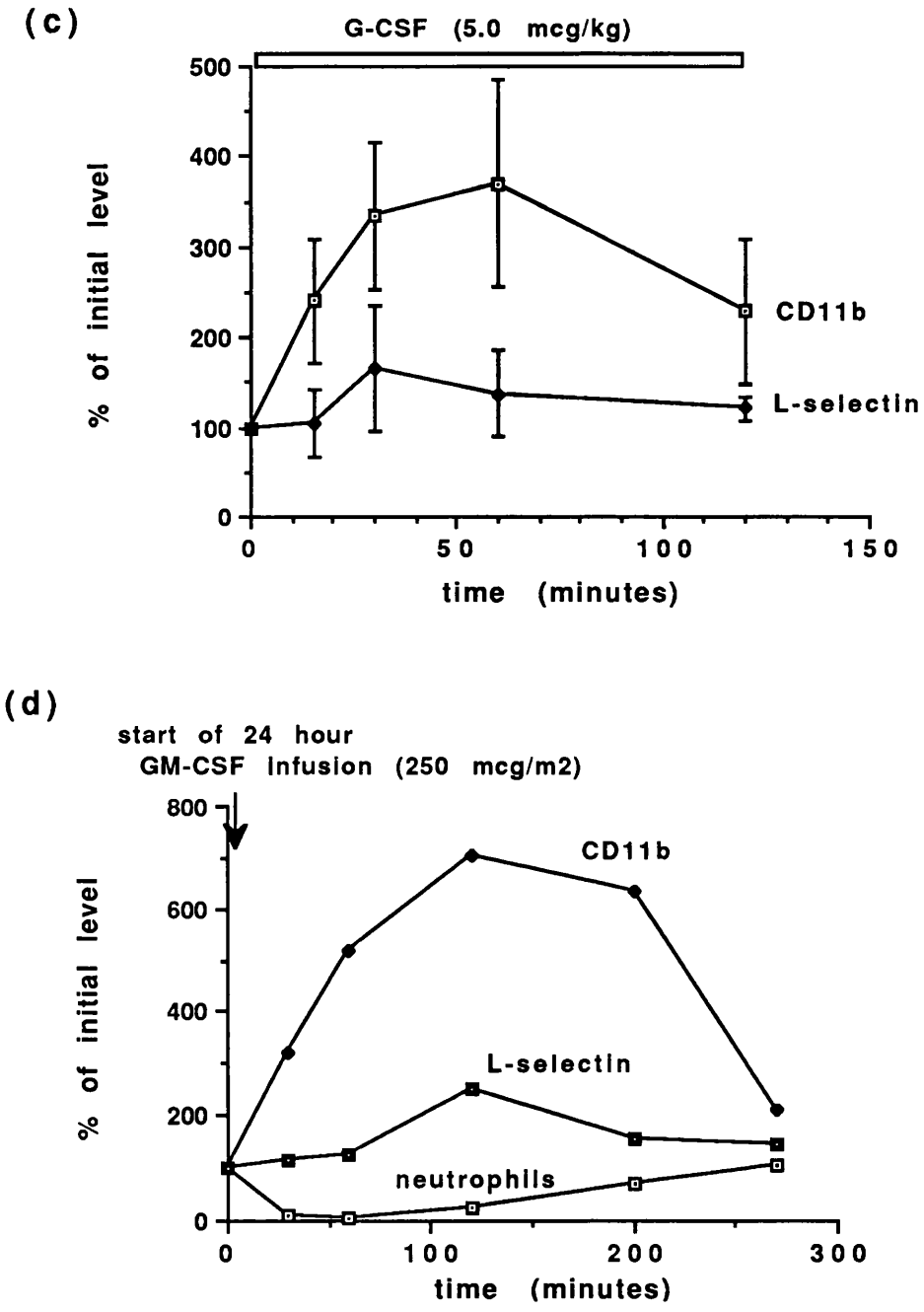


Figure 5.2 (c). Effect of G-CSF infusion on neutrophil CD11b and L-selectin expression in 3 patients. (d) Effect of 24 hour infusion of GM-CSF (250 $\mu g/m^2$) on neutrophil CD11b and L-selectin levels, in 1 patient, who is representative of 2. Data is expressed as for (a) and (b).

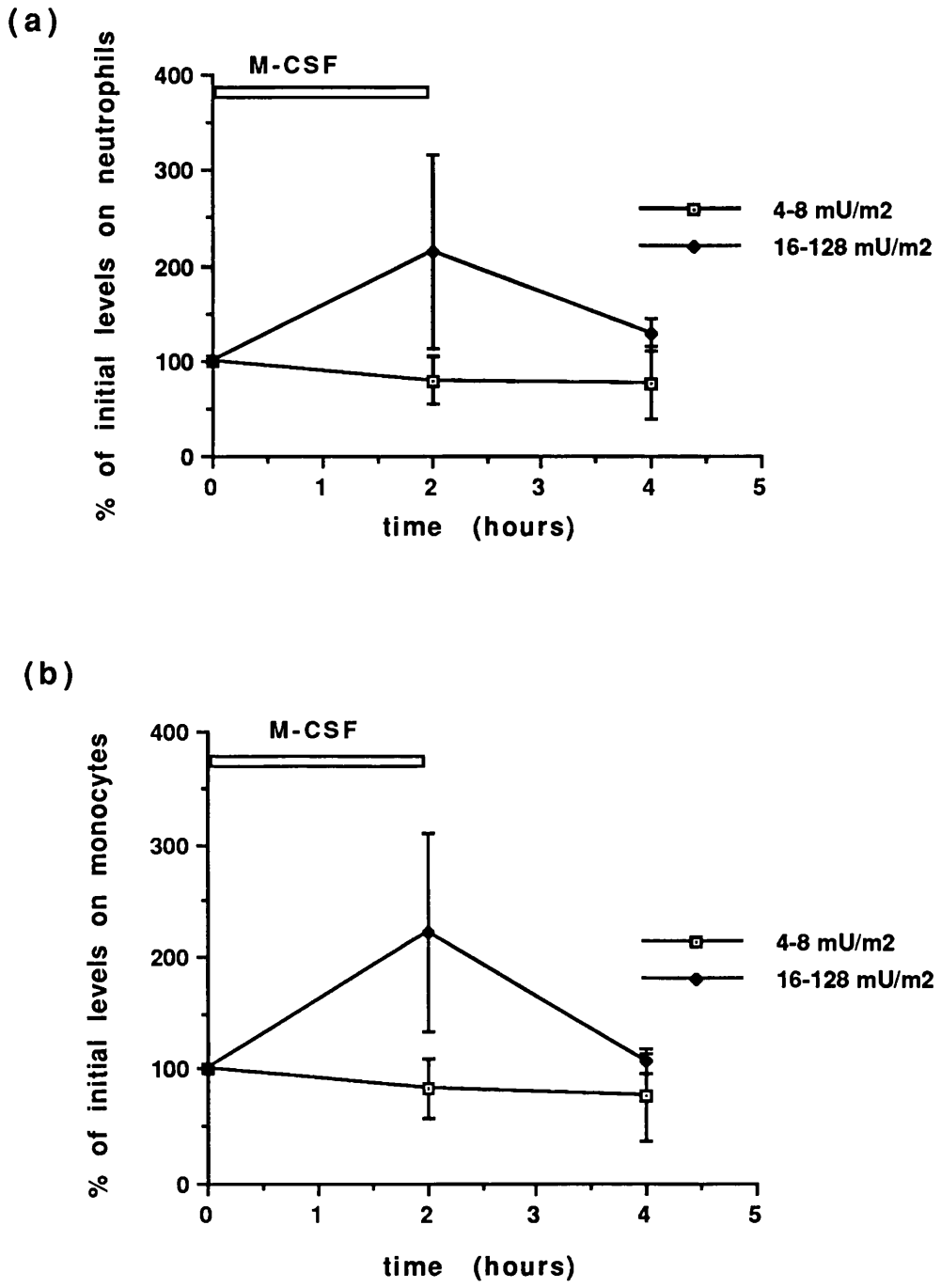


Figure 5.3. Effect of M-CSF, at 2 different dose ranges on CD11b expression on (a) neutrophils and (b) monocytes in 5 patients at each dose range. Data are expressed as for Figure 5.2.

DISCUSSION

Systemic administration of GM-CSF and G-CSF produces a rapid (by 15 minutes) but transient fall in circulating phagocytes, to less than 20% of preinfusion levels, due to the retention of cells in the pulmonary vasculature. Recovery of circulating counts occurs by 120 minutes with GM-CSF, and by 60 minutes with G-CSF. M-CSF, on the other hand, has no consistent effect on circulating phagocytes, except for a slight fall in the monocyte count at 2 hours after completion of a dose of 16 mU/m^2.

Both GM-CSF and G-CSF produce an *in vivo* rise in surface expression of CD11b, on circulating phagocytes, starting at the same time as the counts begin to fall. However, demarginating cells returning into the peripheral circulation continue to express high levels of this molecule, suggesting that quantitative changes in surface levels of CD11b are not directly related to the enhanced neutrophil-endothelial interaction within the lungs. M-CSF produces a slight rise in CD11b expression on circulating neutrophils and monocytes at the end of a 2 hour infusion at doses of 16 mU/m^2 or higher, but this is not accompanied by any reduction in peripheral cell counts. Neutrophil L-selectin levels remain relatively unchanged following GM-CSF and G-CSF.

These effects of GM-CSF and G-CSF on peripheral cell numbers and CD11b expression are in accord with previous reports (Devereux et al, 1989, Socinski et al, 1989, Morstyn et al, 1988, Lindemann et al, 1989, Ohsaka et al, 1989). The prolonged (up to at least 4 hours) elevation of CD11b expression in contrast to the rapid (by 2 hours) recovery of the cell counts has also been noted by others (Socinski et al, 1989) using GM-CSF. One group (Demetri et al, 1990), however, has failed to find any upregulation of neutrophil CD11b levels following G-CSF admnistration by the subcutaneous route. The same group has examined neutrophil L-selectin levels following both G- and GM-CSF admnistration. In contrast to the results presented here, they report that GM-CSF (given subcutaneously) induced loss of surface L-selectin from neutrophils within 1 hour, which persisted for up to 6 days. G-CSF, on the other hand, had no effect on L-selectin expression in vivo. The difference between those results, and the data presented here on GM-CSF cannot be explained, either in terms of the different kinetics of administration of these growth factors (as we have confirmed our results with 24 hour infusions), or in terms of the different antibodies used, as we have confirmed our results using the Lam 1.1 MoAb to L-selectin, which was used by Demetri et al.

There are several ways in which the altered expression of adhesion receptors could be related to the margination of circulating cells. An increase in the numbers of surface

receptors could result directly in an enhanced adhesiveness of circulating cells for vascular endothelium, leading to the retention of adhered cells in the microvasculature of the lungs. Such a direct relationship between CD11b surface receptor density, and phagocyte-endothelial interactions, however, is unlikely, as demarginated cells still express high levels of the receptor. Therefore, if increased expression of CD11b mediates cell margination by increasing the adhesion of circulating cells to endothelium, demargination must be due to the downregulation of another adhesive mechanism, causing the release of adhered cells. The loss of L-selectin from the neutrophil surface following *in vitro* stimulation by GM-CSF, and, to a lesser degree, G-CSF, might be a candidate for the mechanism underlying demargination. However, despite the sustained (up to 2 hours) downregulation of neutrophil L-selectin levels by GM-CSF and G-CSF *in vitro*, demarginated cells *in vivo* do not have low levels of L-selectin. This difference between the *in vitro* and *in vivo* data cannot be due to a fall in circulating levels of GM-CSF to suboptimal levels after the end of the infusion, because the results are similar when GM-CSF is given as a 24-hour infusion. The results presented here do not allow any inference as to whether *in vivo* activation by G- and GM-CSF is able to downregulate neutrophil surface L-selectin in the same way as *in vitro* stimulation does. If, as the *in vitro* data suggests, neutrophil surface L-selectin is shed *in vivo*, then the high levels found on demarginated cells could be explained if the cells, following contact with endothelium, have re-expressed the receptor. It is still not clear what role, if any, the neutrophil L-selectin receptor plays in growth factor mediated neutrophil-endothelial interactions *in vivo.*

If quantitative changes in surface antigen present on cells in the peripheral circulation do not accurately reflect the propensity of these cells to interact with endothelium, perhaps what matters is qualitative changes in these adhesion receptors, as has been discussed in Chapter 3. The functional contribution of adhesion receptors to the adhesive interactions of cells depends, not only on the numbers of receptors expressed, but also on any changes in receptor affinity which may occur following cell activation. Recently, functional activation has also been demonstrated for the leucocyte L-selectin receptor. Both GM-CSF and G-CSF have been reported to produce a rapid (by 5 minutes) increase in the affinity of the neutrophil L-selectin receptor for its carbohydrate ligand, PPME (Spertini et al, 1991), without an actual increase in the number of L-selectin receptors. When neutrophils are stimulated at 37°C, the increased binding of PPME is transient, with a maximum reached at 5 minutes, after which there is a decrease in binding due to the receptor being shed. An analogous situation occurs with the L-selectin receptor on lymphocytes activated by CD3 cross-linking. Interestingly, the binding of PPME is inhibited by the MoAb Lam-1-3, but not by other MoAbs to L-selectin, suggesting that a particular epitope is activated, or exposed following cell stimulation.

Since then, another group has demonstrated that neutrophils stimulated with FMLP show an increased binding of Leu-8 MoAb at 1 minute, but that this is followed by a drop in binding at 3 minutes, which was complete by 10 minutes (Smith et al, 1991).

Finally, there is the possibility that the recovery of cell counts may be, in part, due to release from marrow stores, and hence the demarginated cells may be a different population of rather more immature cells. This possibility is unlikely, as previous work has shown, firstly, that with GM-CSF, the recovery of circulating neutrophil counts parallels the return of radioactivity into the circulation (Devereux et al, 1987), and secondly, that neutrophil lobularity counts were unchanged on demarginating cells. These results were confirmed in a separate study using ^{111}In labelled neutrophils in rabbits receiving injections of FMLP (Worthen et al, 1989). Finally, histological examination of lung biopsies from sheep receiving infusions of zymosan-activated plasma have shown that neutrophils accumulate transiently in the lung, but return into the peripheral circulation after 2-4 hours (Meyrick & Brigham, 1983).

The results presented hitherto show that GM-CSF and G-CSF differ in their actions on the adhesive functions of mature cells. Both growth factors produce a rise in neutrophil CD11b expression which is comparable *in vitro* and *in vivo*. However, although both G-and GM-CSF enhance neutrophil-endothelial interaction *in vivo*, as evidenced by leucocyte margination, the duration of neutropenia following G-CSF is much shorter, with recovery of peripheral counts by 60 minutes, whereas, with GM-CSF, counts are still below preinfusion levels at 120 minutes. This difference exists despite identical protocols of adminstration, and even though the dose of GM-CSF (0.6 μg/kg) is only a tenth of the dose of G-CSF (5 μg/kg). This *in vivo* difference is supported by the *in vitro* results which show that G-CSF does not increase neutrophil adherence to endothelial monolayers, and is much less effective at downregulating L-selectin expression *in vitro*. In general, G-CSF appears to be a less proadhesive agent for neutrophils.

CHAPTER SIX

EFFECT OF MOABS TO CD11B/CD18 ON GM-CSF INDUCED NEUTROPHIL ADHESION *IN VITRO AND IN VIVO*

INTRODUCTION

GM-CSF enhances the adhesion of peripheral blood neutrophils to cultured human endothelial cells *in vitro*, and, to pulmonary vascular endothelium *in vivo* (in man and monkey). In this chapter, the role of the CD11/CD18 receptor complex in mediating these neutrophil-endothelial interactions was explored in functional inhibition studies employing monoclonal antibodies (MoAbs) to CD11b and CD18.

SPECIAL METHODS

Antibodies

Antibodies 44 (anti-CD11b, IgG1, Malhotra et al, 1986) and MHM23 (anti-CD18, IgG1, Hildreth et al, 1983) were purified from ascites fluid as detailed in Chapter 2, and resuspended in sterile PBS for use in functional assays of neutrophil adhesion, or in sterile saline for *in vivo* administration.

In vitro neutrophil adhesion and aggregation

Neutrophil adhesion to endothelial monolayers (carried out in the presence of 5% FCS), and neutrophil aggregation experiments (in serum-free conditions) were performed on freshly isolated neutrophils from the peripheral blood of healthy volunteers as detailed in Chapter 2. Where the effect of MoAbs was studied *in vitro*, neutrophils were preincubated with the antibodies (at stated concentrations) for 20 minutes at room temperature, before being used in functional assays. Antibodies were present throughout the assays. Control neutrophils were incubated with an isotype-matched irrelevant antibody.

In vivo leucocyte margination responses to GM-CSF

5 adult *Macaque fascicularis* monkeys were used in MoAb blocking experiments. Animals were anaesthetised and cannulated as described in Chapter 4. GM-CSF (2μg/kg) was administered intravenously over 5 minutes, and peripheral venous blood drawn for blood counts and cellular adhesion molecule expression as detailed in Chapter 3. Where the *in vivo* effect of MoAbs was studied, purified antibody in sterile saline was administered by slow intravenous bolus 10-20 minutes prior to the injection of GM-CSF.

Determination of antibody levels in plasma

In animals receiving antibody, plasma was obtained from venous blood taken into EDTA by centrifugation at 1500g for 10 minutes. Aliquots of plasma were stored at -20°C until being used in assays. Doubling dilutions of plasma were made in RPMI, and incubated with 1 x 10^5 purified human neutrophils for 45 minutes at 4°C. Appropriate dilutions of the purified antibody stock (of known protein concentration) were also incubated with cells. Following 3 washes, and a further incubation with FITC-RAM, the amount of antigen stained on each cell sample was measured as mean cell flourescence determined by flow cytometry. The antibody level in each plasma sample was determined by comparing the titration curve for that sample with the curve obtained with purified antibody of known concentration (see Appendix 2).

RESULTS

Effect of anti-CD11b and anti-CD18 monoclonal antibodies on neutrophil adhesion and aggregation in vitro

In order to assess the role of the β2 integrin CD11b/CD18 in GM-CSF induced adhesion, purified human neutrophils were preincubated with varying concentrations of MoAbs 44 and MHM23, or an isotype matched leucocyte antibody as control for 10 minutes at room temperature prior to being added to endothelial monolayers with or without GM-CSF 100 ng/mL. Following 30 minutes incubation at 37°C, the percentage of adherent neutrophils was assessed as detailed in Chapter 2. Figure 6.1 shows that GM-CSF (100 ng/mL) produces an increase in neutrophil adherence from 13.8 ± 1.4% (mean ± SE, n=7) with medium alone to 27.4 ± 2.6% with GM-CSF ($p < 0.001$, n=7). MoAb MHM23 (anti-CD18, 10 μg/mL) significantly reduced both unstimulated and GM-CSF induced adherence to 7.8 ± 1.4% ($p < 0.01$, n=7) (by 44%) and 11.3 ± 2.6% ($p<0.001$, n=7) (a reduction in the amount of stimulated adhesion of 74%) respectively. The inhibitory effect of MHM23 on GM-CSF induced neutrophil adhesion was dose dependent, with maximal effect seen at antibody concentrations of 5-10 μg/mL or greater (Figure 6.2a). MoAb 44 (anti-CD11b, 10 μg/mL) had a similar, though lesser, effect on neutrophil adhesion, reducing unstimulated adhesion to 9.7 ± 1.2% (by 30%), and decreasing GM-CSF induced adherence to 17.9 ± 3.3% (by 40%, n=5, $p < 0.01$ for both, Figure 6.1). In view of the smaller inhibitory effect of this MoAb, 2 further experiments were performed on FMLP stimulated neutrophil adherence. Figure 6.2b shows that MoAb 44 blocks both FMLP and GM-CSF mediated neutrophil adhesion in a dose dependent manner, with maximal inhibition at concentrations of 10 μg/mL or higher.

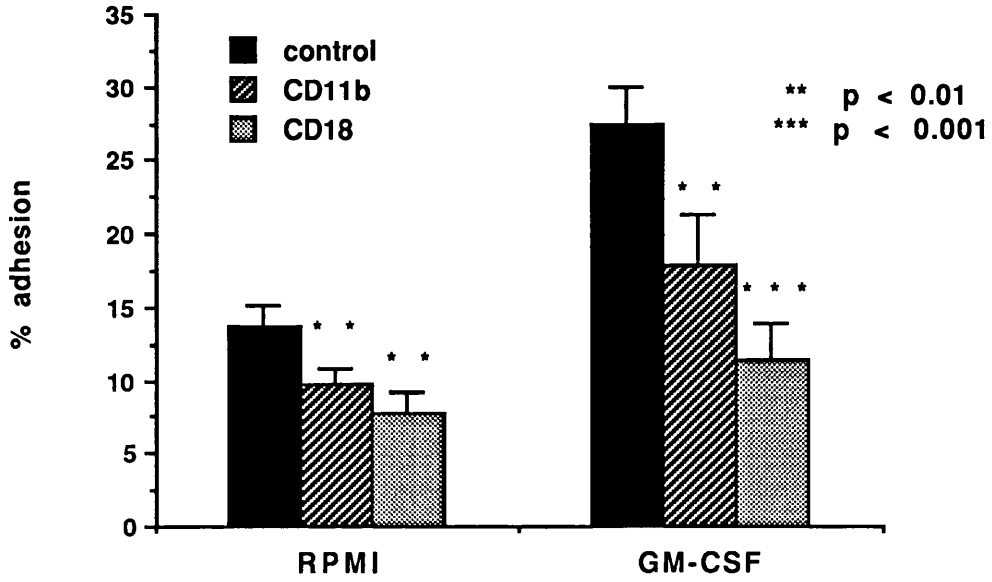


Figure 6.1. Effects of MoAbs to CD11b (44) and to CD18 (MHM23) on the adhesion of neutrophils in the absence (RPMI), or presence of GM-CSF (100 ng/mL). Data are the mean ± SE of 5 experiments with MoAb 44 and 7 experiments with MoAb MHM23.

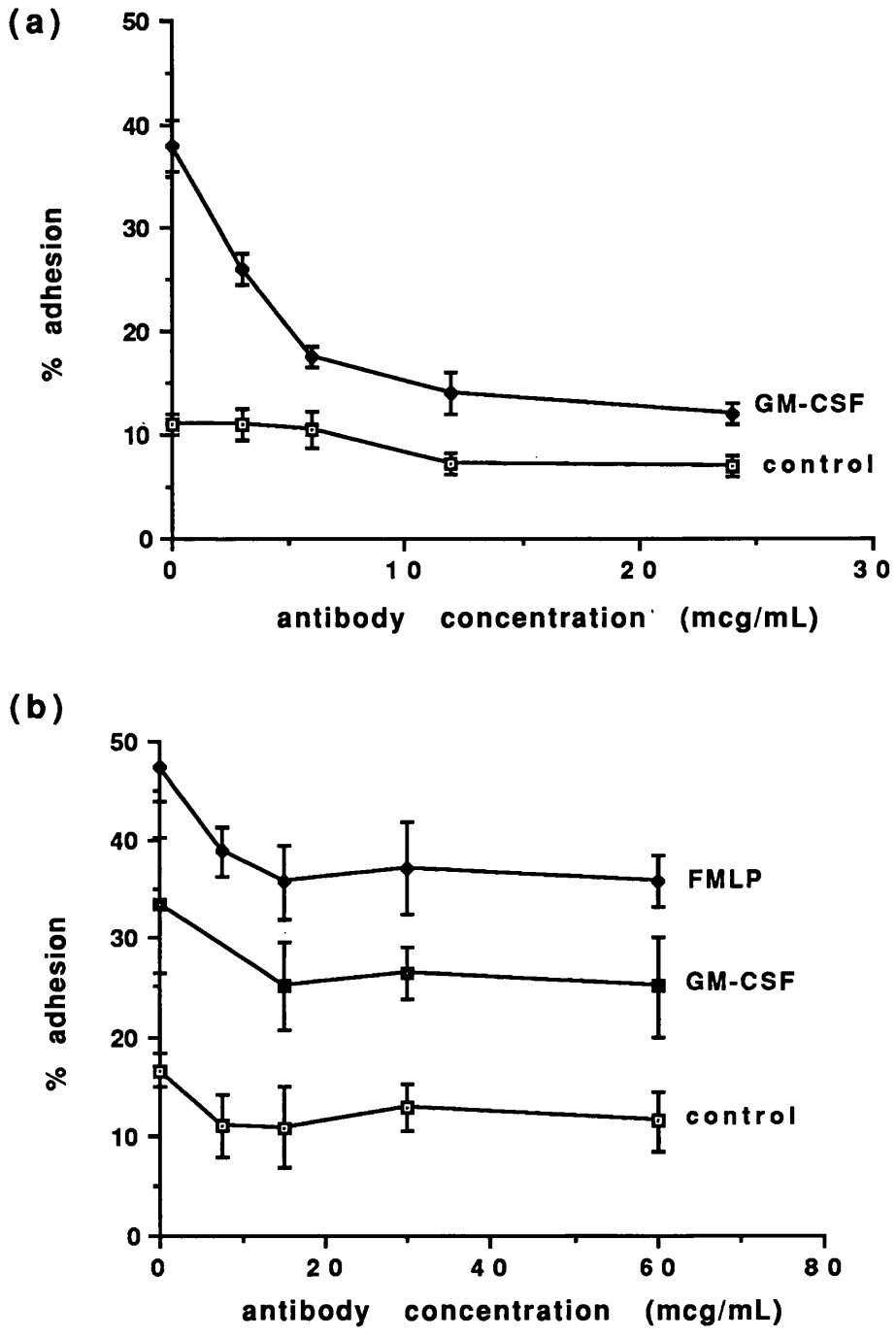


Figure 6.2. (a) MoAb MHM23 (anti-CD18) blocks GM-CSF induced neutrophil adhesion and (b) MoAb 44 (anti-CD11b) blocks GM-CSF and FMLP induced adhesion in a dose dependent manner. Data are the mean ± SD of 6 replicate wells in 1 experiment which is representative of 2 using MoAb MHM23, and 3 using MoAb 44.

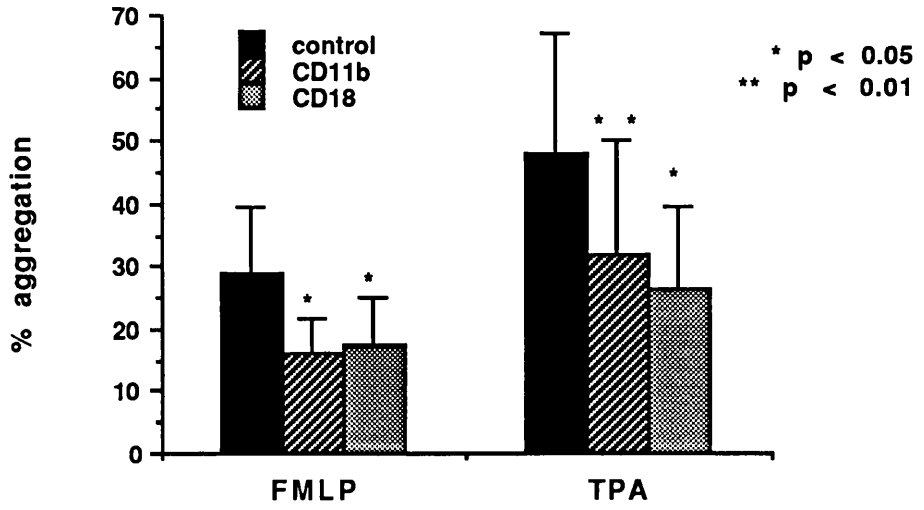


Figure 6.3. Effect of MoAbs 44 and MHM23 on neutrophil aggregation in response to FMLP and TPA. Data is expressed as mean ± SD of 4 experiments with each antibody.

The effect of CD11b and CD18 MoAbs on neutrophil aggregation *in vitro* was studied using purified human neutrophils, stimulated with FMLP 10^{-6} M, TPA 1μg/mL and GM-CSF 10 ng/ml. Both FMLP and TPA induced neutrophil aggregation responses, of 28.8 ± 10.6% and 47.8 ± 19.3% at 3 minutes respectively (n=4, Figure 6.3). Spontaneous aggregation in the absence of agonist was less than 5% at 3 minutes in all experiments. Both MoAbs to CD11b and CD18 were able to inhibit this agonist induced aggregation, and both antibodies were equally effective in this respect (Figure 6.3). FMLP induced aggregation, for example, was reduced by 44.8 ± 12.3% in the presence of anti-CD11b, and by 39.8 ± 11.5% in the presence of anti-CD18 ($p < 0.05$, n=4 for both). There was a similar effect on TPA induced aggregation, i.e., a reduction of 36.3±11.8% by anti-CD11b ($p < 0.001$, n=4) and of 42.8 ± 23.0% ($p < 0.05$, n=4) by anti-CD18.

GM-CSF was found to be a very weak stimulator of neutrophil aggregation; in 5 out of 6 experiments, there was no aggregation in response to GM-CSF (1 ng/mL - 1 μg/mL). In one experiment, GM-CSF at 10 ng/mL induced an aggregation response of 20% at 3 minutes, which was reduced to 10% by CD11b and 7% by CD18 MoAbs.

Effect of anti-CD11b and anti-CD18 monoclonal antibodies on GM-CSF induced neutrophil margination in vivo

Purified antibody in sterile saline was injected intravenously into animals 20 minutes before GM-CSF (2 μg/kg) administration. MoAb 44 was administered to 3 separate animals, at doses of .35, .46 and 1.0 mg/kg. Plasma antibody concentrations were determined as described immediately prior to, and up to 40 minutes following GM-CSF administration. In the animal which received the lowest dose of antibody, antibody concentration in plasma was greater than 7 μg/mL, while in the other 2 animals which received higher doses, antibody levels were between 10-20 μg/mL (see Appendix 2), which is a dose sufficient to produce maximal inhibition of neutrophil adhesion in vitro (see above). Despite this, the antibody had no effect on the neutrophil margination response to GM-CSF (Figure 6.4a). MoAb MHM23 was administered to 2 animals, at 1.2 and 1.0 mg/kg, doses which acheived plasma levels of 15-20 μg/mL (Appendix 2). Unlike MoAb 44, which had no immediate direct effect on neutrophil numbers, MHM23 itself produced a fall in the neutrophil count. However, although administration of the MoAb did not prevent the occurence of a transient leucopenia following the injection of GM-CSF, in both animals, the nadir count was higher than in control animals (Figure 6.4b).

In order to confirm that the MoAbs administered were sufficient to saturate binding sites on cells *in vivo*, peripheral blood neutrophils were incubated with further MoAb *in vitro*, followed by FITC-RAM , and the MCF of these cells compared with that of cells incubated with FITC-RAM alone. There was no difference in the MCF between cells stained in these two different ways, suggesting that the concentration of antibody present *in vivo* was sufficient to saturate surface CD11b or CD18 receptors on peripheral neutrophils.

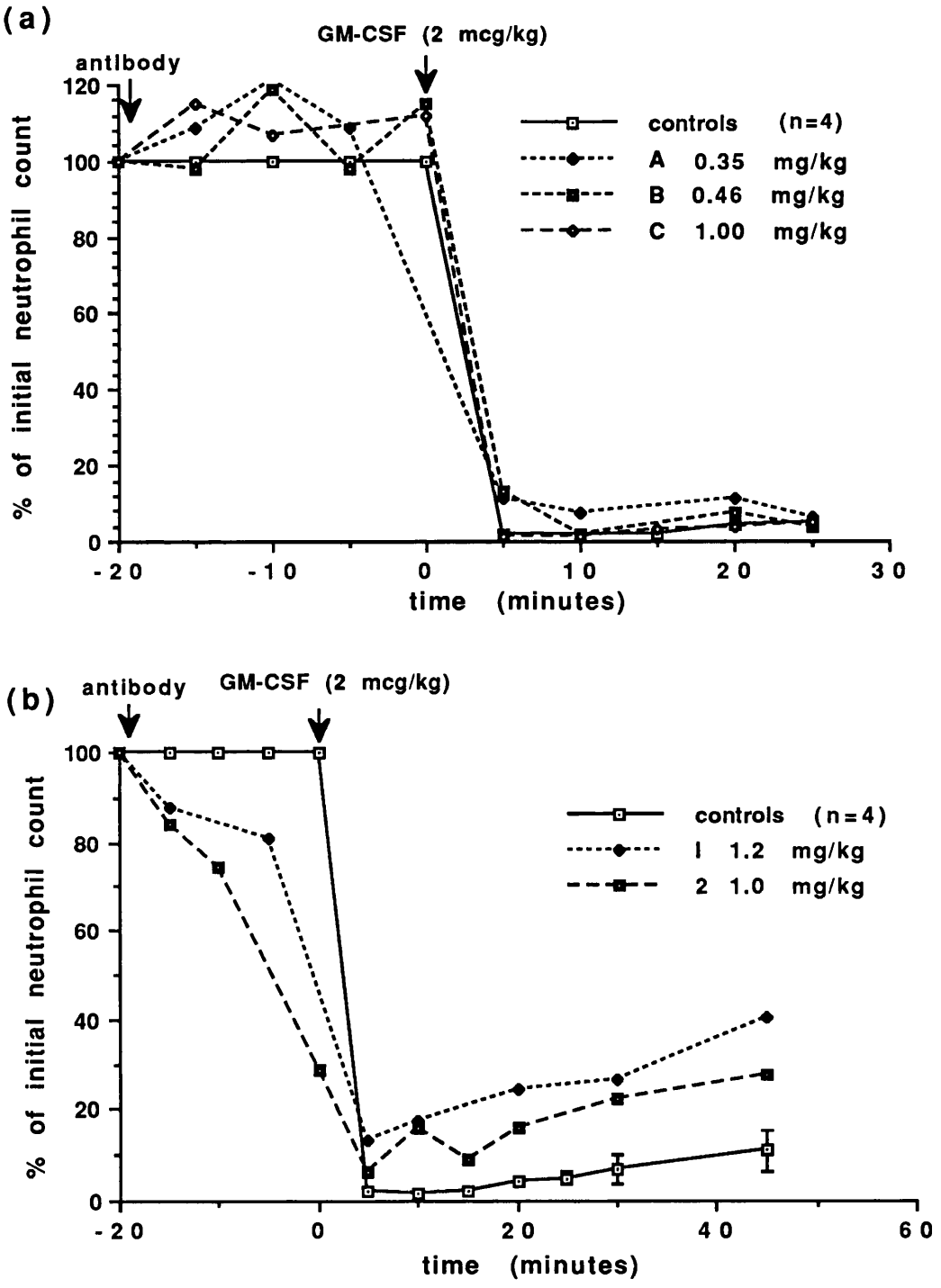


Figure 6.4. Effect of (a) MoAb 44 (anti-CD11b) in 3 animals, and (b) MoAb MHM23 (anti-CD18) in 2 animals, on GM-CSF induced margination in *cynomolgus*. The dose of antibody for each animal is indicated. Data is expressed as a percentage of the initial neutrophil count, and, in controls, is mean ± SD of 4 animals.

DISCUSSION

In vitro, MoAb MHM23 to the common β2-subunit was able to inhibit the GM-CSF induced increment in neutrophil adhesion to endothelial cells by 74%, and also significantly reduced the adhesion of unstimulated cells. This is in accord with previous work using radiolabelled neutrophils (Devereux et al, 1989). MoAb 44 (anti-CD11b), was able to block GM-CSF induced adhesion in vitro by 40%, and also reduced the adhesion of unstimulated cells by 30%. The greater inhibitory effect of MoAb to the β-subunit when compared with MoAb to CD11b on the adherence of activated neutrophils to endothelium *in vitro* is in accord with reports by other workers (Anderson et al, 1986, Lo et al, 1989). *In vitro*, both anti-CD11b and anti-CD18 MoAbs were equally potent in partially inhibiting neutrophil aggregation in response to FMLP and TPA. Similar inhibitory effects of MoAbs to the CD11/CD18 complex on neutrophil aggregation responses to phorbol esters and chemotactic peptides have been demonstrated by other workers (Anderson et al, 1986). In the system used here, neutrophils did not reliably demonstrate an aggregation response to GM-CSF, in contrast to previous reports (Arnaout et al, 1986, Wallis et al, 1986, Phillips et al, 1988). This may be due to the fact that previous work has studied neutrophil aggregation in the presence of cytochalasin B. The studies described here have not employed this agent, in an attempt to avoid nonphysiological conditions.

The data from the *in vivo* studies confirm the greater inhibitory efficacy of anti-CD18 MoAb, which produced a small but significant amelioration of GM-CSF induced neutropenia. On the other hand, anti-CD11b MoAb was without effect. This difference between the inhibitory effects of MoAbs CD11b and CD18 on cytokine induced leucocyte-endothelial adherence in vivo was also reported in a rabbit microcirculation model employing C5a as a proadhesive agonist (Argenbright et al, 1991). Although it must be borne in mind that these results have been obtained using only one MoAb to CD11b, the data suggest that one, or both of the other integrin receptors, CD11a and CD11c, may play a role in the increased adhesion to endothelium *in vivo*.

Although MoAb CD18 did have some effect on GM-CSF induced margination, this effect was small, and hence our studies suggest that the β2 integrins do not represent the major adhesive mechanism underlying GM-CSF induced neutrophil adherence to pulmonary endothelium as demonstrated *in vivo*. The dissociation between the time course of neutrophil margination and the kinetics of the upregulation of neutrophil CD11b/CD18 *in vivo* (Chapters 4 and 5) supports the

notion that these receptors do not represent the major mechanism underlying cytokine induced phagocyte margination *in vivo*. In keeping with these observations, Lundberg & Wright (1990) found that pretreatment of rabbits with anti-CD18 MoAb did not prevent neutrophil margination in lungs following systemic injection of FMLP. Similarly, other workers report that anti-CD18 MoAb did not prevent the rolling of leucocytes along vessel walls, but was able to inhibit neutrophil emigration into inflammatory areas (Lindbom et al, 1990). These observations suggest that distinct processes are involved in the different stages of neutrophil extravasation in inflammation; an early event being leucocyte rolling along endothelium, followed by firm adhesion, which in turn, is a prerequisite for the transendothelial movement of cells (von Andrian et al, 1991). These different cellular adhesive processes may be mediated by different receptor-ligand interactions, a subject which is dealt with in greater detail in Chapters 7 and 8. The greater inhibitory effect of MoAbs to CD11b/CD18 on neutrophil-endothelial adherence *in vitro* than that demonstrated on margination *in vivo* suggest that the CD11b/CD18 receptor participates in neutrophil adhesion under the static conditions of the *in vitro* adhesion assay but is less important in the transient interaction of circulating neutrophils with endothelium in lungs following GM-CSF infusion *in vivo*. This may relate to the different shear stresses involved; β2 integrin-mediated adhesion appears to function mainly at very low flow rates (see Chapter 7).

These observations suggest that the pulmonary sequestration of neutrophils in response to GM-CSF *in vivo* is likely to involve some other mechanism, if not wholly, at least to initiate the interaction of cells with endothelium, following which the β2 integrins might contribute by strenghthening cell-cell adhesion. The retention of neutrophils in the microcirculation is likely to depend, not just on adhesive interactions between circulating cells and endothelium, but also on hydrodynamic forces, the relative sizes of cells and vessels, and the deformability of cells (Downey & Worthen, 1988, Erzurum et al, 1992). Neutrophil stimulation can produce changes in the cytoskeleton (Omann et al, 1987), leading to cell stiffening, and greater retention in 5 μm pores in vitro (Worthen et al, 1989). A diminished ability of cells to deform during transit through capillaries would reduce flow rates, leading to the retention of cells in the microvasculature, and the engagement of adhesion receptors, resulting in neutrophil-endothelial adhesion which is shear stress resistant. In support of this last hypothesis, scanning electron microscopy showed that leucocytes adhere to the endothelium in large vessels within the lungs of animals which have

received GM-CSF, where such cells are not obviously restrained by an inability to deform (Chapter 4, Figure 4.5d).

There remains the possibility that the MoAbs used in these studies, while being effective in blocking adhesion *in vitro*, are directed towards epitopes which are less functionally relevant *in vivo*. In order to circumvent this problem, the following chapter describes studies on the effect of GM-CSF on the adhesive functions of neutrophils from a patient with leucocyte adhesion deficiency (LAD), which only express very low levels of the CD11/CD18 receptor complex.

CHAPTER SEVEN

ADHESIVE RESPONSES OF LEUCOCYTE ADHESION DEFICIENCY (LAD) NEUTROPHILS TO GM-CSF

INTRODUCTION

Leucocyte Adhesion Deficiency (LAD) is a rare inherited disorder characterized by recurrent, life-threatening bacterial infections, lack of pus formation and defective wound healing (Anderson & Springer, 1987, Arnaout, 1990b). There is a striking absence of granulocytes from the infective lesions, despite a persistent neutrophilia (Anderson et al, 1985). Leucocytes from these patients have absent or reduced expression of all three β2 integrins, due to heterogenous mutations in the common β subunit (Kishimoto et al, 1987). The study of this disease has outlined the functional significance of these adhesion molecules *in vivo*. Patients whose cells express less than 1% of normal levels of antigen die in early childhood unless they receive a bone marrow transplant (Anderson & Springer, 1987), while patients whose cell surface expression is 5-20% of normal may survive into adulthood with appropriate treatment of recurrent infections. The major functional defects are those affecting the granulocyte and monocyte series, and the most striking cellular defect seen *in vivo* is the failure of granulocytes to mobilise into skin windows and to extravasate into inflamed tissues. This is consistent with the preponderance of bacterial infection in these patients. Defects in lymphocyte function are variable (Krensky et al, 1985).

This chapter explores the defect in adherence-dependent phagocyte functions of two patients with LAD, focussing in particular on the effect of GM-CSF on the interaction of neutrophils with endothelium, both *in vitro* and *in vivo*, in order to further clarify the role of the β2 integrins in GM-CSF induced phagocyte-endothelial interactions.

PATIENT DETAILS

Patient 1

This Caucasian male, born of a non-consanguinous marriage, was well until the age of 11 when he developed recurrent skin ulcers which progressed rapidly with extensive local tissue destruction, and healed poorly leaving thin dystrophic scars. He died at the age of 20 years following the development of numerous extensive necrotizing ulcers resistant to antibiotic therapy, immunosuppressive drugs, and

debridement and skin grafting. Biopsy of these lesions showed a marked lymphocyte infiltration with a paucity of phagocytes despite the fact that neutrophils were readily apparent within blood vessels. The case history is presented in more detail elsewhere (Davies et al, 1991). During the terminal episode, he received a 5-day course of intravenous GM-CSF which had no effect on his skin lesions.

Patient 2

This Caucasian boy, also of non-consanguinous parents, had a normal perinatal history, but suffered recurrent ear infections in infancy. He suffers from defective wound healing, and has thin dystrophic scars, which are the result of relatively minor trauma, such as insect bites. At the time of testing , he was aged 10 years, and, apart from one episode of pneumonia, has remained relatively free of major systemic complications of the disease.

SPECIAL METHODS

Neutrophil locomotion studies

Whole blood locomotion of neutrophils into micropore membranes

Heparinised blood was diluted in an equal volume of medium and a whole blood migration assay was performed (Al-Hadithy et al, 1981a). Briefly, filter paper was placed in a petri dish and moistened with medium. A strip of micropore membrane (3 micron pore-size, Sartorius) was placed on the filter papers. Plastic caps were filled with diluted blood and inverted onto the micropore membranes. A control sample was set up with each test sample on the same strip. The petri dish was covered and incubated at 37^{o}C for 2 hours in a humidified chamber. The caps were removed and excess red cells were washed off from the membrane. After fixing for 2 minutes in propanol, the membrane strips were stained with haematoxylin, washed under tap water, cleared in xylene and mounted in Ralmount (BDH). Neutrophil migration was determined by measuring the leading front of cells and, in some experiments, the number of cells per field at 50% of the leading front was also counted.

Migration of neutrophils under agarose

Cell migration under agarose was measured by a modification of the method of Nelson et al (1975). Microscope slides were coated with 1.2% agarose containing 0.1% gelatin. 3 wells, 5 mm in diameter, were made in a line with their edges spaced 4 mm apart. 50 μL of a suspension of purified neutrophils (2.5 X 10^{6}/mL in RPMI) were placed in the centre well, and 50 μL of medium or FMLP (10^{-7}M) was placed in the outer wells. The slides were placed in a moist chamber and incubated for 2 hours at 37^{o}C, followed by the addition of

6% glutaraldehyde in PBS to the wells for 5 minutes. The agarose was then carefully stripped off, the slides rinsed, dried and stained with Haematoxylin. Migrating cells were clearly recognisable under light microscopy by their flattened appearance, and the number in a standard sector was counted.

Neutrophil Phagocytosis and Killing of Candida

This assay was performed in whole blood (Al-Hadithy et al, 1981b). Briefly, heparinised blood was diluted in an equal volume of medium. Three concentrations of *Candida guillermondiae* were added to the diluted blood samples in LP3 tubes. The tubes were capped and placed on a rotator at 37^{o}C for 60 minutes. Cytospin preparations were made from each tube and stained with May-Grunwald-Giemsa for microscopy. The number of ingested organisms (which were easily identified as being contained within vacuoles) per 100 neutrophils, and percentage of ingested organisms killed (as evidenced by lack of staining, and a pale, translucent appearance) was quantified under oil microscopy by at least 2 independent observers.

GM-CSF administration to patient 1

Prior to the therapeutic trial of GM-CSF, a test dose was administered, according to the same protocol as for patients on the clinical trial, as described in Chapter 5. Serial venous blood samples were taken into heparinised tubes for immunofluorescence and neutrophil function tests, and into EDTA tubes for blood counts. Full blood counts were done on a S-PLUS STAKR (Coulter Electronics), and white cell differentials were done manually.

RESULTS

Surface expression of β2 integrins on patient cells

(a) Unstimulated cells

The results are expressed as a percentage of the surface expression on cells from at least 3 normal controls examined on the same day. The basal expression of CD11b and CD11c was significantly reduced on peripheral neutrophils and monocytes from both patients, with patient 1 exhibiting a greater defect than patient 2. In patient 1, CD11b and CD11c expression on neutrophils was 10.7 ± 2.5% and 15.5 ± 9.8% respectively (n=5) of normal resting levels, while on monocytes, the corresponding levels were 13.3 ± 4.9% and 6.6 ± 5.6% (n=5) respectively of normal (Figure 7.1a). On cells from patient 2, neutrophil CD11b and CD11c levels were 33 ± 9.8% (n=4) and 29 ± 5% (n=3), respectively, of normal (Figure 7.1b). Monocytes from patient 2 displayed slightly higher levels: CD11b expression was 63 ± 20% of

normal in 3 experiments, and, in one experiment, CD11c expression was 34% of normal control levels. In contrast to these low levels of CD11b and CD11c, the resting surface expression of CD11a was relatively high in both these patients, and approached normal levels in some experiments. In 5 experiments, neutrophils from patient 1 had CD11a levels which were 67.2 ± 16.4% of normal, while the corresponding level on monocytes was 75.2 ± 21.8% of normal (Figure 7.1a). Neutrophil CD11a expression in patient 2 was 80 ± 15% of normal in 4 experiments, and in one experiment, monocyte surface CD11a was 72% of normal. In cells from patient 1, this disparity between the levels of CD11a, and the other two integrin receptors, CD11b and CD11c was reflected in the common beta subunit, CD18, which demonstrated intermediate levels of expression (18.5 ± 3.0% on neutrophils, and 24.5 ± 5% on monocytes, n=3, Figure 7.1a).

(b) Effect of stimulation

Cells were examined for the surface expression of CD11b and CD11c following incubation of whole blood aliquots for 45 minutes at 37°C with GM-CSF, FMLP, TPA or medium as control. Samples from at least 2 normal individuals were included in each incubation with each agonist. In both patients, phagocyte CD11b expression was upregulated by each agonist, the proportional increase being similar to controls (approximately 2-fold increase), but even after stimulation, the level of antigen on the cells from patient 1 remained less than 30% of the resting levels in control (unstimulated) cells (Figures 7.2a, b). The maximal stimulated expression of CD11b on neutrophils from patient 2 was considerably higher; following incubation with GM-CSF and FMLP, CD11b expression rose from 33 ± 9.8% to 70.6 ± 14% (n=4) and 122 ± 60% (n=3), respectively, of levels on control unstimulated cells (Figure 7.2c). Even so, the maximal stimulated levels on patient's cells remained about 30% of the maximal stimulated levels on control cells, for each agonist studied. Stimulation of monocytes from patient 2 with GM-CSF and FMLP resulted in a similar degree of upregulation of CD11b expression (data not shown).

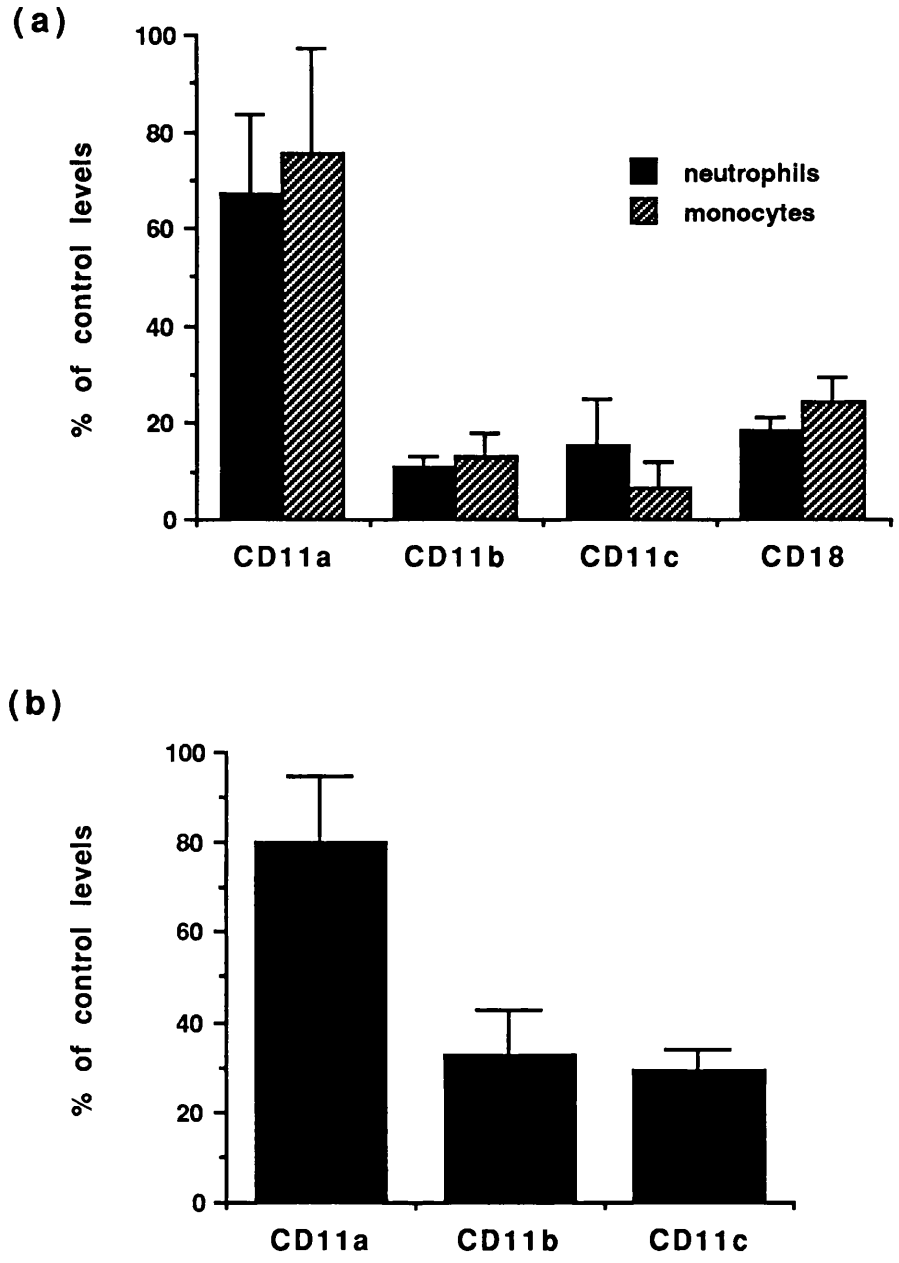
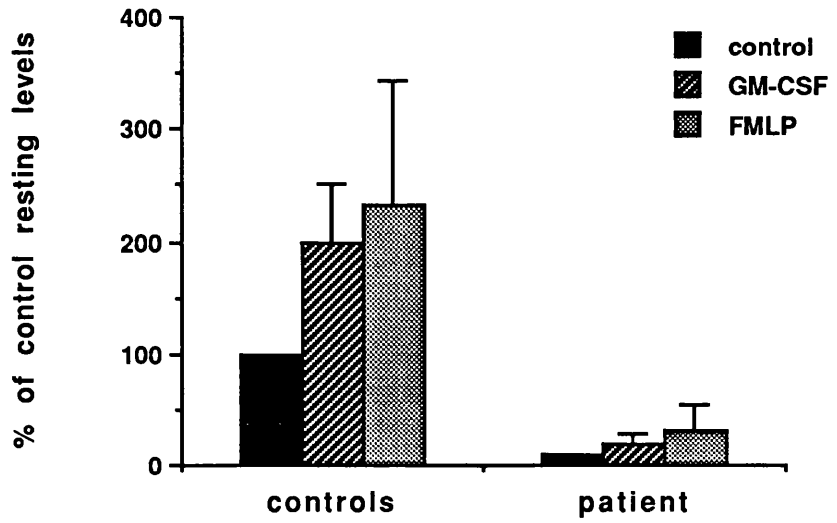


Figure 7.1. β2 integrin expression on (a) unstimulated neutrophils and monocytes from patient 1 and (b) unstimulated neutrophils from patient 2, measured as MCF of cells, and expressed as a percentage of MCF of cells from normal controls examined on the same occasion. Data is mean ± SD of the number of experiments as stated in the text.

(a)



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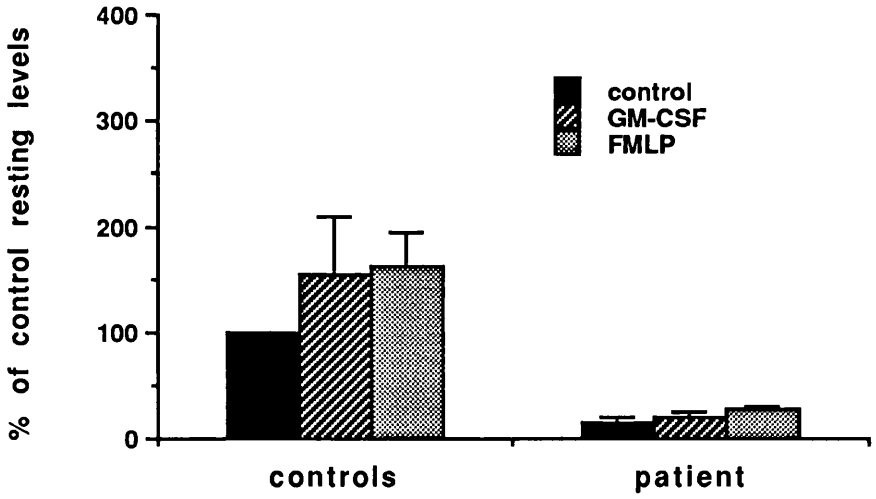


Figure 7.2. Effect of stimulation with GM-CSF (10 ng/mL) and FMLP (10^{-6} M) on CD11b expression of (a) neutrophils (n=5) and (b) monocytes (n=4) from patient 1. Data, expressed as percentage of the levels on unstimulated control cells, is mean ± SD.

(c)

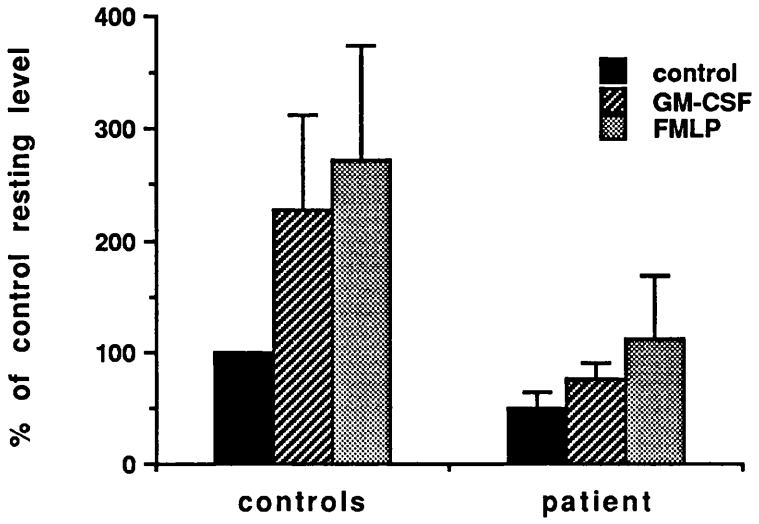


Figure 7.2 (c). Effect of stimulation with GM-CSF (10 ng/mL, n=4) and FMLP (10^{-6} M, n=3) on CD11b expression on neutrophils from patient 2. Data, expressed as percentage of levels on unstimulated control cells, is mean ± SD.

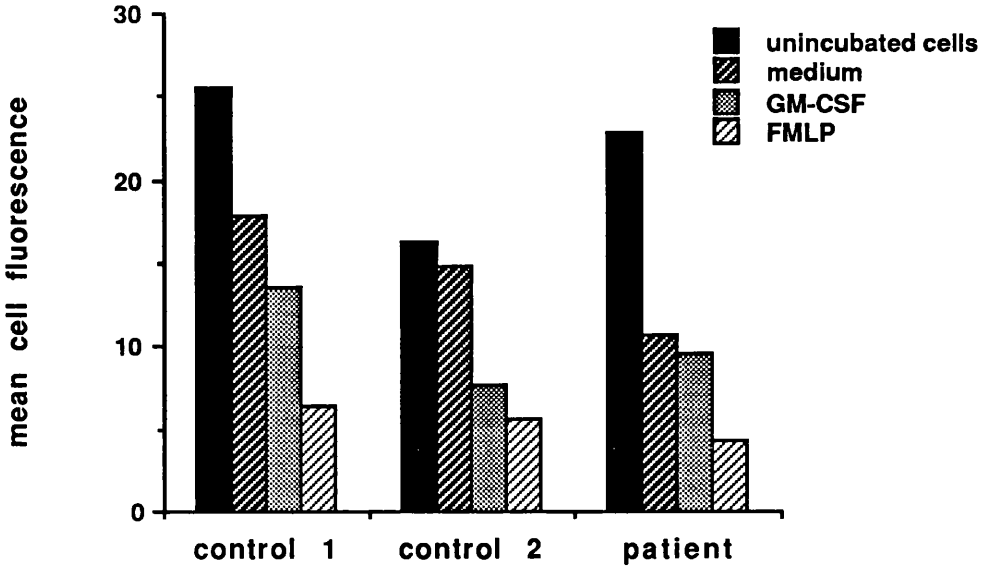


Figure 7.3. L-selectin expression on neutrophils from patient 2 and 2 controls (1 experiment). Antigen expression, expressed as MCF, is given for unincubated cells kept at room temperature, and for cells incubated with medium alone, GM-CSF (10 ng/mL), or FMLP (10^{-6}M) for 45 minutes at 37°C. Data is the mean of duplicate samples.

Expression of L-selectin on LAD neutrophils

The surface expression of L-selectin on neutrophils from patient 1, examined on 3 occasions, was normal (data not shown). L-selectin expression on neutrophils from patient 2 was also normal, and was downregulated by GM-CSF and FMLP in a similar fashion to control cells (Figure 7.3).

Neutrophil adherence studies

Adherence to cultured human umbilical vein endothelial cells (HUVEC)

The adherence of LAD and normal control neutrophils to cultured human endothelial cells was examined in the presence, or absence of stimulation by GM-CSF or TPA, and under different serum conditions. In both patients, the adherence of unstimulated neutrophils to endothelial monolayers was not different from that of cells from normal controls examined on the same day. In patient 1, basal adherence was 14.7 ± 3.3% (mean ± SE, n=3), as compared with 16.3 ± 5.2% (n=3) for control cells (Figure 7.4). The adherence of unstimulated neutrophils from patient 2 (10.2 ± 1.6%, n=3) was also comparable to that of cells from normal controls (11.3 ± 3.5%, n=3).

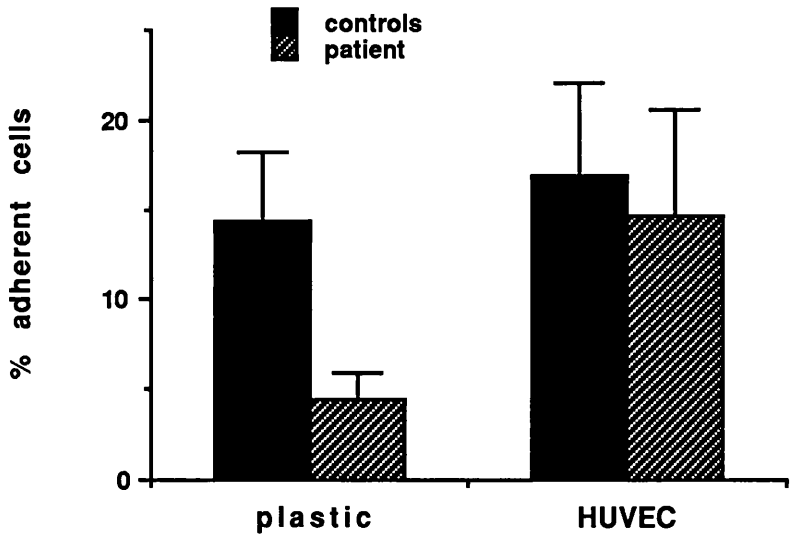


Figure 7.4. Percentage adherence of unstimulated neutrophils from patient 1, and from normal controls to plastic and to HUVEC, expressed as mean ± SE of 3 experiments.

The effect of GM-CSF and TPA on neutrophil adherence to endothelium was examined under different serum conditions. Neutrophils from normal donors demonstrate an increased adherence to HUVEC in the presence of GM-CSF, and this response is independent of serum conditions (Figures 7.5 and 3.5). In 2 experiments, neutrophils from patient 1 did not respond to GM-CSF under low serum conditions (while cells from normal donors showed an increase to 156 ± 17% (n=4) of baseline in the same experiments), but, in the presence of 50% foetal calf serum (FCS), exhibited an enhanced adherence to HUVEC (140%, 142%), similar to that demonstrated by cells from normal controls (132 ± 16%, n=4, Figure 7.5). The adherence response of neutrophils from patient 2 was examined in 2 experiments. In 1 experiment, the LAD neutrophils did not respond to GM-CSF, in either serum-free medium, or in the presence of 50% serum (Figure 7. 6) while, in the same experiments, control neutrophils showed an increase to 137.5%, and 146.7% of baseline, respectively. In a second experiment using radiolabelled neutrophils and in the presence of 5% FCS, cells from this patient were able to increase adherence to HUVEC (to 125% of baseline) in response to GM-CSF (Figure 7.7).

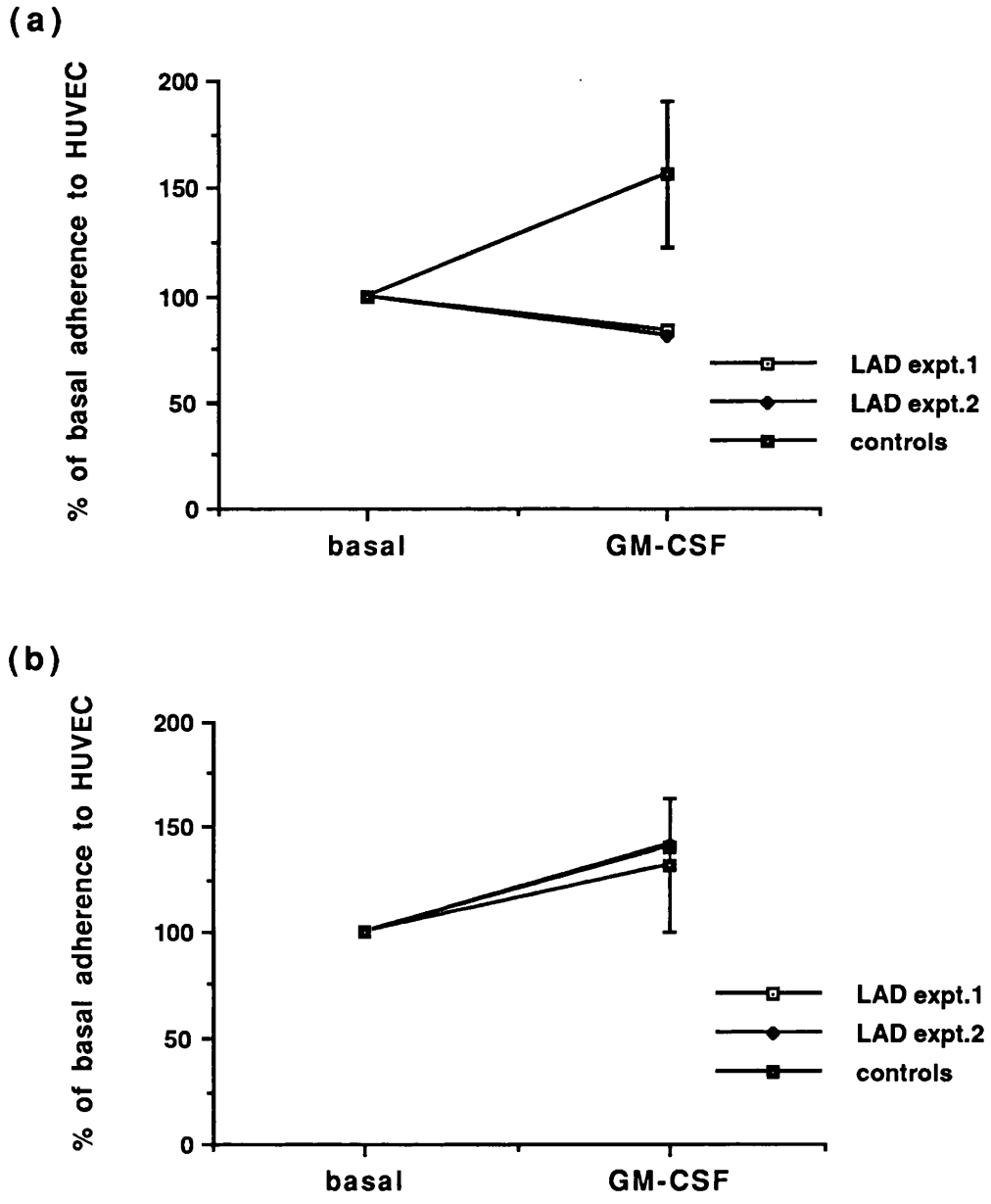


Figure 7.5. Effect of GM-CSF (100 ng/mL) on adherence of neutrophils from patient 1 and normal controls to HUVEC in the presence of (a) 2% serum, and (b) 50% serum. Data, expressed as percentage of basal adherence (adhesion of unstimulated cells), is given for each of 2 experiments with LAD cells, and, for controls, is mean ± SE of 4 experiments.

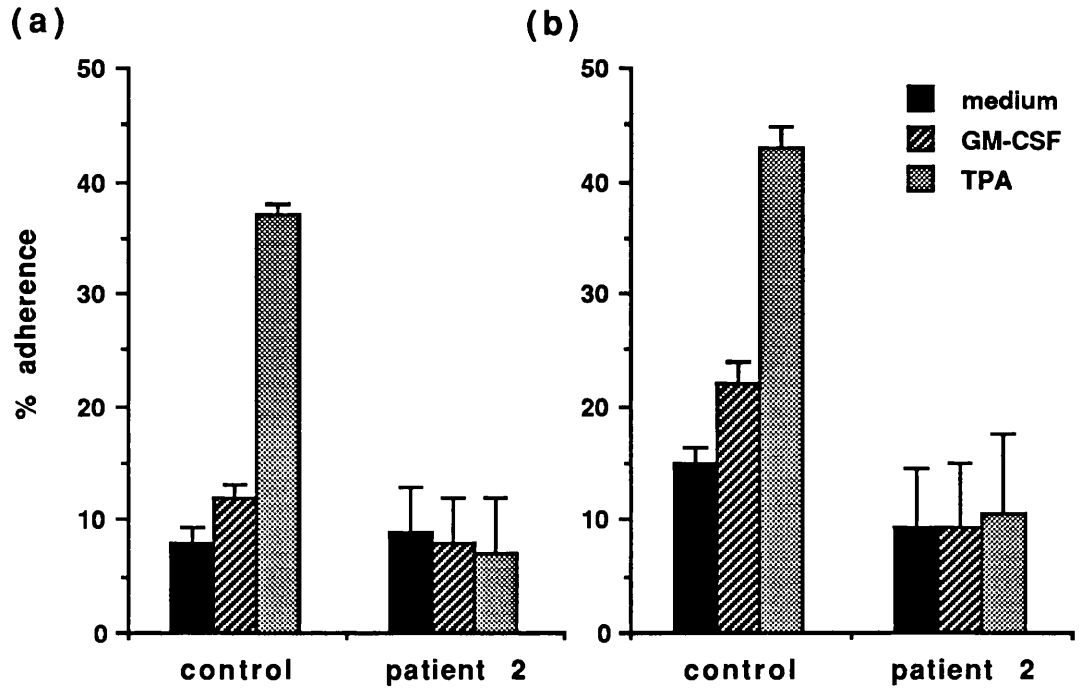


Figure 7.6. Adherence of neutrophils from patient 2 and from a normal control to HUVEC under unstimulated conditions, and in the presence of GM-CSF (100 ng/mL), and TPA (1 μg/mL) (a) in absence of serum, and (b) in 50% serum. Data is mean ± SD of 6 replicate points of 1 experiment.

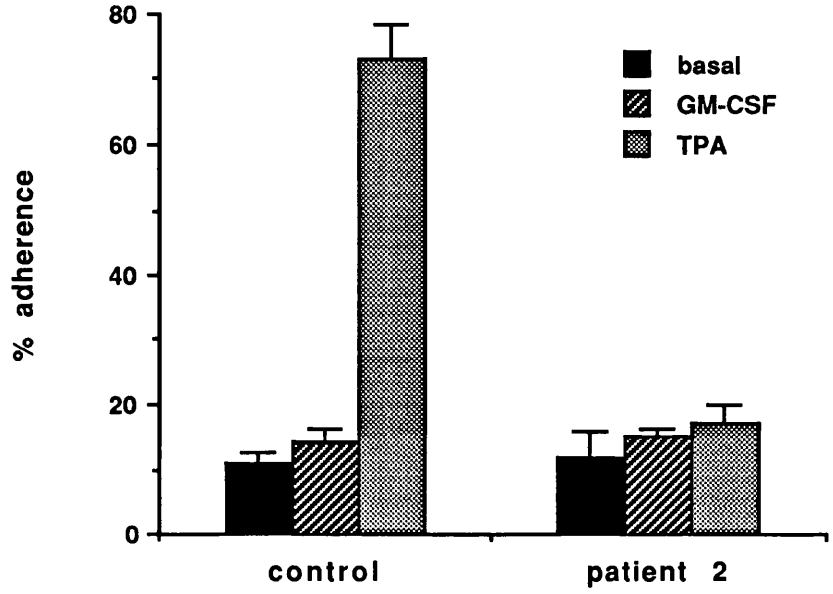
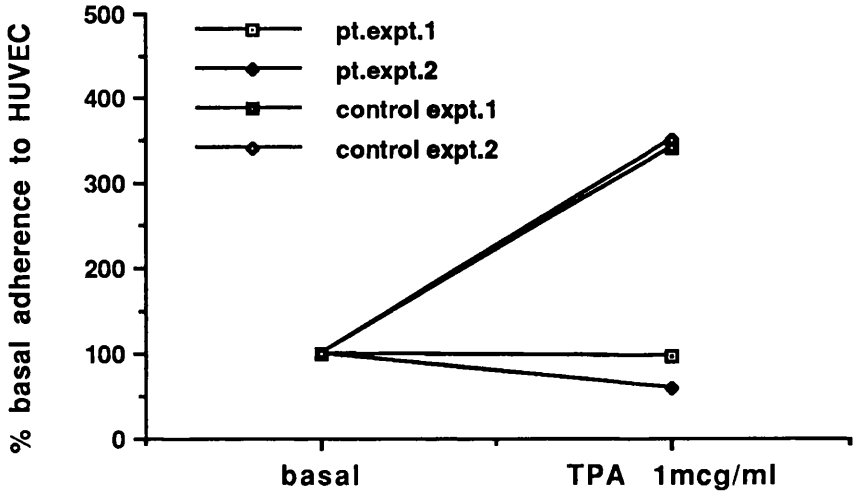


Figure 7.7. Effect of GM-CSF (100 ng/mL) and TPA (1 μg/mL) on the adherence of radiolabelled neutrophils from patient 2 and a normal control to HUVEC. Data is mean ± SD of 6 replicate points of 1 experiment.

The most striking defect in the adherence of LAD neutrophils was seen in the presence of TPA. TPA (1 μg/mL) stimulation of normal neutrophils results in a marked (more than 3-fold) increase in adhesion to HUVEC (from 11.2 ± 2.6% to 45.5 ± 7%, mean ± SE, n=5). This response, like that to GM-CSF, is independent of serum concentration. In 2 experiments conducted in the presence of 50% serum, neutrophils from patient 1 showed no adherence response to TPA (Figure 7.8a), despite the fact that TPA (at the same dose) induced upregulation of surface CD11b and CD11c on these cells (from 4% to 23%, and from 15% to 63% respectively, of the levels found on unstimulated neutrophils from normal donors), and GM-CSF augmented adhesion of these cells to HUVEC. In one experiment, LAD neutrophils from patient 2 showed no response to TPA, regardless of serum concentration (Figure 7.6). When radiolabelled cells were used in a second experiment (Figure 7.7), LAD cells from patient 2 were able to increase adherence from 12% to 17% in response to TPA, but this response is markedly subnormal, when compared with control cells examined on the same day, which showed an increase in adherence from 11% to 73% with TPA.

(a)



(b)

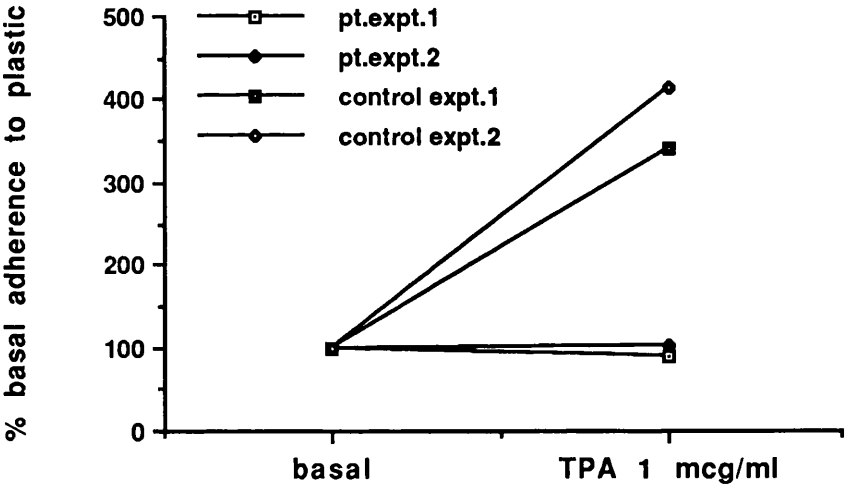


Figure 7.8. Effect of TPA (1 μg/mL) on the adherence of neutrophils from patient 1 and a normal control to (a) HUVEC, and (b) plastic. Data, expressed as percentage of basal adherence, is given for each of 2 experiments with patient and control cells.

Adherence to plastic

LAD neutrophils from patient 1 were examined for adherence responses to plastic. Under unstimulated conditions, there was a marked defect in the adherence of LAD neutrophils to plastic, the percentage of adherent cells being 4.4 ± 1.5% (n=3) as compared to 14.4 ± 3.8% (n=5) in cells from normal individuals (Figure 7.4). This contrasts with the normal adherence of these cells to HUVEC (see above). GM-CSF did not increase neutrophil adherence to plastic, either in controls, or in the patient (112 ± 7% of baseline for control neutrophils, and 92 ± 21% of baseline for LAD neutrophils). Once again, TPA had no effect on the adherence of the LAD cells, in contrast to its marked proadhesive effect on control cells (Figure 7.8b).

Neutrophil Locomotion

LAD neutrophils from patient 1 demonstrated a significant defect in locomotion as assessed by the migration of cells through 3 micron pore-size membranes in the whole blood locomotion assay. The leading front of cells, done on 5 separate occasions, was 49.0 ± 9.1% of normal controls tested at the same time ($p < .001$, Table 7.1). There were slightly fewer neutrophils from the LAD patient entering the membrane (cell counts at 50% of leading front being 28 ± 16, n=4 compared with 33 ± 8, n=4 for controls). However, when the counts were standardised for the peripheral neutrophil count (60-80 X 10^9/L in the patient compared with 4-8 X 10^9/L in controls), the proportion of cells entering the membrane was less than 10% of controls. The addition of GM-CSF at the start of the migration assay had no significant effect on cell migration (n=3, data not shown).

Neutrophils from patient 2 demonstrated a similar defect in migration assays across micropore membranes. In 4 experiments, leucocytes were separated from heparinised whole blood by Dextran sedimentation, and resuspended in Eagle's MEM with 30% autologous plasma for migration assays across micropore membranes. In these experiments, cell migration was assessed, both as the leading front, and as the proportion of migrating cells. The latter was quantitated by counting the number of cells which had moved into the membrane as compared with the number remaining on the surface in any one X400 field, and the number of migrating cells was expressed as a percentage of the total number of cells in that field. The leading front of migration achieved by LAD neutrophils was 50 ± 5% of the levels achieved by control cells (Table 7.1). In addition, the percentage of cells which had moved into the membrane (28 ± 4%, n=4) was significantly lower than that for control cells (80 ± 4%, n=4). In one experiment, cells were preincubated with GM-CSF (10 ng/ml) for 40 minutes prior to the migration assay. This led to a slight decrease in

migration of both patient and control cells, measured both as the leading front, and as the percentage of migrating cells (data not shown).

In one experiment, cells from patient 2 were tested in an under-agarose technique. In this assay, the migratory defect of LAD neutrophils was even more pronounced. Leucocyte suspensions in plasma-free medium were placed in a well in an agar plate, and the number of cells moving in either direction towards wells in which FMLP or medium as control had been placed was quantified. When the effects of GM-CSF were investigated, GM-CSF (100 ng/mL) was placed in the centre well with the cell suspension at the start of the assay, but was not present in the agar, or the adjacent wells. In this assay system, normal unstimulated cells do not migrate under the agar, but do so in response to chemotactic/chemokinetic stimulation, such as FMLP (Table 7.2). GM-CSF, in this system, appears to act as a chemokinetic stimulus, increasing random migration and also the directed migration towards FMLP. LAD neutrophils from patient 2 demonstrated a marked defect of stimulated migration in this system (Table 7.2).

Finally, the migration of LAD neutrophils across confluent endothelial monolayers cultured on 3 micron-pore filters was examined. Full details of this technique, including culture conditions and details of the migration assay are given in Chapter 8. The results obtained for LAD neutrophils are given in full in Chapter 8, and are only summarized briefly here. In this system, neutrophils from normal individuals demonstrate a baseline unstimulated migration of 7.4 $\pm$ 1.3% (n=7). Inclusion of GM-CSF in the assay medium, whether with the cells, or in the lower well, or both, results in a significant increase in the percentage of migrating cells, to 14.5 $\pm$ 0.3% ($p < .005$, n=5). Similarly, FMLP 10^{-8}M, when placed in the lower well, increases neutrophil migration to 30 $\pm$ 5.5% ($p < .005$, n=7). In one experiment, the transendothelial migration of neutrophils from patient 2 was studied. LAD neutrophils demonstrated normal migration in the absence of stimulation (10.8% as compared to 7% for control cells), but were unable to increase migration in response to GM-CSF (12% in the presence of GM-CSF), or to chemotactic stimulation with FMLP (in this experiment, FMLP was able to increase the migration of control cells from 7% to 40%, but had no effect on the migration of LAD neutrophils, Figure 8.5b, page 143).

Table 7.1. Migration of LAD and normal neutrophils through 3 micron pore membranes.

	Leading Front (μ)	% of cells migrating
patient 1	45 ± 5.3	ND
controls	92 ± 8.4	ND
patient 2	44 ± 4.0	28 ± 4
controls	88 ± 4.0	80 ± 4

Data is the mean ± SD of 5 experiments with cells from patient 1, and 4 experiments using cells from patient 2. A different control subject was used for each occasion of testing in each patient.

Table 7.2. Migration of neutrophils from patient 2 and a normal control in the under agarose assay

	unstimulated cells		GM-CSF stimulated cells	
	medium	FMLP	medium	FMLP
control	0.4 ± 0.5	177 ± 19	56 ± 13	260 ± 9
patient 2	0	5.3 ± 1.5	5.6 ± 2.0	13.0 ± 4.4

Duplicate slides were set up for each condition. On each slide, the number of cells migrating outward toward each of the side wells was counted in 3 standard sectors under light microcscopy, and data expressed as mean ± SD of the 6 replicate counts for each condition.

Neutrophil Phagocytosis and Killing of Candida

Experiments were performed on whole blood samples. The number of candida ingested per 100 LAD neutrophils was markedly reduced (5 ± 1.9 per 100 LAD cells cf. 90 ± 8.3 per 100 control cells), but this may be influenced by the higher neutrophil count of the patient, with correspondingly lower candida:neutrophil ratios. Killing of ingested Candida was also markedly reduced, with only 24 ± 8.8% of ingested organisms killed as compared with 71 ± 6.5% in controls (n=4, $p < 0.001$). In normal individuals, decreased candida:neutrophil ratios giving lower ingestion rates leads to increased percentage killing of ingested organisms (Al-Hadithy et al, 1981). Therefore, taking into account the grossly elevated neutrophil counts in the patient, LAD cells may be killing even less efficiently than the figures imply. There was no effect of GM-CSF when added to the reaction mixture *in vitro* (n=2). Neutrophils from patient 2 were similarly defective in this assay, demonstrating levels of phagocytic/killing activity of about 50% of normal controls (data not shown).

Effect of in vivo GM-CSF infusion on phagocyte function

In patient 1, prior to the therapeutic trial with GM-CSF, a test dose of 30 $\mu g/m^2$ in 100 mL 0.9% NaCl was infused into a peripheral vein over 2 hours. Within 5 minutes of commencement of the infusion, peripheral granulocyte and monocyte counts fell dramatically (Figure 7.9a); granulocyte counts were still low at the end of the infusion. Coinciding with the fall in circulating cells, the levels of CD11b on granulocytes increased to over 300% of initial levels, although this was still only 30% of normal resting levels (Figure 7.9b). There was no significant change in neutrophil L-selectin expression.

Phagocytosis and killing of Candida was examined before and after the 2-hour test infusion of GM-CSF, and also before and after the patient had received 3 daily infusions of GM-CSF. On both occasions, *in vivo* administration of GM-CSF increased percentage killing of ingested Candida. At a 1:3 volume dilution of candida in whole blood, percentage killing of ingested candida increased from 21% prior to GM-CSF administration to 39% at the end of the test infusion. Similarly, when cells were tested after 3 days of GM-CSF at 250 $\mu g/m^2$ daily, percentage killing of ingested organisms had increased from 16% prior to GM-CSF therapy to 35%.

There was no change in the migration of LAD neutrophils, examined before and after the infusion (data not shown).

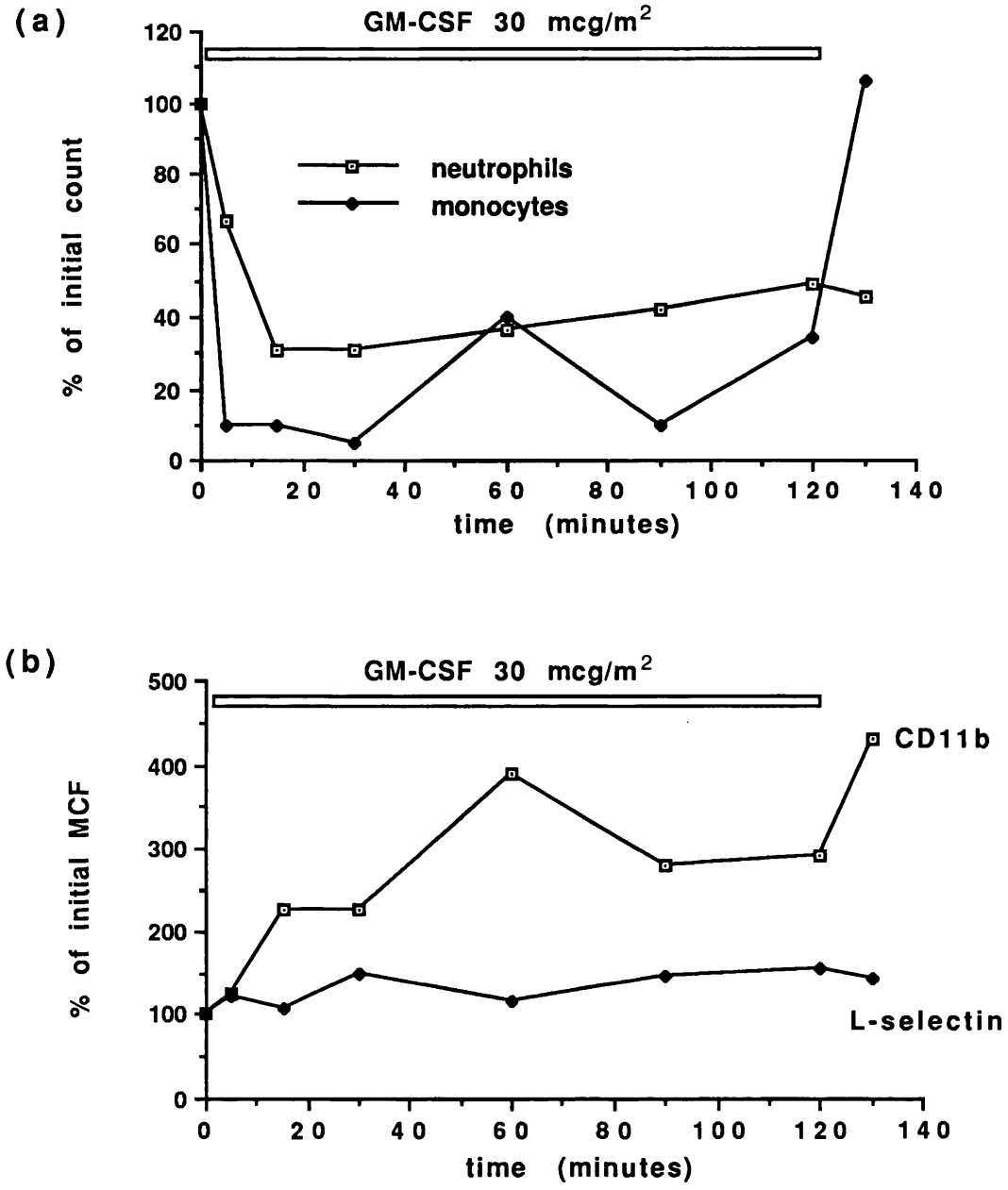


Figure 7.9. Effect of GM-CSF (30 μg/m^2 over 2 hours) on (a) neutrophil and monocyte counts, and (b) neutrophil surface CD11b and L-selectin/LAM-1 in patient 1

DISCUSSION

The cells used in this series of experiments were from 2 patients with partial LAD. Patient 1 had more severe clinical manifestations of the disease, and correspondingly, phagocyte levels of CD11b and CD11c were lower, being 10-15% of that seen in normal individuals. Phagocytes from patient 2 have slightly higher levels of CD11b/11c expression (about 30%), in keeping with the milder phenotype expressed by this patient. In both patients, CD11a levels were relatively normal, suggesting that the CD11a subunit has a higher affinity than the other two α subunits for the common β subunit, which is present at limiting concentrations. This disparity of expression between CD11a and CD11b has been noted previously in other patients with the moderate phenotype of the disease (Anderson et al, 1985). GM-CSF and other neutrophil agonists were able to upregulate the surface levels of CD11b/11c on patients' phagocytes, although the maximal stimulated levels on cells from patient 1 remained less than a third of normal resting levels. Phagocytes from patient 2 were able to increase surface levels of CD11b/11c on maximal stimulation to within the levels expressed by normal resting cells, but which were still only 30% of the expression on maximally stimulated control cells. Surface levels of L-selectin were normal on neutrophils from both patients, and, in cells from patient 2, were downregulated by GM-CSF and FMLP *in vitro*, in a similar fashion to control cells. These results are in accord with previous reports which demonstrate that stimulation with FMLP can upregulate surface levels of CD11b on neutrophils from patients with the moderate phenotype of the disease (Arnaout et al, 1984), but has no effect on neutrophils from patients with severe LAD (Anderson et al, 1985).

The defect in adhesive functions of the cells from these patients was confirmed in functional assays. In accord with previous studies (reviewed in Anderson & Springer, 1987), we have demonstrated defective phagocytosis/kiling of Candida, suggesting that these phagocyte functions are in part dependent on CD11b and CD11c. One explanation for this dependence would be that these integrin receptors are involved in the cytoskeletal organisation of phagocytic cells, and, hence, required for cell motility. The reduced killing of ingested organisms does not necessarily imply reduced respiratory burst activity, but could reflect the slower rate of phagocytosis. In further studies, the superoxide production by GM-CSF primed LAD neutrophils in response to soluble agonists (FMLP and TPA) was normal.

Under unstimulated conditions, LAD neutrophils from patient 1 showed markedly defective adhesion to plastic but virtually normal adhesion to human umbilical vein endothelial cells. This suggests that adhesion to plastic is more dependent on the $\beta 2$ integrins than adhesion to endothelium, although stimulation with GM-CSF, while upregulating these receptors in both normal and LAD neutrophils, did not

significantly augment adhesion to plastic. This difference in the adhesion of unstimulated LAD neutrophils between plastic surfaces and endothelial cells has been noted by others (Buchanan et al, 1982). The normal adherence of unstimulated LAD neutrophils to endothelium was confirmed by experiments using cells from patient 2. Incubation of control neutrophils with GM-CSF significantly augments adhesion to endothelial cells, and this response is independent of serum concentration in the assay medium. The adhesive response of LAD neutrophils to GM-CSF is variable, and, in cells from patient 1, only seen in the presence of high serum levels. The reasons for this serum dependence when CD11b and CD11c levels are limiting are not clear but CD11b has been shown to bind fibrinogen and Factor X (Altieri et al, 1988a,b), which could play an intermediary role in the adhesion to endothelium, and similar bridging mechanisms may be involved with other adhesion molecules. LAD neutrophils from patient 2 showed no response to GM-CSF in one experiment, regardless of serum concentration, but demonstrated increased adhesion in the presence of GM-CSF in a second experiment in which radiolabelled cells were used.

The defective interaction of LAD neutrophils with endothelium seen *in vitro* was most pronounced in adhesion assays carried out in the presence of TPA. Phorbol esters are some of the most potent agonists for enhancing *in vitro* neutrophil adhesion to both plastic and endothelium, yet adherence of LAD cells was completely unaffected by exposure to TPA in all experiments except one in which radiolabelled cells from patient 2 demonstrated a modest, but still subnormal, response to TPA. The strikingly defective response of LAD cells to TPA contrasts with the variable, and perhaps serum dependent, response to GM-CSF, and further emphasizes the difference between GM-CSF and TPA mediated neutrophil adhesion to endothelium, which is discussed in Chapter 3. These results suggest, not only that adhesion to plastic and to endothelium are mediated by different mechanisms, but that augmentation of adhesion by GM-CSF and phorbol esters also involve distinct processes. GM-CSF-induced adhesion can clearly occur in the presence of low levels of CD11b and CD11c whereas TPA-induced adhesion appears to require higher levels.

The results presented here are, broadly speaking, in accord with other studies which have also shown, using cells from some LAD patients (probably with the moderate form of the disease) that, while unstimulated cells adhere normally to endothelium and artificial surfaces, there is a failure to augment adhesion in response to agonists, in particular, phorbol esters (Anderson et al, 1985, Harlan et al, 1985, Tonnesen, 1989). The differential behaviour of unstimulated and stimulated LAD cells in these *in vitro* adhesion systems is consistent with published data which shows that, while

the adherence of unstimulated neutrophils to endothelium is only partially dependent on β2 integrin receptors (and this dependence is mainly mediated by CD11a), the enhanced adherence in response to neutrophil mediators is almost entirely mediated by β2 integrins, this time, largely by CD11b (Zimmerman et al, 1985, Harlan et al, 1985, Smith et al, 1989, Lo et al, 1989b). In keeping with these observations, the LAD cells used in these studies display disproportionately high surface levels of CD11a (thus explaining the normal adherence of unstimulated cells) in comparison to the low levels of CD11b/CD11c (see above).

The finding that GM-CSF-induced adhesion of LAD neutrophils from patient 1 to endothelium was normal in the presence of high serum levels was confirmed by *in vivo* administration of GM-CSF, which caused rapid phagocyte margination, comparable to that seen in normal individuals (Devereux et al, 1989). These observations suggest that high levels of CD11b are not required for this form of adhesion, in keeping with the results presented so far. This is further supported by the disparity between the kinetics of the *in vivo* rise in surface CD11b, and the changes in peripheral phagocyte numbers which have been discussed previously (Chapter 5). Qualitative, rather than quantitative changes in the adhesive function of these adhesion molecules may be what is relevant. However, it is unlikely that a conformational change in such a small number of receptors as expressed by the resting LAD phagocytes in our studies (10% of normal) could lead to normal GM-CSF-induced margination. This raises the possibility that other adhesion receptors are involved in mediating the 'pro-adhesive effects' of GM-CSF in vivo, either CD11a which is expressed normally in the LAD neutrophils used in this study, and is upregulated in response to GM-CSF, or other adhesion molecules such as L-selectin, which is expressed at normal levels on LAD neutrophils. The possible contribution of changes in cell deformability to GM-CSF margination has already been mentioned in Chapter 6.

The migratory defect of LAD neutrophils into micropore membranes demonstrated in this study is similar to that described in previous reports on partial LAD neutrophils (Anderson & Springer, 1987), i.e. about 50% of the response of normal cells. It is interesting that although the neutrophils from patients 2 express about twice as many CD11b and CD11c receptors as granulocytes from patient 1, the defect in migration is very similar. The far more pronounced defect revealed in the under agarose migration assay suggests that cell movement in this system may be more dependent on the β2 integrins, and may relate to a fundamental difference between two- and three-dimensional movement of cells (Schmalstieg et al, 1986). The normal migration of unstimulated cells across endothelium demonstrated in these

studies is also in keeping with previous studies on LAD neutrophils, one of which used a similar system of endothelial monolayers on filters (Harlan et al, 1985), and another which examined the migration of cells under endothelium cultured on coverslips (Buchanan et al, 1982). Harlan et al (1985) also demonstrated that neutrophils pretreated with MoAb to CD18 displayed normal movement across endothelium in the absence of stimulus. In contrast, LAD neutrophils were unable to migrate across endothelium in response to a chemotactic stimulus (Harlan et al, 1985 and this study), or in response to endothelial activation (Smith et al,1991).

It thus appears that LAD neutrophils are capable of interacting normally with endothelium under basal conditions, and are able to increase their adhesiveness in response to some (GM-CSF) but not all stimuli. The failure of neutrophils to extravasate into tissues *in vivo* may be related to the reduced migratory activity seen *in vitro*, in particular the failure to migrate across endothelium in response to stimulation. Recent studies examining the interaction of neutrophils with endothelium under conditions of flow have yielded results which are in keeping with these observations. Under the range of flow rates estimated to exist in postcapillary venules (where margination of cells occurs), and which generate wall shear stresses of 1-10 dynes/cm^2 (Atherton & Born, 1973), the interaction of neutrophils with endothelium was found to be largely β2 integrin-independent, but to be dependent on the selectin family, L-selectin on neutrophils, and ELAM-1 on endothelium (Lawrence et al, 1990, Lawrence & Springer, 1991). This interaction, in which leucocytes roll along the endothelial lining of postcapillary venules, can also be demonstrated on artificial lipid bilayers containing purified selectin receptor, CD62, (Lawrence & Springer, 1991). In contrast, the adhesion of activated neutrophils to endothelium is CD18-dependent, can only take place at sheer stresses of about 0.5 dynes/cm^2, and leads to the immobilisation of neutrophils on endothelium (Lawrence & Springer, 1991, Smith et al, 1990). Using an *ex-vivo* preparation of exteriorized rabbit mesenteric vessels, and intravital microscopy, one group has demonstrated that anti-L-selectin MoAb inhibits leucocyte rolling, and subsequent cell arrest on endothelium, while anti-CD18 MoAb was only effective in blocking the arrest (firm adhesion) of rolling cells (von Andrian et al, 1991). In addition, a recombinant soluble chimaera consisting of L-selectin linked to human IgG Fc regions (LEC-IgG) also inhibited leucocyte rolling in the same system (Ley et al, 1991). Hence leucocyte rolling, mediated, at least in part, by selectin receptors on both leucocytes and endothelium may represent the inital interaction between circulating leucocytes and activated endothelium lining the venules in inflamed tissues. The rolling neutrophils are then activated by signalling molecules (such as PAF and IL-8) expressed on inflamed endothelium, this leads to the functional

upregulation of CD18, and the subsequent binding via CD18 facilitates transendothelial migration (von Andrian et al, 1991).

In contrast to these different forms of attachment of neutrophils to endothelium, the transendothelial migration of neutrophils is heavily dependent on the β2 integrins, even at higher shear forces, and both CD11a and CD11b appear to be required (Smith et al, 1989). These observations would explain why LAD neutrophils, while demonstrating a normal interaction with endothelium under unstimulated conditions, such as the margination of cells in postcapillary venules, are unable to adhere to endothelium when activated by local cytokines in inflammatory areas, where vascular flow may reach near stasis, and to migrate across endothelium and localise in an infective focus.

The *in vivo* studies of Lundberg & Wright (1990) and Lindbom et al (1990) referred to in Chapter 6 further support these ideas. In the first study, pretreatment of rabbits with anti-CD18 MoAb prevented neutrophil emigration into intradermal inflammatory sites, but had no effect on the margination of neutrophils in lungs following systemic injection of FMLP. In the second study, anti-CD18 MoAb inhibited the emigration of neutrophils into inflammatory areas, but did not prevent the rolling of leucocytes along vessel walls (Arfors et al, 1987, Lindbom et al, 1990). The role of the CD11/CD18 receptor in neutrophil migration across endothelium is explored in Chapter 8.

The question that arose in the late stages of patient 1's illness was whether systemic GM-CSF would be of value. *In vitro* studies showed that GM-CSF increased the expression of leucocyte integrins, adhesion to endothelium and, as expected, could prime neutrophils for agonist-induced respiratory burst activity. A test infusion was therefore performed, confirming the *in vivo* increase in adhesion (margination), priming of the respiratory burst, and also showing augmentation of phagocytosis and killing, which had not been demonstrable *in vitro*. This last, however, should be interpreted with caution, bearing in mind that only 2 experiments were performed, both of which were in the context of the patient's changing clinical state. On the other hand, GM-CSF could further reduce the migration of phagocytes into an inflammatory focus (Addison et al, 1989, Peters et al, 1988), which would counteract the beneficial effects. Ultimately, when the patient's skin lesions were progressing despite conventional therapy, and the patient was septic, a therapeutic trial of GM-CSF was commenced. This made no impact on the lesions and shortly afterwards, he died. Whether GM-CSF or alternative

cytokines might have made an impact at an earlier stage in the evolution of such lesions is unresolved.

CHAPTER EIGHT

STUDIES ON NEUTROPHIL MIGRATION ACROSS HUMAN ENDOTHELIUM *IN VITRO* : EFFECT OF GM-CSF AND THE ROLE OF CD18 AND L-SELECTIN RECEPTORS

INTRODUCTION

The results presented hitherto demonstrate that GM-CSF increases neutrophil adhesion to endothelium under static conditions *in vitro*, and, when administered *in vivo*, produces a transient increase in the number of neutrophils interacting with vascular endothelium in the lungs. The former effect is inhibited by MoAbs to CD11b/CD18, while the latter *in vivo* effect is not. Studies on CD18 deficient neutrophils were, broadly speaking, in agreement with these findings.

The adhesion of circulating neutrophils to activated endothelial cells lining venules of inflamed tissues is only the first step of leucocyte extravasation. The subsequent migration of neutrophils across endothelium, and into extravascular tissues is under the control of soluble mediators, which act on both leucocytes and endothelial cells. Inflammatory mediators which act directly on leucocytes can act at various stages in the recruitment of phagocytic cells into infective foci. Apart from promoting adhesion to endothelium, some also act as chemoattractants, for example, FMLP, leukotriene B4 (LTB4), and platelet activating factor (PAF), to induce the migration and localisation of neutrophils to inflammatory sites (Palmblad et al, 1981, Wardlaw et al, 1986). In addition, inflammatory mediators which activate neutrophils, such as GM-CSF and IL-8, are synthesized and secreted by cytokine activated endothelium, along with other neutrophil activating factors such as PAF, and leukotriene C_4 (Fibbe et al, 1989, Strieter et al, 1989, Zimmerman et al, 1990). Endothelial activation by IL-1 and TNF also induces the expression of various endothelial adhesion receptors, such as ICAM-1 and ELAM-1, thus promoting the adherence of leucocytes (Pohlman et al, 1986, Pober et al, 1986, Bevilacqua et al, 1989). The endothelium thus participates actively in the recruitment and activation of leucocytes into inflammatory areas. *In vitro*, pretreatment of cultured endothelial monolayers with IL-1 and TNF increases the transendothelial migration of neutrophils (Moser et al,1989, Furie & McHugh, 1989, Smith et al, 1991, Luscinkas et al, 1991), but less is known of the direct effect of neutrophil activating agents on cell migration.

The recent clinical interest in GM-CSF derives, not only from its ability to increase neutrophil numbers, but also from its ability to enhance the functional activity of mature cells. However, it is not clear that activating circulating cells by administering inflammatory cytokines sytemically will be of clinical benefit. Systemic administration of GM-CSF reduces the migration of neutrophils into skin windows (Peters et al, 1988, Addison et al, 1989), suggesting that exogenously administered GM-CSF present within the vascular compartment may decrease the ability of neutrophils to extravasate into inflammatory sites. A similar effect has been demonstrated for IL-8 in rabbits (Hechtman et al, 1991). However, when injected intradermally, IL-8 induced local neutrophil accumulation. *In vitro*, IL-8 has been shown to induce the migration of neutrophils across endothelium, but only in the presence of a gradient (Huber et al, 1991). The enhanced neutrophil migration appeared to be quite separate from, and not related to, the effect on adhesion to endothelium. GM-CSF was initially characterized by its ability to inhibit neutrophil migration in an agarose assay (Weisbart et al, 1979), but has since been reported to act as a chemoattractant in Boyden chambers (Wang et al, 1987). The way in which these inflammatory cytokines act to control the extravasation of neutrophils may, therefore, depend upon their secretion locally, in infective foci. Hence a general increase in the adhesiveness of circulating cells activated by systemically administered GM-CSF may not necessarily lead to an increase in the number of neutrophils available for transendothelial migration, but may, paradoxically, inhibit the movement of cells into extravascular tissues.

Recently, much interest has focussed upon the relative participation of the different adhesion receptors in the adhesive interactions which underly the processes of neutrophil extravasation. One proposed model suggests that the initial rolling attachment of circulating neutrophils to inflamed endothelium is mediated by selectin receptors, on both leucocytes and endothelial cells (von Andrian et al ,1991, Lawrence & Springer, 1991). Activation of rolling neutrophils produces functional upregulation of CD11/CD18 receptors which bind to endothelial cell surface ligands to strengthen adhesion, and to mediate subsequent cell migration into the extravascular tissues. *In vitro*, MoAbs to CD18 are able to block neutrophil migration across cytokine activated endothelium, while MoAbs to L-selectin are without effect (Smith et al, 1991). On the other hand, anti-MEL-14 (murine homologue of L-selectin) MoAb blocks neutrophil extravasation *in vivo* (Jutila et al, 1989), while histological studies in the mouse show that neutrophils which have extravasated into local inflammatory areas *in vivo* express low levels of MEL-14 and high levels of CD18 (Lewinsohn et al, 1989). Shedding of neutrophil L-selectin receptors which occurs as the result of activation by locally released mediators,

such as PAF, IL-8, or GM-CSF, may be required for the process of cell migration across the endothelium.

An *in vitro* model of the vascular endothelium was set up in order to address two questions. Firstly, the specific effect of GM-CSF on the migration of neutrophils across endothelium was investigated, under different conditions of endothelial activation. Secondly, this system was designed to allow the recovery of migrated cells, which were then examined for quantitation of surface antigens, thus addressing directly the question of the way in which changes in surface antigen expression relate to the process of cell migration.

SPECIAL METHODS

Culture of HUVEC on millipore filters

HUVEC were grown on collagen coated polycarbonate filters (6.5 mm diameter, 3 micron millipore membranes, Costar, UK). These filters, mounted on inserts, were placed in tissue culture wells (24 well plate, Costar UK), such that the upper chamber was separated from the lower chamber by the endothelium-covered filter. In initial experiments carried out to optimize the culture conditions for HUVEC on millipore filters, it was found that only first passage cells reliably formed confluent monolayers. This was achieved by prior coating of the filters with fibronectin, and with the use of a higher concentration of ECGS (100 μg/mL) than that required for cell growth on solid surfaces. Cells were seeded at 50-75% of confluent density onto fibronectin-coated filters, and used within the next 48 hours. The formation of confluent monolayers was confirmed by phase microscopy; only those filters which were completely covered by a confluent monolayer of cells were used for migration studies. The confluency of endothelial monolayers established by these culture conditions was further confirmed by the demonstration that gradients of soluble factors could be maintained across the monolayers (see RESULTS section).

Isolation and labelling of neutrophils

Neutrophils were purified from venous blood taken into EDTA (2 mM) by double density centrifugation (Histopaque 1119 and 1077, Sigma Diagnostics), and washed twice in PBS with 5 mM glucose (PBS/glucose). Neutrophils obtained by this method were >95% pure and >99% viable by trypan blue exclusion. Purified neutrophils, at 15-20 X 10^6 /mL in PBS/glucose, were incubated with ^{51}Cr (1 mCi/mL, 2 μCi/10^6 cells) at 37°C for 60 minutes, with gentle agitation. Following 3 washes with large volumes of PBS/glucose, neutrophils were resuspended at 2-4 X 10^6 cells/mL in IMDM with 20% FCS for migration studies.

Migration Assay

Confluent endothelial monolayers on Transwell filters were washed twice with warm medium and the filters placed in wells with fresh medium (IMDM/20% FCS) before being used in migration experiments. In some wells, FMLP (10^{-8} M) was placed in the lower chamber. Where the effect of endothelial activation was examined, confluent monolayers were preincubated with IL-1β (10 U/ml) or medium as control for 4 hours, and washed 3 times before the assay. 100 μL of labelled neutrophils (2-4 X 10^5 cells) were placed in the upper chamber, and the wells incubated for 2 hours at 37°C in 5% CO_2 in a humidified chamber. Duplicate or triplicate wells were used for each data point. At the end of the incubation, the Transwell inserts were removed, and the lower surface of each filter was swabbed with a cotton wool tip, which was counted, together with the collected contents of the lower chamber, in an automated gamma counter (LKB, Milton Keynes, UK). For each experiment, spontaneous release of ^{51}Cr by the labelled cells was determined by incubating aliquots of the neutrophil suspensions for the same length of time, following which the cells were centrifuged down and the supernatant removed and counted. In all experiments, spontaneous release was less than 5%, that is, more than 95% of counts were cell associated. In order to determine the total radioactivity, measured as counts per minute (cpm) added to each well, 100 μL aliquots of neutrophil suspension were also counted, in triplicate. Percent migration was calculated by dividing the cpm of each well by the total number of counts added.

Phenotyping of migrated and nonmigrated cells

In order to obtain sufficient cells for phenotyping, larger Transwell filters (1 cm diameter, placed in 12-well tissue culture plates, Costar UK) were used in these experiments. Confluent monolayers were pretreated with IL-1, or medium as control, and washed as detailed above. 6-10 X 10^5 neutrophils were placed in the upper chamber and, folowing a 2 hour incubation at 37°C, migrated cells were carefully collected from the lower chamber. Nonmigrated cells were collected from the upper chamber; adherent cells were scraped off mechanically, with a soft plastic tip. At least half the adherent cells were removed by this means. Cells were washed once in PBS before being placed in microtitre plates, and incubated with Mo-1-FITC (anti-CD11b), Leu-8-FITC (anti-L-selectin), or the relevant isotype-matched control FITC-conjugated antibody for 45 minutes at 4°C. Following 3 washes, cells were fixed in 1% paraformaldehyde, and analysed on the EPICS flow cytometer (Coulter Electronics, UK). Antigen density was measured as mean cell fluorescence on a linear scale. A negative control was included for each sample analysed, and each sample was stained with the positive antibody in duplicate.

RESULTS

Endothelial monolayers on Transwell filters as a model for studying transendothelial migration

In preliminary experiments, the capacity of confluent endothelial monlayers to maintain gradients of soluble factors was assessed by adding ^{125}I-GM-CSF (100 ng/mL) to the lower chambers of Transwell filter systems, sampling the medium in both upper and lower compartments at various time points, and measuring the radioactivity in the samples in a gamma counter. At 2 hours , only 25% of the total radioactivity was detected in the upper chambers, and at 3 hours, this had increased to 33%, demonstrating that the endothelial monolayer is able to exclude soluble factors, and hence to function as a barrier, at least during the period of the migration assay (2 hours). In contrast, when filters alone were used, the radioactivity was equally distributed between upper and lower chambers by 30 minutes.

The ability of confluent endothelium on filters to induce neutrophil migration was assessed by comparing the migration of unstimulated neutrophils across filters with and without endothelium. In 4 experiments, the random migration of neutrophils across filters was 5.7 ± 0.8%, as compared with 10.3 ± 2.0% across endothelium ($p < .05$), thus illustrating that the presence of endothelial cells induces the directional movement of neutrophils across the filters.

In 12 experiments, the migration of neutrophils across endothelium under unstimulated conditions was 7.7 ± 0.9%. Neutrophil migration could be further increased, either by the presence of a chemotactic gradient, or by pretreating the endothelial cells with IL-1β . When FMLP (10^{-8} M) was placed in the lower chamber, neutrophil migration across the endothelial monolayers was increased to 362 ± 55% of baseline ($p < .001$, n=7). Similarly, the pretreatment of endothelial cells with IL-1β (10 U/ml) for 4 hours enhances neutrophil migration to 221 ± 19% of baseline ($p < .005$, n=7, Figure 8.3a).

GM-CSF increases neutrophil migration across cultured human endothelial monolayers

When GM-CSF (100 ng/mL) was placed in the upper chamber only, the percentage of migrating neutrophils was increased from 7.7 ± 0.9% to 12.5 ± 1.5% ($p < .0005$, n=12). This effect of GM-CSF on neutrophil migration is dose dependent, with maximal effects achieved at concentrations of 1-10 ng/mL (Figure 8.1a). In order to determine if this effect of GM-CSF is related its properties as a

chemoattractant, as previously reported in the Boyden chamber system (Wang et al, 1987), chequerboard experiments were performed to analyse the effect of varying the concentration gradient of GM-CSF across the endothelium. Figure 8.1b shows that, when used at 100 ng/mL or greater, GM-CSF is equally effective, whether placed in the upper or lower chamber. Furthermore, when present only in the upper chamber (at concentrations ranging from 1-100 ng/mL) , GM-CSF is at least as effective in enhancing migration, as it is when placed only in the lower chamber. Hence GM-CSF induced migration across cultured endothelium *in vitro* appears to be independent of the presence of a positive gradient, suggesting that this is not, primarily, a chemotactic effect.

Scanning electron microscopy illustrates the different stages of GM-CSF induced neutrophil migration across endothelium (Figure 8.2). Activated neutrophils adherent to the endothelial surface undergo shape change (Figure 8.2a) and pseudopod extension in preparation for transmigration (Figure 8.2c). Finally, the outline of a migrated neutrophil can be discerned lying just beneath the endothelial monolayer (Figure 8.2c).

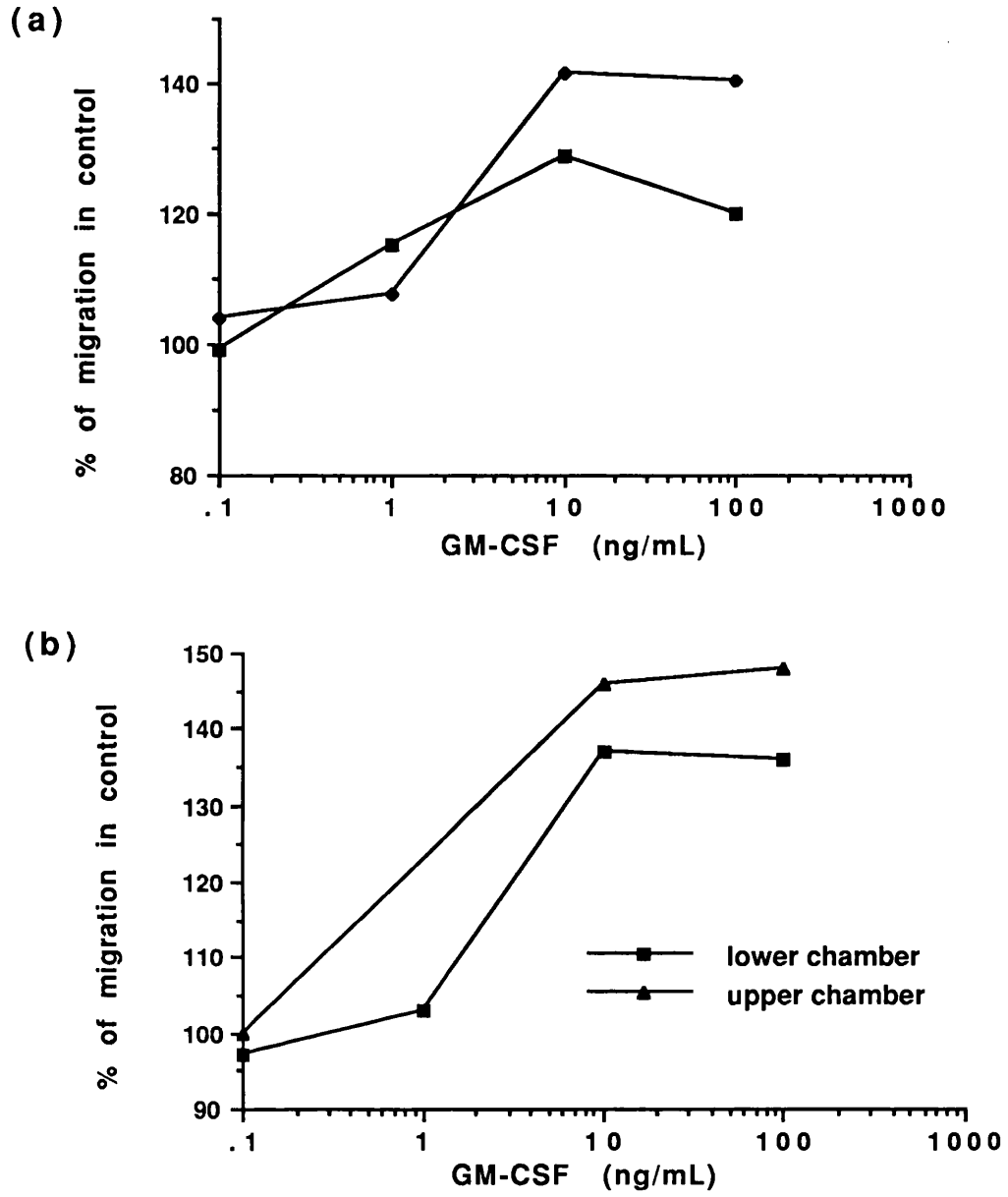


Figure 8.1 (a). GM-CSF increases neutrophil migration across untreated endothelium in a dose dependent manner. Data, expressed as percentage of migration of unstimulated cells, is given for 2 experiments. (b) GM-CSF induced migration is independent of concentration gradients. Different concentrations of GM-CSF were placed in upper or lower chambers at the start of the migration assay. Data is given for 1 experiment which is representative of 2.

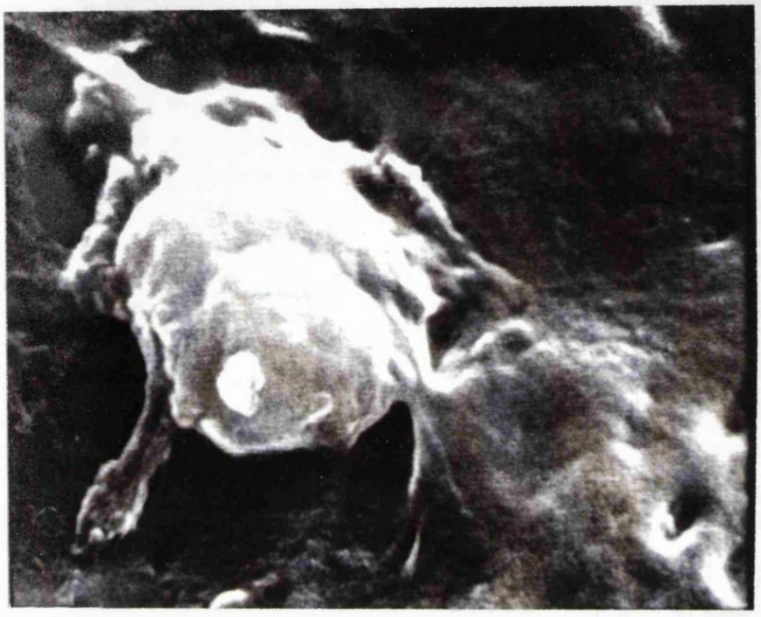


Figure 8.2a. Scanning electron microscopy of GM-CSF activated neutrophil adhering to the surface of endothelium.

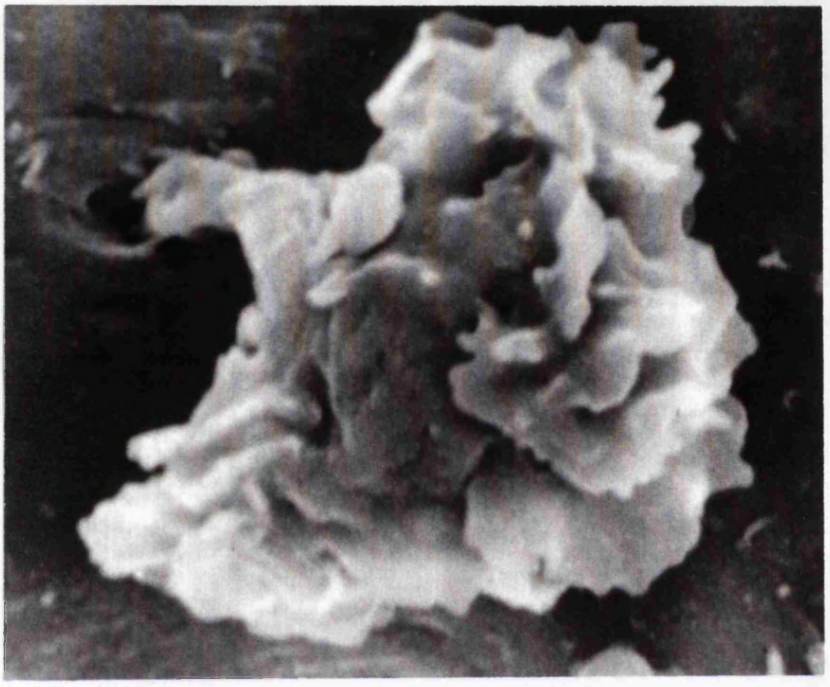


Figure 8.2b. An adherent neutrophil shows signs of activation (ruffling) and pseudopod extension, in the early phase of transmigration.

Figure 8.2c. A transmigrated neutrophil can be discerned lying just beneath the endothelial monolayer.

GM-CSF decreases neutrophil migration across IL-1 activated endothelium

In 7 experiments, the effect of pretreating the endothelial monolayers with IL-1β (10 U/mL for 4 hours) on neutrophil migration was assessed. In these experiments, baseline migration under unstimulated conditions was 7.6 ± 1.2%, and GM-CSF was able to increase this to 11.6± 1.6% ($p<0.0005$, $n=7$, Figure 8.3a). Pretreating the endothelium with IL-1 also enhances the migration of unstimulated neutrophils to 16.8 ± 1.4% (221 ± 19% of baseline , $p<0.005$, $n=7$). In the presence of GM-CSF, however, the enhanced migration produced by IL-1 is almost completely abolished (the migration of GM-CSF stimulated neutrophils across IL-1 activated endothelium is 107.8 ± 16% of that across untreated endothelium, as compared with an increase to 221 ± 19% of baseline in the absence of GM-CSF, $p<.0005$, $n=7$, Figure 8.3a). Figure 8.3b illustrates this differential effect of GM-CSF on neutrophil migration, showing that in each of the 7 experiments carried out, GM-CSF increased the percentage of neutrophils migrating across untreated endothelium, but decreased the percentage of neutrophils migrating across IL-1 treated endothelium in 6 of these experiments, while, in the seventh, there was no change.

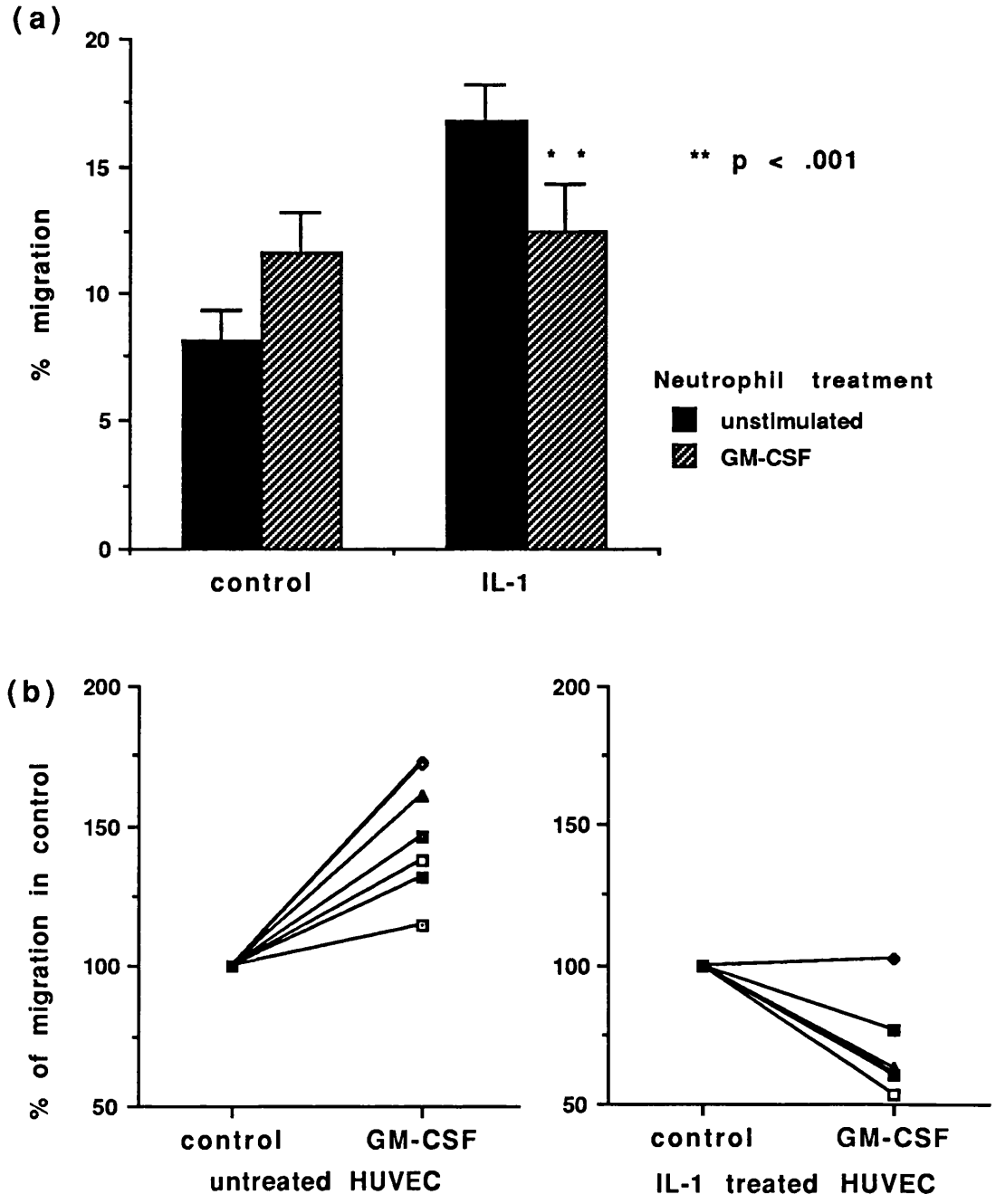


Figure 8.3. Differential effect of GM-CSF on neutrophil migration across untreated, and IL-1 treated endothelium in 7 experiments; expressed as (a) percent migration, mean ± SD, and (b) a percentage of the migration of unstimulated neutrophils in each experiment.

GM-CSF increases neutrophil adherence to unstimulated endothelium

The divergent effects of GM-CSF on neutrophil migration across cytokine-activated, as opposed to unstimulated, endothelium might relate to a differential effect on adhesion. GM-CSF increases the adhesion of neutrophils to unstimulated endothelium (Devereux et al, 1989, Chapter 3), but the effect on adhesion to cytokine activated endothelium has not previously been reported. Figure 8.4 shows that the neutrophil adhesion to cultured human endothelial cells is markedly enhanced by pretreatment of the endothelium with IL-1β (10 U/mL). When neutrophils are incubated with GM-CSF (100 ng/mL) during the adhesion assay, adhesion to unstimulated endothelium is increased from 6.3 ± 1.1% to 11.4 ± 3.8% ($p < .05$, $n=4$). In contrast, there is no effect of GM-CSF on neutrophil adhesion to IL-1 activated endothelium (Figure 8.4a,b). More importantly, GM-CSF stimulation does not decrease the percentage of neutrophils adhering to IL-1 activated endothelium, suggesting that the inhbitory effect of GM-CSF on neutrophil migration across IL-1 stimulated endothelium is independent of any effect on adhesion.

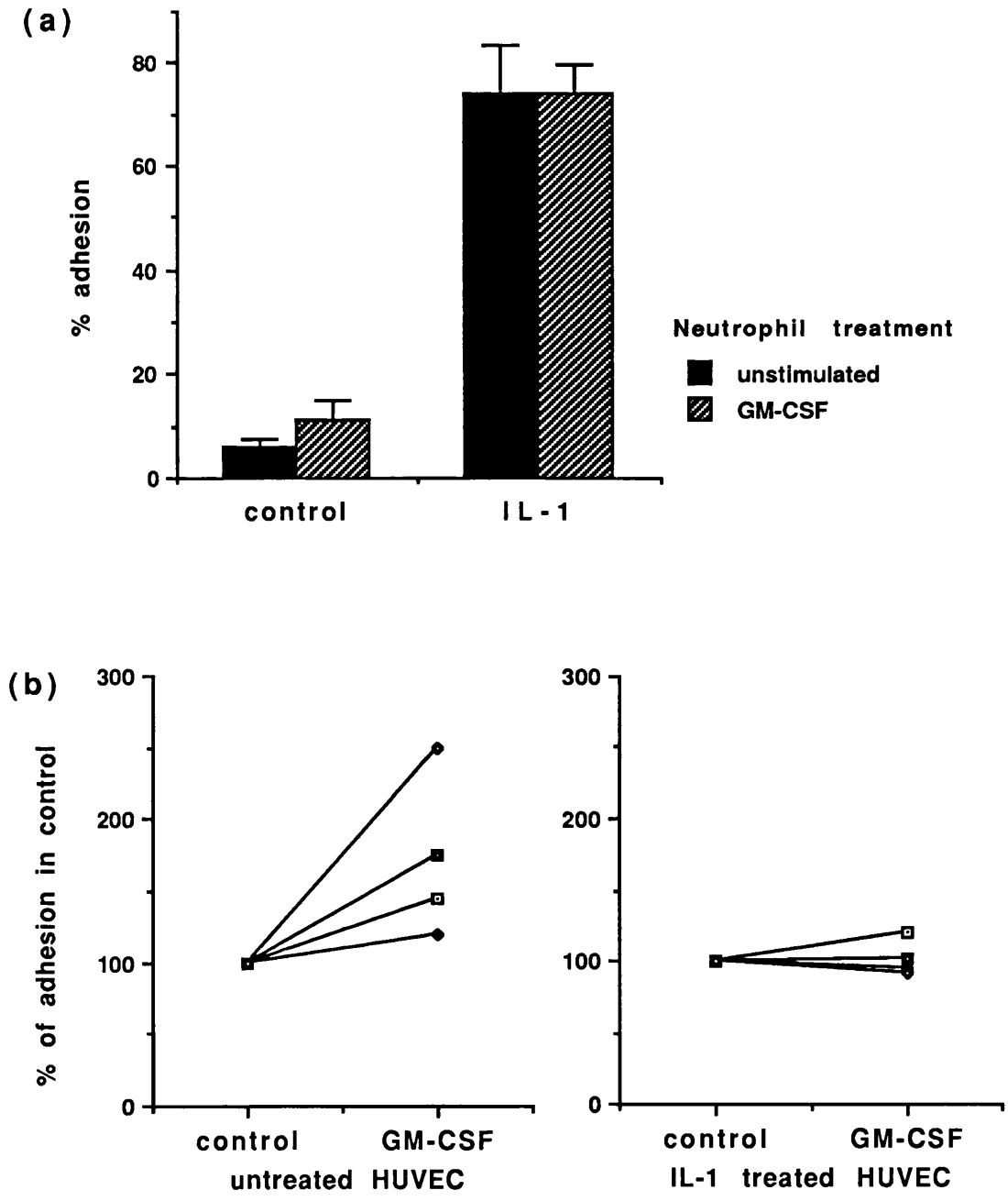


Figure 8.4. GM-CSF increases neutrophil adhesion to unstimulated endothelium, but has no effect on the enhanced adhesion to IL-1 activated endothelium. Neutrophil adhesion is expressed (a) as the percent of adherent neutrophils (mean ± SD of 4 experiments), and also (b) as a percentage of the adherence of unstimulated neutrophils for each of the same 4 experiments.

Neutrophil migration is inhibited by anti-CD18 MoAb but not by anti-L-selectin MoAb

Neutrophils were incubated with MoAbs MHM23 (anti-CD18), DREG 56 (anti-L-selectin) or an isotype-matched control MoAb for 10 minutes at room temperature before being added to endothelial monolayers, with or without GM-CSF (100 ng/mL) for migration assays. MHM23 was able to block neutrophil migration under both unstimulated, and stimulated conditions (Figure 8.5a). The migration of unstimulated cells was reduced by 40 ± 15% of control ($p < .05$), n=3), while GM-CSF stimulated migration was reduced by 86 ± 11% ($p < .025$, n=3). Similarly, the migration of neutrophils across IL-1 activated endothelium was reduced by 66 ± 19% in the presence of anti-CD18 MoAb ($p < .05$, n=3, Figure 8.5a). In contrast, DREG 56 (anti-L-selectin) MoAb had little effect on the migration of unstimulated cells (87.8 ± 10% of control across resting endothelium, and 130 ± 59% of control across IL-1 activated endothelium), but there was a small effect on GM-CSF induced migration, which did not reach significance (Figure 8.5a).

CD18 deficient neutrophils fail to migrate across endothelium in response to chemotactic stimulation

The importance of CD18 in the transendothelial migration of neutrophils was confirmed in one experiment on CD18 deficient neutrophils. Neutrophils from a patient with partial leucocyte adhesion deficiency (patient 2, Chapter 7), which expressed 30% of normal levels of CD11b, were used in a migration assay. Under unstimulated conditions, the migration of these CD18 deficient neutrophils was within normal limits (Figure 8.5b). However, these cells showed no increase in migratory capacity, in response either to stimulation with GM-CSF, or to the presence of a chemotactic gradient of FMLP (Figure 8.5b).

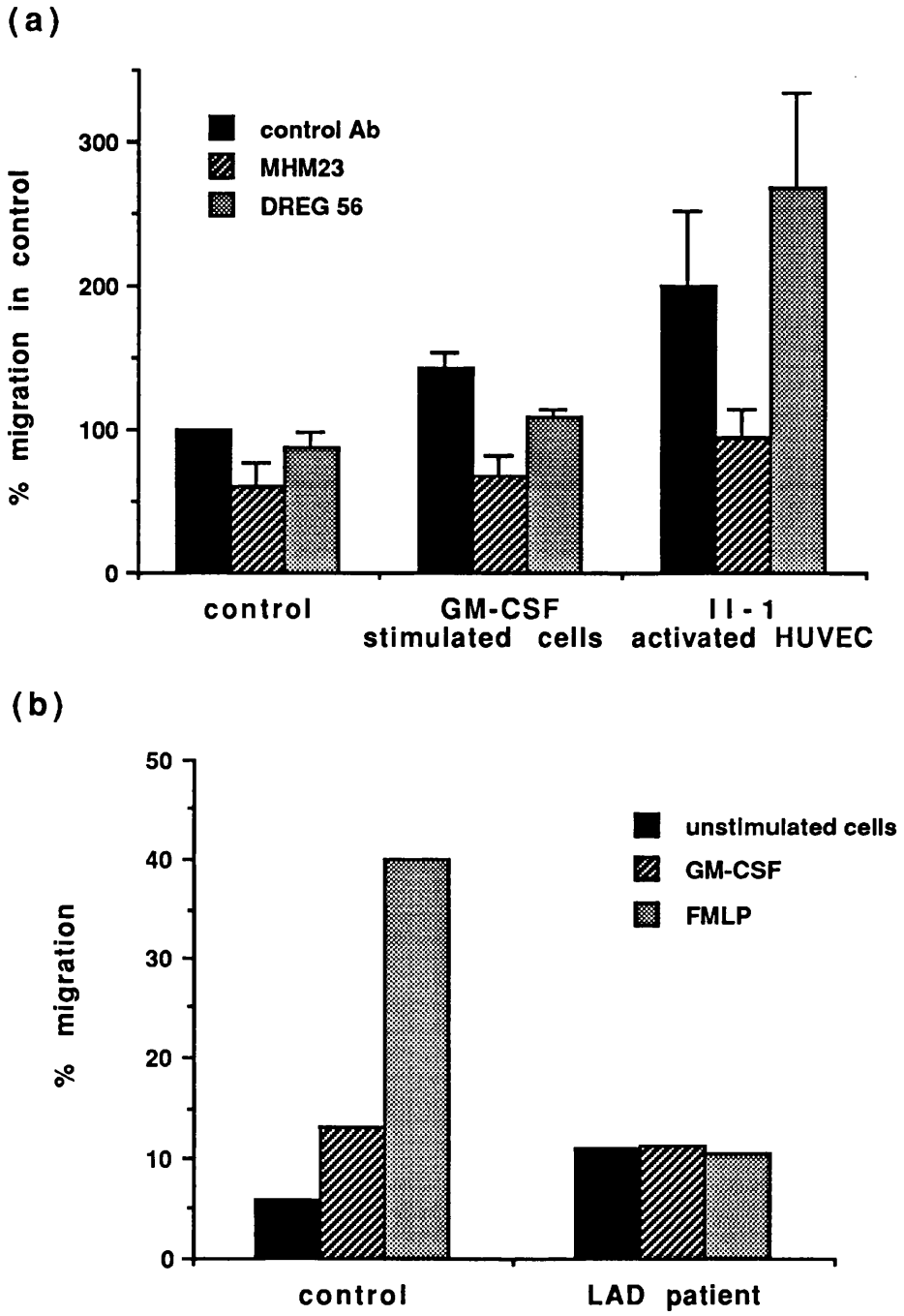


Figure 8.5. (a) Effect of anti-CD18 (MHM23) and anti-L-selectin (DREG 56) MoAbs on neutrophil migration. Data, expressed as percentage of the migration in controls, is mean ± SD of 3 experiments. (b) Transendothelial migration of CD18 deficient neutrophils. Data, mean of duplicates, is given for 1 experiment.

Migrated neutrophils express iow levels of L-selectin

Neutrophils which had migrated across IL-1 activated endothelium were compared with nonmigrating cells, with respect to their surface expression of CD11b and L-selectin. Control neutrophils (2-4 X 10^6/mL in medium), placed in polypropylene tubes were included in parallel incubations in migration assays. All cell populations (control neutrophils as well as migrated and nonmigrated cells harvested from Transwell filters) were washed in PBS prior to being examined for CD11b and L-selectin expression. Figure 8.6a shows that migrating cells express significantly lower levels of L-selectin than nonmigrating cells. L-selectin levels on neutrophils that have migrated across IL-1 treated endothelium were 11.1 ± 0.6% of the levels on control cells (incubated in polypropylene tubes), while levels on nonmigrated neutrophils were 72.3 ± 10% of control ($p < .005$, n=4). Similarly, neutrophils which have migrated across endothelium in reponse to stimulation with GM-CSF express 18.7 ± 3.2% of control L-selectin levels, as compared with nonmigrating cells, which expressed 65.4 ± 15% of control levels ($p < .05$, n=4). In contrast, there is no difference in the levels of CD11b between migrating and non migrating neutrophils (Figure 8.6b).

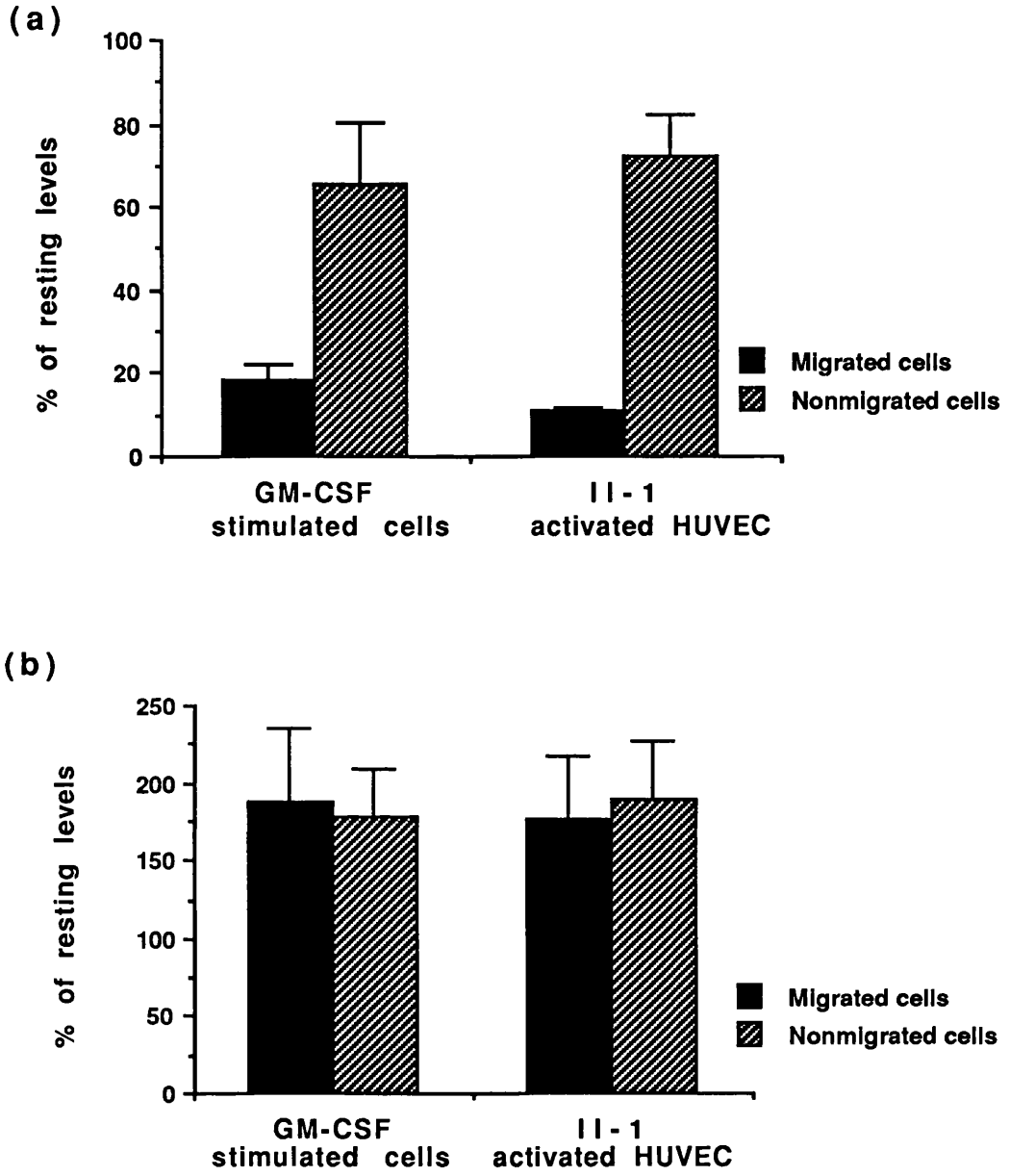


Figure 8.6. Comparison of surface expression of (a) L-selectin and (b) CD11b on migrated and nonmigrated cells after a 2 hour migration assay, in the presence of GM-CSF, or across IL-1 activated endothelium. Data, expressed as a percentage of the MCF on control neutrophils, is the mean ± SE of 4 experiments.

DISCUSSION

The central new finding reported in this chapter is that GM-CSF is able to regulate the migration of neutrophils across cultured human endothelium *in vitro*. GM-CSF significantly enhances neutrophil migration across unstimulated endothelium in a dose dependent manner, with maximal effect (162% of baseline) achieved between 1 and 10 ng/mL. This effect of GM-CSF does not appear to be a chemotactic one, as it is independent of concentration gradients across the endothelial monolayer. In contrast to the effects seen with unstimulated endothelium, GM-CSF inhibits neutrophil migration across IL-1 activated endothelium, such that, in the presence of GM-CSF, the enhanced neutrophil migration caused by pretreatment of endothelium with IL-1 is almost completely abolished.

The enhancement of transendothelial neutrophil migration in response to chemotactic stimulation with FMLP (Furie et al, 1991), or to IL-1 treatment of the endothelium as demonstrated here agrees with previous reports (Harlan et al, 1985, Furie & McHugh, 1989, Smith et al, 1989, Luscinkas et al, 1991). The demonstration that GM-CSF directly stimulates neutrophils to migrate across endothelium *in vitro* is also not unexpected, in that GM-CSF is a chemokinetic and chemotactic agent (Wang et al, 1987). GM-CSF induced migration across endothelium as demonstrated here is independent of concentration gradients, and therefore would appear to be related either to the chemokinetic effect of the growth factor, or to an effect on the interaction of neutrophils with endothelium. The finding that GM-CSF induced neutrophil migration is dependent upon CD18 suggests that this effect may rely on similar adhesive mechanisms as those which mediate neutrophil migration across cytokine activated endothelium, or in response to chemotactic stimulation, both of which are also heavily dependent on CD18 (Smith et al, 1989, Furie et al, 1991, Luscinkas et al, 1991).

The effect of GM-CSF to enhance neutrophil migration across unstimulated endothelium may relate to the upregulation of CD11/CD18 receptors, and an enhanced adhesiveness of cells for endothelium, as demonstrated earlier (Chapters 3 and 5). Both the adhesive and migratory responses of neutrophils to GM-CSF can be blocked by anti-CD18 MoAb, suggesting that these effects might be mediated by the binding of neutrophil surface CD11/CD18 receptors to ligands on endothelial cells. On the other hand, the inhibition of neutrophil migration across IL-1 activated endothelium appears not to be directly related to adhesion, because GM-CSF stimulation has no effect on neutrophil adhesion to IL-1 activated endothelium. The lack of effect of GM-CSF on neutrophil adhesion to cytokine activated endothelium

suggests that the adhesive mechanisms upregulated by GM-CSF on the neutrophil are already maximally upregulated by exposure to IL-1 activated endothelium. Hence the effects of GM-CSF on neutrophil migration across endothelium may be distinct from any effects on adhesion. A similar finding has been reported for IL-8; neutrophils preincubated with IL-8 display increased adhesion to endothelium, as well as enhanced transendothelial migration (Huber et al, 1991). These effects seem to be separate as migration, but not adhesion, requires the continued presence of IL-8, and is inhibited by subsequent exposure to IL-8 antiserum. These authors suggest that IL-8 acts primarily as a chemoattractant to induce neutrophil migration across cultured endothelium.

The differential effects of GM-CSF on neutrophil migration across endothelium under different conditions of endothelial activation are compatible with the results of skin window studies. Although GM-CSF activates neutrophils to adhere to, and move across unstimulated endothelium, in artificial inflammatory areas such as the skin window, GM-CSF may act to inhibit the movement of circulating neutrophils across cytokine activated endothelium. The stimulation of circulating cells with exogenously administered GM-CSF could therefore decrease the ability of these cells to migrate into skin windows. The main role of GM-CSF as an inflammatory cytokine may relate to its production locally, in infective foci, where it serves to attract and activate phagocytic cells. Thus, inappropriately high concentrations of GM-CSF in the vascular compartment might reduce the host defence response.

These findings are relevant to the clinical application of GM-CSF to augment neutrophil functions *in vivo*. When present together with other inflammatory cytokines such as TNF, only picomolar concentrations of GM-CSF are required in order to prime neutrophil functions such as superoxide production; that is, these cytokines are highly synergistic in their effects on neutrophil function (Khwaja et al, 1992, Yuo et al, 1991). Hence, much lower *in vivo* doses of GM-CSF may be sufficient to enhance neutrophil function, than have been used hitherto to accelerate haematological recovery. At these lower doses, GM-CSF may not reach high enough concentrations in the circulation (10 ng/mL) to inhibit neutrophil entry into inflammatory areas. In this regard, it is noteworthy that GM-CSF is undetectable in the serum, not only of normal subjects, but also in infection and stress, where levels of G-CSF are elevated (Sallerfors & Olofsson, 1991).

The system used in this study was selected because it offers the opportunity, not only of accurately quantitating neutrophil migration, but also of recovering and studying the phenotype of migrated cells. Various systems have been used to study the

transendothelial migration of neutrophils. Neutrophils migrating across cytokine-treated endothelial monolayers grown on solid surfaces can be visualized and quantitated by microscopy (Smith et al, 1989). More physiologically relevant models of neutrophil migration have employed endothelial monolayers grown on collagen gels (Luscinkas et al, 1991, Hakkert et al, 1991) or on amniotic membranes (Furie & McHugh, 1989, Furie et al, 1991), so as to allow migrating neutrophils to continue to move into a three-dimensional matrix, once they have traversed the endothelial barrier. The Transwell system of culturing endothelial cells on collagen coated millipore filters inserted into tissue culture wells provides a model in which intravascular and extravascular compartments are separated by endothelium supported on a three-dimensional substratum. Human umbilical vein endothelial cells (HUVEC) cultured on Transwell filters have been shown to secrete a continuous basement membrane, with barrier functions similar to those demonstrated for venules *in vivo* (Huber & Weiss, 1989). In this system, confluent endothelial monolayers are able to exclude soluble macromolecules, and to support neutrophil migration such that the percentage of cells migrating across filters with cultured endothelium was significantly greater than that across filters alone. The latter observation suggests an effect of the endothelium on the directed movement of neutrophils across the filters and supports the use of this system as a model for studying neutrophil extravasation *in vitro*.

The way in which the enhanced migratory capacity of neutrophils relates to changes in surface expression of adhesion receptors remains unclear. IL-1 activated HUVEC are able to upregulate neutrophil surface CD11b/CD18 (Smith et al, 1991, Huber et al, 1991), which is a crucial receptor for cell migration, as evidenced both by functional inhibition studies, and by studies on CD18 deficient cells. (Smith et al, 1991, Hakkert et al, 1989, Luscinkas et al, 1991, Furie & McHugh, 1989, Furie et al, 1991, Harlan et al, 1985). These *in vitro* observations are in keeping with the major clinicopathological feature in LAD, which is the virtual absence of neutrophils in inflammatory areas, and with the results of animal studies, where prior administration of anti-CD18 MoAb *in vivo* inhibits neutrophil extravasation into inflammatory foci (Arfors et al, 1987, Noursargh et al, 1989, Lundberg & Wright, 1990). However, the exact relationship between the amount of surface receptor expressed, and the ability of neutrophils to migrate across IL-1 activated endothelium still remains unclear. Although anti-CD18 MoAb is able to block neutrophil migration, the results presented here show that the absolute level of CD11/CD18 does not dictate the migratory capacity of cells.

IL-1 activated endothelium is also able to induce a fall in neutrophil surface LAM-1 levels (Smith et al, 1991) and there is recent evidence that both this effect, and the rise in CD11/CD18 are mediated by endothelial synthesis and secretion of IL-8 (Huber et al, 1991). In contrast to the effect of MoAb to CD18, there was little effect of MoAb to L-selectin on the migration of unstimulated cells, either across unactivated endothelium, or IL-1 activated endothelium. The lack of effect of MoAb to L-selectin on neutrophil migration in this model confirms the reports of other workers using static assay systems (Kishimoto et al, 1991) Under conditions of flow, however, L-selectin may participate in the attachment of circulating cells to endothelium (Smith et al, 1991). Following this initial attachment, the shedding of L-selectin may be necessary for transmigration across endothelium, and hence the degree of L-selectin loss may relate directly to the ability of cells to transmigrate. The data presented here, showing that neutrophils which have migrated across endothelium *in vitro* express far lower L-selectin levels than nonmigrating cells confirm and extend previous histological findings on extravasated neutrophils in the mouse (Kishimoto et al, 1989). Alternatively, the magnitude of L-selectin loss may simply be a marker of cell activation, cells which have transmigrated belong to a more activated subpopulation, and hence express lower levels.

The finding that migrating cells express lower L-selectin levels as compared to nonmigrating cells may explain some observations on the *in vivo* effects of inflammatory mediators such as GM-CSF. *In vitro*, GM-CSF stimulation causes upregulation of neutrophil surface CD11/CD18, and downregulation of L-selectin (Griffin et al, 1990, Chapter 3). When administered *in vivo*, circulating cells show upregulation of CD11/CD18, but there is no change in the L-selectin levels on these cells (Chapter 5). This may be because the subpopulation of cells which have shed the L-selectin receptor have, in fact, migrated into the extravascular tissues.

Finally, the *in vitro* model used in these studies provides a valuable system for studying the regulation and mechanisms of neutrophil migration across endothelium and other cellular barriers.

CHAPTER NINE

EFFECT OF GM-CSF AND G-CSF ON CULTURED HUMAN ENDOTHELIAL CELLS *IN VITRO*

INTRODUCTION

The studies presented so far illustrate the proinflammatory effects of GM-CSF and, to a lesser extent, G-CSF, which are able to modulate the adhesive and migratory properties of mature phagocytic cells, thus playing a part in the recruitment of leucocytes into inflammatory areas. As the endothelium is now recognized to play an active role in this process, the next set of studies will address the question of the possible effects of G- and GM-CSF on the vascular endothelium itself. Vascular endothelium is a multifunctional organ which can respond to a wide variety of stimuli to participate actively in the maintenance of the haemostatic balance, the regulation of haemopoiesis, and the control of immune and inflammatory phenomena. Endothelial cells engage in reciprocal interactions with other cells, mediated by cytokines and other signalling molecules, as well as by cell-cell adhesion pathways. Stimulated endothelial cells produce cytokines such as IL-1, IL-6, PAF, platelet-derived growth factor (PDGF), as well as GM-CSF, G-CSF and M-CSF (Libby et al, 1986, McIntyre et al, 1986, Fibbe et al, 1989, Zsebo et al, 1988). In addition, the growth and function of cultured endothelial cells can be modulated by cytokines such as gamma-interferon (γ-IFN), IL-1 and tumour necrosis factor (TNF) (Saegusa et al, 1990). These cytokines also increase the surface expression of adhesion receptors, enhance the adhesiveness of endothelium for leucocytes and influence the haemostatic and fibrinolytic activities of endothelial cells (Bevilacqua et al, 1986a,b, Schleef et al, 1988, Pober, 1988).

Recently, human endothelial cells themselves have been reported to express receptors for GM-CSF and to proliferate and migrate in response to GM-CSF and G-CSF (Bussolino et al, 1989, 1991). The production of these cytokines by the activated endothelium could thus create an autocrine positive feedback loop, and play a crucial part in modulating the interactions of phagocytes with endothelial cells. This chapter summarizes the results of *in vitro* studies on the effect of G- and GM-CSF on the proliferation and function of cultured human umbilical vein endothelial cells (HUVEC).

SPECIAL METHODS

Cell culture

HUVEC were isolated and maintained in culture as described in Chapter 2. The endothelial cell line, EAhy926 (Edgell et al, 1983) was kindly provided by Dr. C. J. Edgell, University of North Carolina, Chapel Hill, U.S.A.

Proliferation Assays

Passaged (1-7) HUVEC and primary cultures were seeded ($2x10^3$ cells/well) in 96-well fibronectin-coated microtitre plates. Cytokines were either present at time of seeding or added to cultures at 50% confluence after 48 hours serum starvation. A standard MTT assay (Hansen et al, 1989) was used to quantitate cell numbers. Briefly, cells were rinsed twice with medium before incubating with 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT, 1mg/mL in medium, 100 µL/well) for 2 hours at 37°C. The incubation solution was aspirated and 100 µL of dimethyl sulphoxide (DMSO) was added to each well, to solubilize the formazan crystals. The change in absorbance was read at 570 nm on a MR 700 Microplate Reader (Dynatech Laboratories).

The validity of this assay as a measure of cell proliferation was tested in two ways. Firstly, the assay was calibrated by seeding known numbers of cells in 96-well tissue culture plates. Eight wells were used for each data point. After a 2-hour incubation to allow the cells to adhere, the wells were rinsed twice with medium and on half the wells, an MTT assay was carried out. On the other 4 wells of each data point, cells were removed by trypsin/EDTA treatment and counted on a Neuebauer counting chamber. The number of cells removed from each well was within 5-10% of the number seeded. The calibration curve of absorbance units against cell numbers derived from 3 separate experiments is shown in Appendix 3. Secondly, in 2 parallel experiments, the results of ^{3}H-thymidine incorporation were compared with those from the MTT assay. Microtitre plates were set up for proliferation assays as stated in Methods. Half the wells were pulsed with ^{3}H-thymidine (1 µCi per well) from 7-24 hours after cytokines were added. The cells in the other half of the plate were allowed to grow for 48 hours before the MTT assay was carried out. The results of ^{3}H-thymidine uptake were comparable to the cell numbers obtained from the MTT assay using the calibration curve.

Assays of endothelial cell associated procoagulant activity (PCA)

Subconfluent HUVEC (passages 1 and 2) in 96-well microtitre plates were washed twice with medium and then stimulated with cytokines in medium with 10% FCS. The surface expression of procoagulant activity (PCA) was measured by a 2-stage amidolytic assay using the chromogenic substrate S2222 (Surprenant & Zuckerman, 1989) which measures the conversion of Factor X to its activated form. Briefly, plates were washed three times with medium and then incubated for 3 minutes at 37°C with 100 µL/well of M199 without phenol red containing F VII 1 u/ml and F X 1 u/ml. S2222 1 mM was added (50 µL/well) and incubation continued until optimum colour development (10-20 minutes). The change in optical density at 410 nm was quantified using a MR 700 microplate reader (Dynatech Laboratories). The concentration of procoagulant activity was calculated from a standard curve obtained using rabbit thromboplastin (1/100 000 dilution rabbit thromboplastin =1 U PCA)

Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 Assays

Confluent HUVEC (passages 1 and 2) in 24-well plates (Costar) were washed and stimulated in triplicate wells with cytokines in 1% FCS. After 24 hours, culture medium was removed, centrifuged for 3 minutes at 15,000g to remove cell debris, and aliquots frozen at -70°C after the addition of 0.01% Tween 80. Monolayers were then washed once, treated with 0.5% Triton X-100 and cellular extracts frozen at -70°C.

Total human tPA (uncomplexed and inhibitor-bound) was determined using enzyme immunoassay (Biopool, Sweden). Background binding was eliminated by the use of quenching antibodies. Briefly, each test sample was incubated in paired adjacent wells. To one well was added an excess of the coating antibody in solution, in order to block binding of tPA antigen. The other well contained an irrelevant isotype-matched antibody. Hence the difference in assay response between the two wells would be highly specific for tPA antigen. The concentration of tPA antigen in each sample was extrapolated from a standard curve obtained using single-chain recombinant human tPA. The interassay variability was less than 10%. PAI-1 antigen (both active and 'latent' forms) was assayed by enzyme immunoassay, utilizing a similar double antibody principle to control for immunological specificity (Biopool, Sweden). The concentration of PAI-1 in each sample was calculated from a standard curve obtained using PAI-1 standard plasma supplied by Biopool, Sweden. The interassay variability was 8%.

GM-CSF Receptor Assays

The binding characteristics and biological activity of the batch of ^{125}I-GM-CSF used for receptor studies are described in Appendix 4. HUVEC (III-IV passage) were grown to confluence as described, and detached using trypsin/EDTA or EGTA alone. After 2 washes with RPMI 1640/ 20% FCS, cells were resuspended in RPMI 2% FCS at $4x10^7$cells/ml. 10^7 cells were incubated with varying concentrations of ^{125}I-GM-CSF (specific activity $4.2\text{-}6.8x10^5$ cpm/ng) for 4 hours at 23°C. Each sample was Incubated in the presence, or absence, of a 50-fold excess of unlabelled GM-CSF, in order to control for non-specific binding. Triplicate samples were layered on to chilled 20% glycerol in RPMI, centrifuged at 7000g for 60 seconds and snap frozen in liquid nitrogen. The associated radioactivity of the cell pellet was measured in an automated gamma counter (LKB, Milton Keynes, U.K.). An identical protocol was carried out using human peripheral neutrophils purified by dextran sedimentation of whole blood, followed by centrifugation over Lymphoprep 1077 (Nycomed, Oslo, Norway).

RESULTS

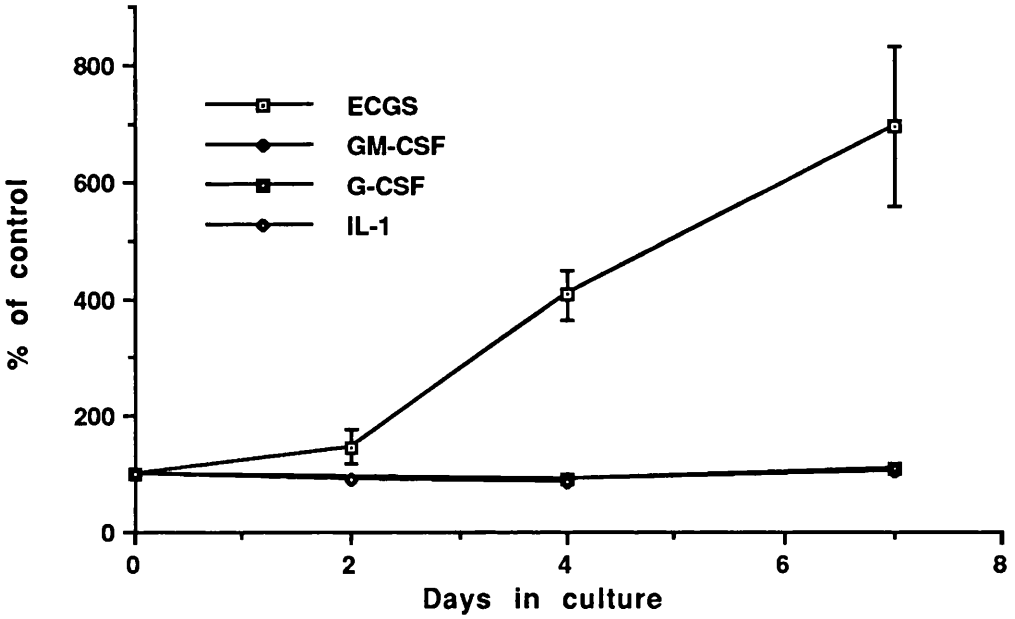
Cell proliferation

Cells were cultured in the continuous presence of ECGS (50 μg/mL), IL-1 β (5-10 U/mL), GM-CSF (1 ng/mL - 1 μg/mL), G-CSF (1 ng/mL - 1 μg/mL), or diluent as control in medium with 20% FCS. Quantitation of cell numbers was done at various time points using an MTT assay, converting the absorbance values into actual cell numbers using the calibration curve (Appendix 3). The data for passaged and primary cells was combined as there was no difference between the two. When present continuously, ECGS (50 μg/mL) increased endothelial cell proliferation by 305 ± 45% ($n=5$, $p < 0.01$) as compared with controls at 4 days, this enhancement rising to 596 ± 137% ($n=5$, $p < 0.01$) after 7 days in culture (Figure 9.1a). Neither GM-CSF nor G-CSF (1 ng/mL-1 μg/mL) had any effect on cell growth. IL-1 β (5 U/mL) produced a small decrease in growth at 4 days to 85 ± 11% (n=5, NS). In an attempt to maximise any response to added cytokines, a second series of experiments was carried out, whereby cultures were grown up to 50% confluence in full culture medium, then 'starved' for 48 hours in 1% FCS with 0.5% bovine serum albumin (BSA, fraction V, Sigma). Following this, cells were treated with cytokines in the presence of 2% FCS, and cell proliferation assessed at various times using the MTT assay. Data from primary and passaged cells were

pooled. ECGS enhanced proliferation of HUVEC by 342 ± 63.2% (n=10, $p < 0.01$) over control cells at 4 days (Figure 9.1b), but there was no effect of GM-CSF (1-100 ng/mL), G-CSF (1-100 ng/m l) or IL-1 β (1-10 U/mL) in this assay system.

Procoagulant activity

The effect of cytokines on the expression of procoagulant activity (PCA) by HUVECs was studied by incubating confluent cultures with cytokines in medium with 10% FCS for varying periods of time. Unstimulated HUVECs contained low levels of PCA (3.22 ± 0.44 PCA U/well, n=10). After 4 hours incubation with IL-1 β (10 U/mL), procoagulant activity increased to 1721 ± 376% of control levels ($p < 0.005$, n=7), while TNF-α (10 U/mL) produced a more modest rise of 247 ± 71% of control levels (n=4) (Figure 9.2). IFN-γ (50 U/ml) and GM-CSF (1-100 ng/mL) had no direct effect on PCA. IFN-γ, however, was able to augment the PCA production by endothelial cells in response to IL-1 β. Figure 9.3, which is representative of 2 experiments, shows the effect of a 16-hour preincubation with IFN-γ (50 U/mL) prior to addition of IL-1β (1-10 U/mL) for a further 4 hours. GM-CSF (1-100 ng/mL) had no effect. In 2 further experiments, GM-CSF was added simultaneously with IL-1 β, and incubation carried on for 4 - 24 hours. No modulation of IL-1 β induced PCA expression was observed.



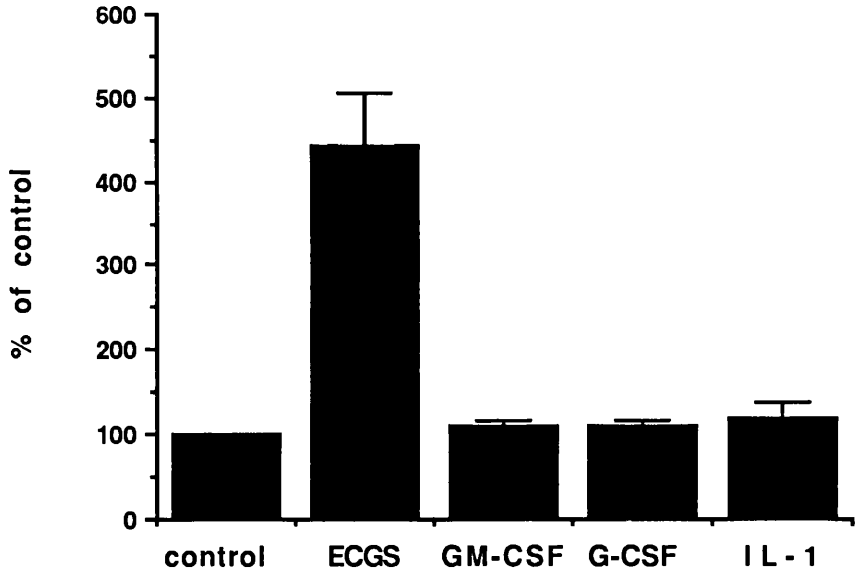


Figure 9.1. Effect of cytokines on the proliferation of (a) primary (n=3) and passaged (n=10) HUVEC, assessed at days 2 (n=5), 4 (n=4) and 7 (n=4), and (b) serum starved cells, assessed at day 4 (n=10). Data is expressed as % of cell numbers in control wells at each time point (mean ± SD).

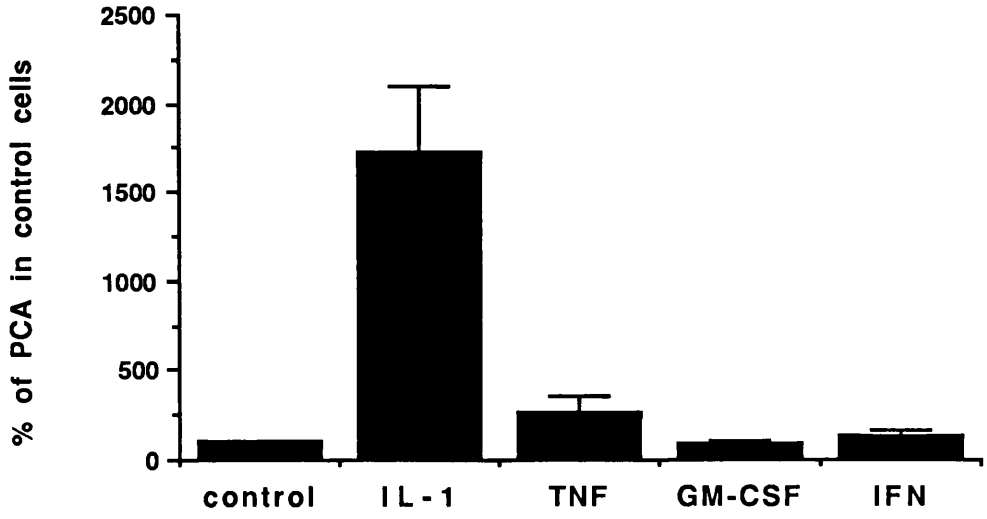


Figure 9.2 Effect of cytokines on procoagulant activity (PCA) of HUVEC. Data, expressed as percentage of PCA in control cells, is the mean ± SE of 7 experiments with IL-1β and GM-CSF, 4 experiments with TNF and 3 experiments with IFN -γ.

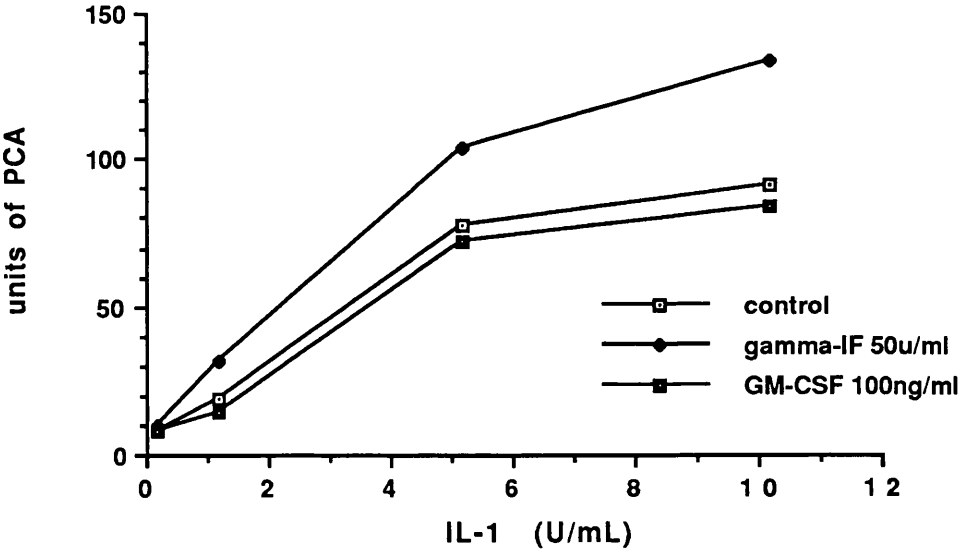


Figure 9.3. Priming of IL-1 β-induced procoagulant activity. IFN -γ, GM-CSF or diluent as control was added to cells for 16 hours prior to the addition of Il-1 β at various doses for 4 hours. Data from 1 experiment, representative of 2.

Tissue Plasminogen Activator and Plasminogen Activator Inhibitor Expression

The influence of cytokines on the fibrinolytic properties of HUVEC was studied by 24 hour stimulation of confluent monolayers of cells in the presence of 1% FCS. This concentration of serum was chosen because it has been reported to result in near optimal t-PA expression by primary cultured endothelial cells (Levin & Loskutoff, 1984)

The unstimulated level of t-PA antigen secreted by HUVEC is low (2.4 ± 0.2 ng/10^5 cells, n=5) and increases to 314 ± 72% of control levels ($p < 0.025$, n=5) following 24 hours incubation with thrombin (4 U/mL) (Figure 9.4). IL-1 β (10 u/ml) produced a small but insignificant decrease to 85 ± 9% of control levels (n=3) while GM-CSF (1-100 ng/mL) and G-CSF (1-100 ng/mL) had no effect on tPA expression. Cell associated t-PA antigen levels were below the level of detection of the assay.

Secreted PAI-1 antigen expression by HUVEC was increased to 275 ± 44% and 180 ± 12% of controls ($p < 0.0005$, n=5 for both) following 24 hours incubation with IL-1β (10 U/mL), and thrombin 4 U/mL respectively (Figure 9.5). TNF-α (200 U/mL), over the same time period, produced an increase to 241 ± 44% of control levels ($p < 0.005$, n=3). Similarly, cell-associated PAI-1 antigen was increased to 266 ± 108% (n=3, N.S. by Student's t-test) and 195 ± 54% (n=3, N.S.) of control levels following 24 hours incubation with IL-1 and thrombin, respectively. Neither GM-CSF nor G-CSF had any effect on secreted or cellular PAI-1.

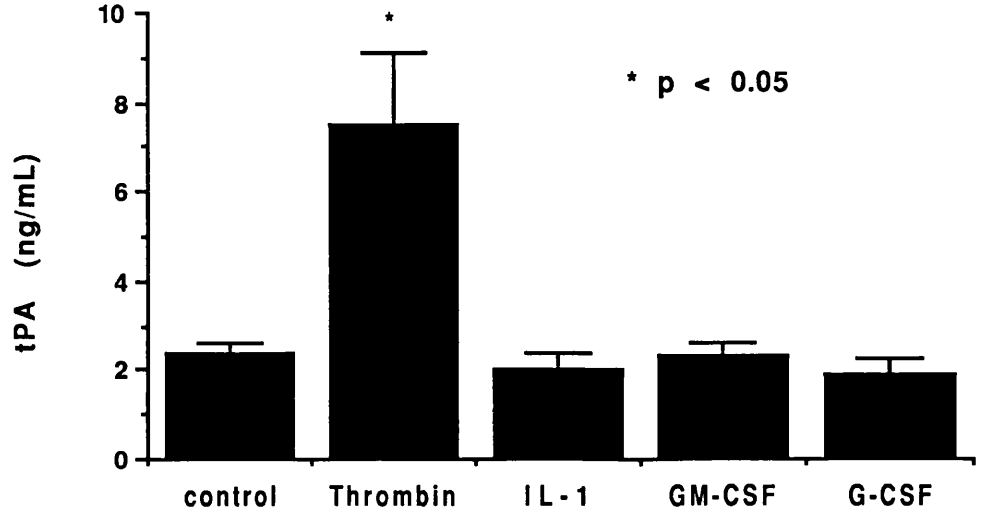


Figure 9.4. Effect of cytokines on HUVEC t-PA secretion. Results are the mean ± SE of 5 experiments.

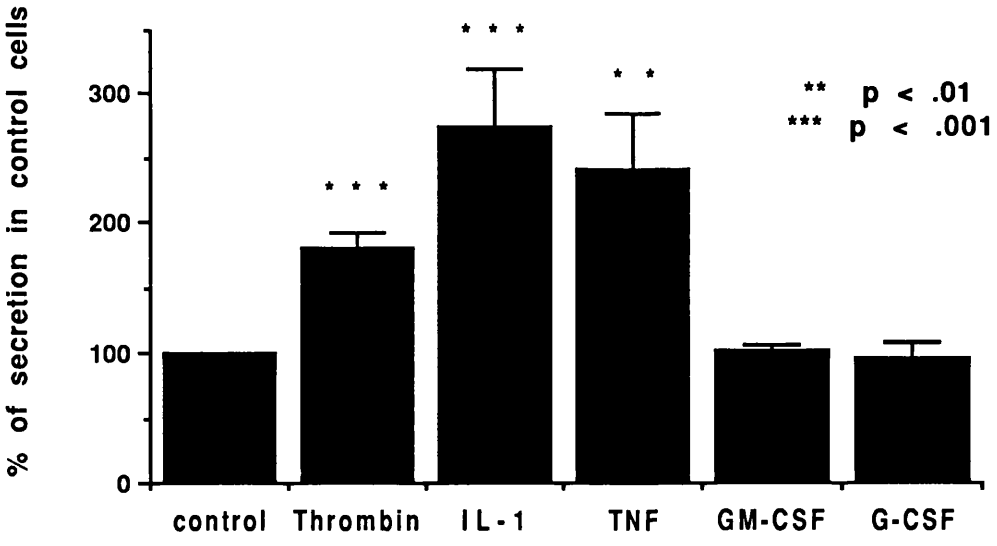


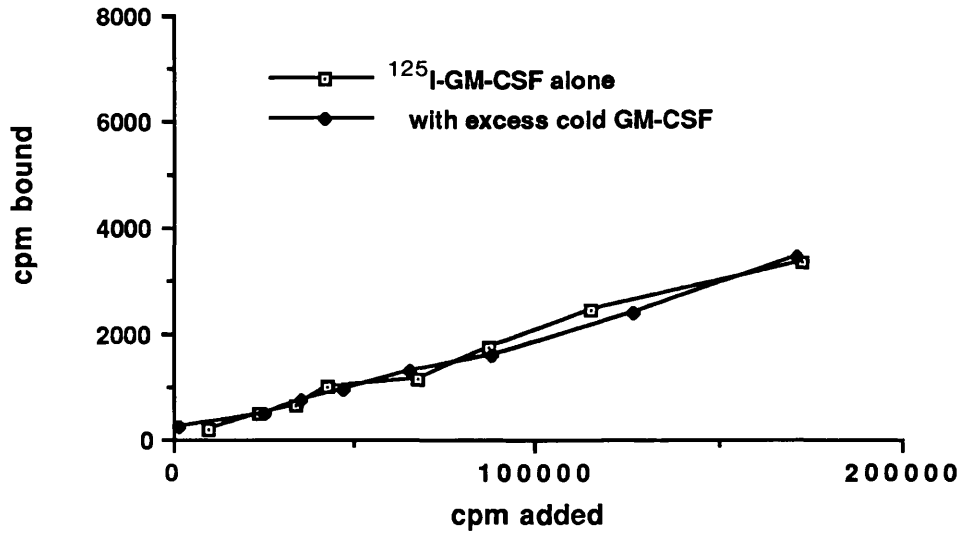
Figure 9.5. Effect of cytokines on HUVEC PAI-1 secretion. Results are mean ± SE of 5 experiments , expressed as percentage of secreted antigen in control cells.

GM-CSF Receptor Assay

In view of the lack of effect of GM-CSF on HUVEC in our system, HUVEC were examined for the presence and number of GM-CSF receptors. HUVEC at third passage, pooled from at least 3 umbilical cords, were detached by treatment with trypsin/EDTA and used in a binding assay with ^{125}I-GM-CSF as detailed in Methods. In 2 experiments, no specific binding of ^{125}I-GM-CSF was seen (data not shown). In case trypsin treatment might have cleaved off any GM-CSF receptors on the cells, 2 further experiments were performed using HUVEC grown on gelatin (0.05%)-coated flasks, and removed by treatment with 1 mM EGTA (10 minutes at 37^{o}C). Again, no specific binding of ^{125}I-GM-CSF to endothelial cells was seen (Figure 9.7a), while peripheral blood neutrophils exhibited significant specific binding (Figure 9.7b). Parallel experiments revealed the presence of 802 ± 78 (mean ± SEM) high affinity receptors per neutrophil, with an affinity constant of 57 ± 15 pM. Using 3 X 10^6 cells per data point, 10 receptors per cell can be calculated to give specific binding of ^{125}I-GM-CSF 50% above the background binding to neutrophils. Endothelial cells exhibit higher levels of nonspecific binding of ^{125}I-GM-CSF than neutrophils do, but 25 receptors per endothelial cell should give binding values 50% above background.

The endothelial line, EAhy926 (Edgell et al, 1983) has also been reported to express GM-CSF receptors, and to respond to this cytokine (Bussolino et al, 1989). This cell line was therefore examined for the presence of GM-CSF receptors, using identical assays to those described above. In two experiments, these cells did not demonstrate any specific binding of ^{125}I-GM-CSF (data not shown).

(a)



(b)

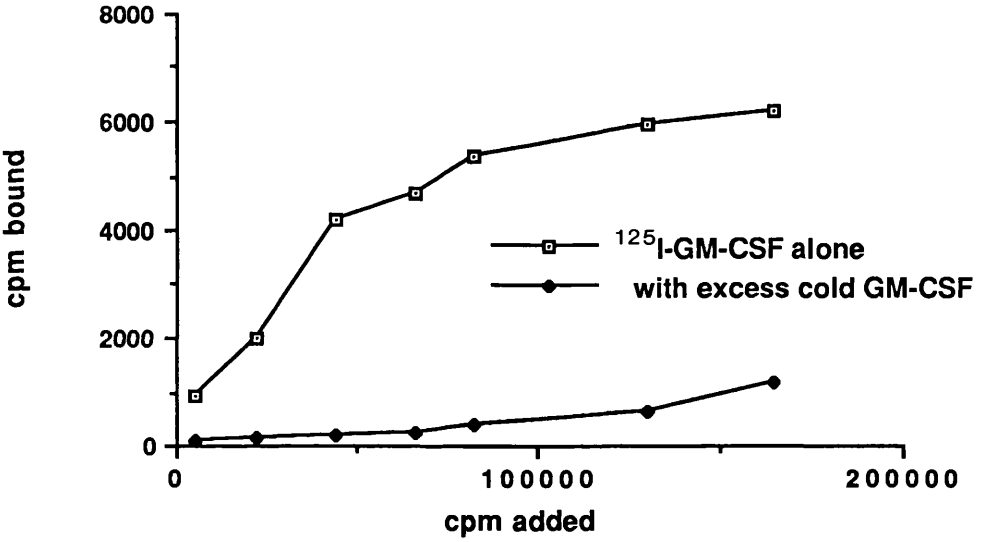


Figure 9.6. Binding of ^{125}I-GM-CSF to (a) HUVEC and (b) peripheral blood neutrophils, in the presence or absence of a 50-fold excess of unlabelled GM-CSF. Results are representative of 4 experiments (2 on trypsinized cells, and 2 on EGTA-detached cells) on HUVEC and 3 experiments on neutrophils.

DISCUSSION

The HUVEC used in these studies display a range of responses to cytokines. Proliferation is enhanced in response to ECGS, but not to IL-1 β. IL-1 β and TNF-α directly enhance cell-associated PCA while γ-IFN, although not having any direct effect on its own, primes the IL-1 β response. This confirms previous reports (Bevilacqua et al, 1986, Pober, 1988, Saegusa et al, 1990). The secretion of t-PA by HUVEC is enhanced by thrombin, but not by IL-1 β and TNF-α. All 3 agonists, however, increase both secreted and cellular PAI-1 antigen. Again, these observations are in keeping with those of other workers (Bevilacqua et al, 1986b, Schleef et al, 1988)· In these responses, the HUVEC in this culture system behave in a similar manner to that reported for other cultured human endothelial cells.

There was no effect of GM-CSF or G-CSF on these cells, in all the parameters studied above. The failure of cultured HUVEC to proliferate in response to GM-CSF and G-CSF in these studies contrasts with the reports by Bussolino et al (1989) on multiply passaged human umbilical cord endothelial cells. In addition, culturing cells up to the eighth passage in the continuous presence of GM-CSF (10-100 ng/mL) did not induce any responsiveness to the growth factor (data not shown). Other groups, using non-human cells derived from different tissues, for example, rat Peyers Patches (Chin et al, 1990) and mice bone marrow (Fei et al, 1990), have also reported an effect of GM-CSF on endothelial cell growth. However, these studies employed very different isolation techniques and culture conditions, and as some of these reports do not show statistical data for controls, it is difficult to assess their relevance to the work presented here.

The lack of effect of GM-CSF on HUVEC cell-associated PCA has been noted by others (Surprenant & Zuckerman, 1989). RhG-CSF has been reported to increase plasminogen activator activity in bovine aortic endothelial cells (Kojima et al, 1989), but had no effect on t-PA or PAI-1 expression of HUVEC in these studies, using similar doses of the same recombinant product. These differences in results between studies using cells from different sources are not surprising, as the amount and type of plasminogen activator produced can vary with the origin of the endothelial cells as well as the conditions of stimulation (Levin & Loskutoff, 1982, Wojta et al, 1989). Furthermore, the functional assays for plasminogen activator activity employed by Kojima et al

(1989) do not distinguish between urokinase-type and tissue-type plasminogen activators, while the immunological assays used here are specific for tissue plasminogen activator.

In keeping with the lack of response to GM-CSF, these studies failed to demonstrate GM-CSF receptors on either primary human endothelial cells or the endothelial cell line, EAhy926, despite the fact that, in the same experiments, neutrophils were shown to express 802 ± 78 high affinity receptors. This again contrasts with reports from Bussolino et al (1989) who found 423 ±187 high affinity receptors on human endothelial cells. The reason for this discrepancy is not clear, but it suggests that the expression of GM-CSF receptors by cultured endothelial cells and the ability of these cells to respond to these growth factors are critically dependent on culture conditions. There is no evidence, from the studies presented here, that either GM-CSF or G-CSF has any direct effect on the growth and function of cultured human endothelial cells.

CHAPTER TEN

EFFECT OF GM-CSF AND M-CSF ON VASCULAR ENDOTHELIAL FUNCTION *IN VIVO*

INTRODUCTION

In vitro studies show that cultured human endothelial cells do not respond directly, in terms of proliferation or function, to GM-CSF or G-CSF and, furthermore, do not express receptors for GM-CSF. However, in the physiological setting, the enhanced phagocyte adhesion produced by systemic administration of these growth factors *in vivo* might have indirect effects on the integrity and function of vascular endothelium. Pulmonary sequestration of cells following an intravenous administration of zymosan-activated serum in sheep is associated with endothelial cell damage and increased pulmonary vascular permeability (Meyrick & Brigham, 1983). In this context, it is relevant that side effects resembling the capillary leak syndrome have been described in patients receiving GM-CSF (Antmann et al, 1988, Brandt et al, 1988, Lieschke et al, 1990). Apart from its function in containing the vascular compartment, vascular endothelium also participates actively in the maintenance of the haemostatic/fibrinolytic balance, displaying a range of anti-thrombotic, prothrombotic, fibrinolytic and anti-fibrinolytic properties, which are mainly under the control of cytokines, but which may be modified by interactions with other cells (Gimbrone, 1986). Perturbation of endothelial cell function might, by producing an imbalance in this system, account for the large vein thromboses which have been reported in association with GM-CSF at high doses (Antman et al, 1988, Nissen et al, 1988, Jost et al, 1990).

The following studies were carried out to investigate the effects of systemic infusions of GM-CSF and M-CSF on the haemostatic/fibrinolytic balance *in vivo*, focussing in particular on markers of endothelial activation.

PATIENTS AND SAMPLE COLLECTION

Patients with Hodgkin's disease or non Hodgkin's lymphoma undergoing high dose chemotherapy with autologous bone marrow transplantation were treated with GM-CSF or M-CSF as part of a randomised clinical trial (see Chapter 5 for details). Prior to commencing on myeloablative therapy, these patients were given a two hour infusion of the growth factor (GM-CSF 30 $\mu g/m^2$, or M-CSF 16-128 mU/m^2) as a test dose. Venous blood samples were taken into trisodium citrate, and centrifuged immediately at 2000g for 30 minutes. Platelet poor plasma was stored in aliquots at -70°C until used in assays. For assays of tissue plasminogen activator (t-PA) activity, citrated blood was acidified immediately by adding to an equal volume (500 µL) of 0.2M acetic acid. After spinning for 2 minutes at 2000g, 600 µL of the supernatant was added to 40 µL of 1M HCl, and frozen immediately at -70°C until use. All samples were only thawed once.

SPECIAL METHODS

Materials

The following reagents were obtained from KABI Diagnostica (KABI/VITRIUM Ltd., Brune End, Buckinghamshire, UK): S-2251 chromogenic substrate, t-PA stimulator (human fibrinogen fragments), t-PA standard, human plasminogen, and t-PA/PAI deleted plasma. Rabbit anti-human von Willebrand factor (vWF) and HRP-conjugated rabbit anti-human vWF were obtained from DAKO, UK Ltd. The following materials for the D-dimer immunoasssay were obtained from Agen Biomedical Ltd., Brisbane, Australia: 96-well microtitre plate precoated with mouse anti-D-dimer MoAb, peroxidase conjugated mouse anti-fibrin degradation products (FDP) MoAb and purified human D-dimer. The following materials were obtained from Technoclone, Austria: 96-well microtitre plate precoated with anti-u-PA MoAb, peroxidase-conjugated anti-u-PA MoAb, u-PA standard.

Tissue plasminogen activator (t-PA) activity assay

This assay is based on the activity of plasmin on the chromogenic substrate, S-2251 (Nilsson et al, 1987), and was carried out in microtitre plates. Briefly, samples of acidified plasma (thawed rapidly at 37°C) were added to the reaction mixture consisting of human plasminogen, the chromogenic substrate S-2251, t-PA stimulator (fibrinogen fragments) in Tris buffer, and incubated for 4 hours at 37°C. The reaction was stopped by the addition of 20% acetic acid, and the amount of plasmin generated, which is proportional to the amount of t-PA in the sample, was quantitated by measuring the change in optical density at 405/490 nm on a

microplate reader (MR 700, Dynatech Laboratories). The t-PA activity in each sample (expressed in IU/mL) was determined from the standard curve obtained using recombinant t-PA, diluted in t-PA depleted plasma. All reagents were obtained from KABI Diagnostica. The lower limit of detection of this assay is 0.10 IU/mL, and the assay is linear between 0.25 to 10 IU/mL. The intraassay variability of the assay is 5%, with an interassay variability of 8%. The fibrinolytic activity of t-PA is expressed in International Units (IU), assessed by calibration against the International Standard for t-PA from human melanoma cells (lot 83/517, National Institute for Biological Standards and Control, London, UK).

Tissue plasminogen activator antigen concentrations

These were determined by enzyme immunoassay, using citrated plasma samples, as detailed in Chapter 9.

Plasminogen activator inhibitor (PAI) activity assay

This assay, also carried out in microtitre plates, is in principle similar to the one described above for t-PA activity, except that a fixed amount of t-PA is added in excess to each plasma sample at the start of the assay. This binds rapidly with PAI, and hence the amount of plasmin generated in the reaction mixture is proportional to the residual t-PA, and inversely proportional to the PAI activity in the sample. The amount of plasmin is determined by measuring the amidolytic activity of plasmin on the chromogenic substrate S-2251, as detailed above. All reagents were obtained from KABI Diagnostica. One IU of inhibitor is defined as the amount which inhibits one IU of t-PA/mL plasma under these conditions. The assay is linear between 2.5-40 IU/mL, the intraassay variability is 5%, and interassay variability is reported as being less than 10%.

Von Willebrand factor antigen (vWF Ag) assay

An enzyme immunoassay was used on citrated plasma. Briefly, microtitre plates (Costar 3590) were coated with rabbit anti-human vWF (Dako, 1/1000 in PBS buffer, pH 7.2) overnight at 4°C. The following morning, the wells were washed using PBS with 0.05% Tween (PBS/Tween), and standards and plasma samples were incubated in duplicate wells for 1 hour at room temperature. After washing with PBS/Tween, HRP-conjugated rabbit anti-human vWF (Dako, 1/1000 in PBS/Tween) was placed in each well. The plate was incubated for 1 further hour at room temperature, and washed before the addition of substrate (0.8 mg/mL OPD in 0.1M citrate phosphate buffer pH 5.0, with 0.015% hydrogen peroxide). The plate was placed in the dark, and, when optimum colour development had occurred, the reaction was stopped by addition of 2M sulphuric acid. The absorbance was measured

at 490 nm on the microplate reader, and the sample values obtained from the standard curve. The standard used was the British standard for coagulation factors (National Institute for Biological Standards and Control, London, UK). The interassay variability was 8%.

D-Dimer immunoassay

Citrated plasma samples were assayed for crosslinked fibrin degradation products using an enzyme immunoassay (Dimertest EIA, Agen Biomedical Ltd., Queensland, Australia, Rylatt et al, 1983). Briefly, plasma samples and standards were placed in duplicate wells of a microtitre plate precoated with mouse anti-D-dimer MoAb and incubated at room temperature for 1 hour. The wells were washed with PBS/Tween, incubated for 1 hour with peroxidase conjugated mouse anti-fibrin degradation products (FDP) MoAb, and washed once more before addition of substrate (ABTS in citrate buffer pH 5.0, with 0.003% hydrogen peroxide). Once optimum colour development had occurred, the reaction was stopped with 2M sulphuric acid, and absorbance read at 420 nm on the microplate reader. D-dimer concentrations in samples were calculated from the standard curve, obtained using purified human D-dimer (Agen Biomedical Ltd.) as standard. The assay is linear in the range 0.02-5.0 μg/mL, the intraassay variability was 5%, and the interassay variability is less than 10%.

Urokinase-type plasminogen activator (u-PA) assay

Plasma samples were assayed for the presence of u-PA using an enzyme immunoassay (Darras et al, 1986) which measures both single and double chain u-PA. All reagents were obtained from Technoclone, Austria. Briefly, standards (Technoclone) and EDTA plasma samples were placed in duplicate wells in a microtitre plate precoated with anti-u-PA MoAb, and incubated for 2 hours at 37°C. The plate was washed, peroxidase-conjugated anti-u-PA antibody was added to the wells, and incubation carried out for a further 2 hours at 37°C. At this stage the plate was washed, and ABTS substrate pippetted into the wells. The reaction was stopped after 1 hour incubation at room temperature by adding 0.32% sodium fluoride, and absorbance of wells read at 405/490 nm using the microplate reader. The concentration of u-PA in plasma samples was obtained from the standard curve, which was linear between 0.6-10 ng/mL. The intraassay variability was less than 5%, and the interassay variability is given as being less than 10%.

RESULTS

4 patients who received GM-CSF (30 $\mu g/m^2$), and 4 patients who received M-CSF (2 at 16 mU/m^2, and the other 2 at 128 mU/m^2) were tested.

Tissue plasminogen activator (tPA) activity

T-PA activity was measured in 3 of the 4 patients receiving GM-CSF (30 $\mu g/m^2$), and in all 4 patients who received M-CSF. Preinfusion levels of t-PA activity were 0.35 ± 0.10 IU/mL in the 3 patients receiving GM-CSF, and 0.61 ± 0.3 IU/mL in the patients receiving M-CSF. At the end of the 2 hour infusions of GM-CSF, plasma t-PA activity had risen to 445 ± 139% of basal levels ($p < 0.01$), and remained elevated (201 ± 46% of preinfusion levels, $p < .01$) at 2 hours afterwards (Figure 10.1a). Figure 10.1b shows the absolute levels ot t-PA activity in each of these 3 patients, expressed in ng/mL. In contrast, in patients receiving M-CSF, there was no significant change in plasma tPA activity, at either dose level (Figure 10.1a). All infusions were carried out at the same time each day, in order to control for possible diurnal variation in plasma t-PA actitivy.

Tissue plasminogen activator antigen levels

Plasma t-PA antigen concentration was measured in all 4 patients receiving GM-CSF, and all 4 patients receiving M-CSF. In patients receiving GM-CSF, t-PA antigen levels rose from a preinfusion level of 4.4 ± 2.0 ng/mL to 6.8 ± 3.6 ng/mL (150 ± 32%, $p< .05$) at the end of the 2 hour infusion (Figure 10.2). In patients receiving M-CSF, t-PA antigen levels did not change significantly from the preinfusion levels of 5.7 ± 3.0 ng/mL at any of the time points measured.

Plasminogen activator inhibitor (PAI) activity

In the 4 patients receiving GM-CSF, there was a 52 ± 25% ($p < .05$) fall in plasma PAI activity from a preinfusion value of 14.5 ± 3.2 IU/ml to 6.8 ± 2.9 IU/ml at the end of the 2 hour infusion, and at 2 hours afterwards , PAI levels were still only 50 ± 23% of initial levels ($p < .05$, Figure 10.3). Patients receiving M-CSF did not demonstrate any consistent change in PAI-1 acitivity (Figure 10.3).

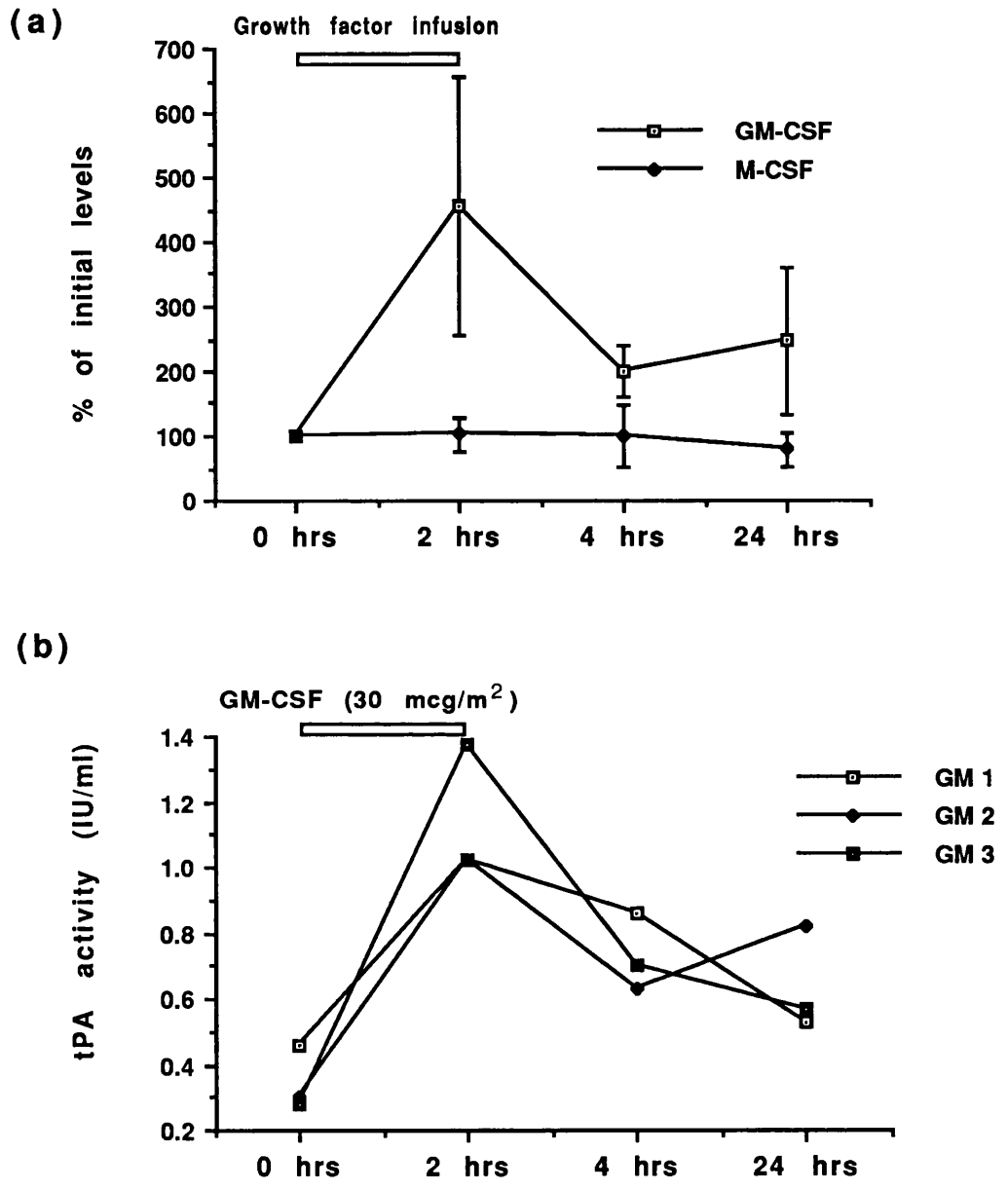


Figure 10.1. Effect of growth factors on plasma t-PA activity.
(a) Mean ± SD of t-PA activity, expressed as percentage of initial levels, in 3 patients receiving GM-CSF (30 $\mu g/m^2$), and 4 patients receiving M-CSF (2 at 16 mU/m^2, and 2 others at 128 mU/m^2).
(b) T-PA activity in ng/mL in each of the 3 patients who received GM-CSF.

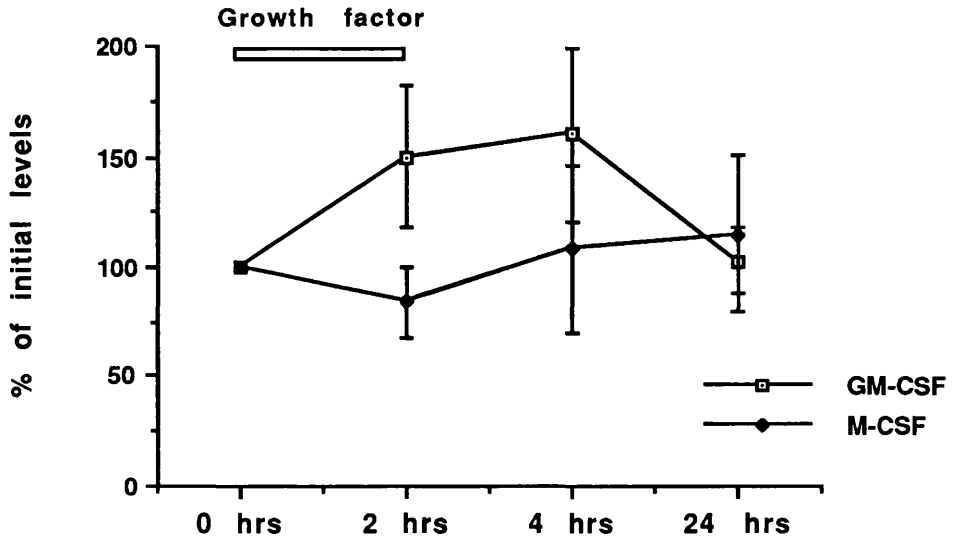


Figure 10.2. Effect of growth factors on plasma t-PA antigen, expressed as percentage of initial levels, mean ± SD of 4 patients each receiving GM-CSFand M-CSF.

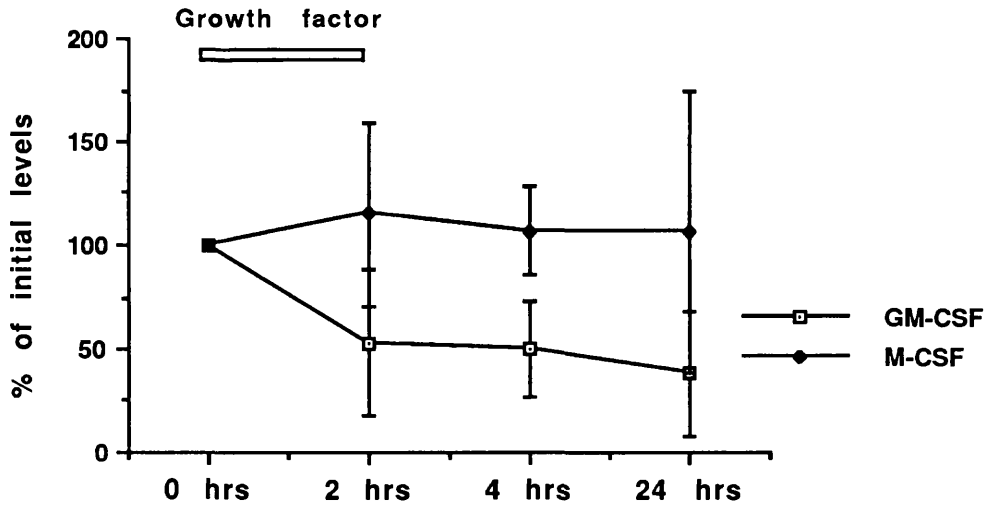


Figure 10.3. Effect of GM-CSF (4 patients) and M-CSF (4 patients) on plasma PAI activity, expressed as percentage of initial levels, mean ± SD.

4. Plasma von Willebrand factor (vWF) concentration

Plasma vWf antigen levels were measured in 4 patients receiving GM-CSF, and 2 patients who received 128 mU/m^2 of M-CSF. There was no significant change in plasma vWf Ag levels, in either group of patients (Figure 10.4) .

5. Plasma D-dimer concentration

Neither growth factor (GM-CSF in 4 patients, or M-CSF in the 2 patients receiving 128 mU/m^2) had any significant effect on plasma D-dimer levels (Figure 10.5).

6. Urokinase-type plasminogen activator (u-PA)

U-PA antigen levels were measured in 4 patients each who received GM-CSF, and M-CSF. Preinfusion levels were 0.8 ± 0.3 ng/mL and 0.8 ± 0.2 ng/mL in patients receiving GM-CSF and M-CSF respectively. In neither group of patients was there any significant change in u-PA antigen levels following growth factor infusions (Figure 10.6). In patients receiving GM-CSF, plasma u-PA levels showed a small rise to 125±41%, and 131±23% of preinfusion levels at 2 and 4 hours, but these differences did not reach significance.

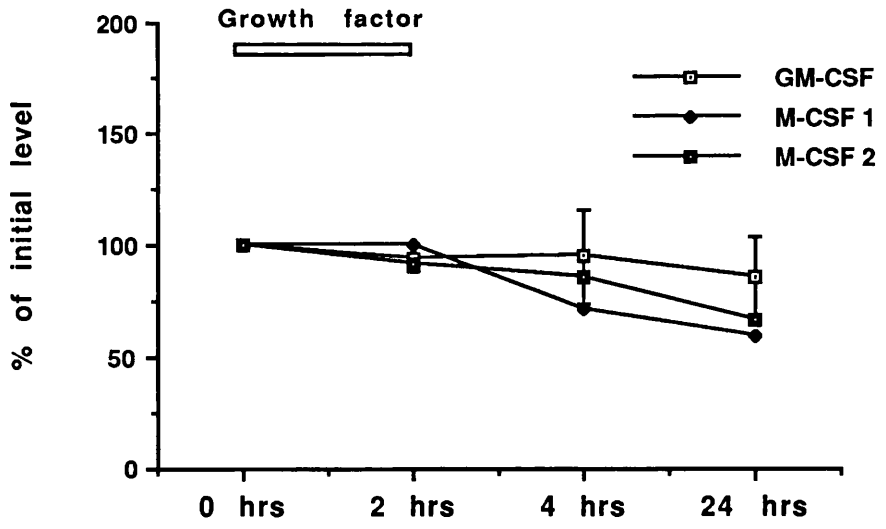


Figure 10.4. Plasma vWF antigen levels, expressed as percentage of initial levels, in patients receiving GM-CSF (mean ± SD, n=4) and M-CSF (n=2, expressed individually).

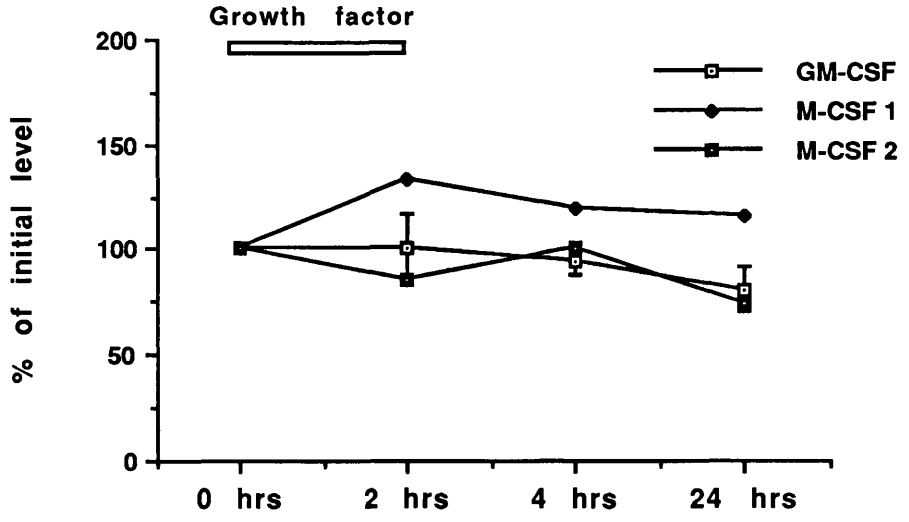


Figure 10.5. Effect of GM-CSF (mean ± SD of 4 patients) and M-CSF (n=2) on plasma D-dimer concentrations, expressed as percentage of initial levels.

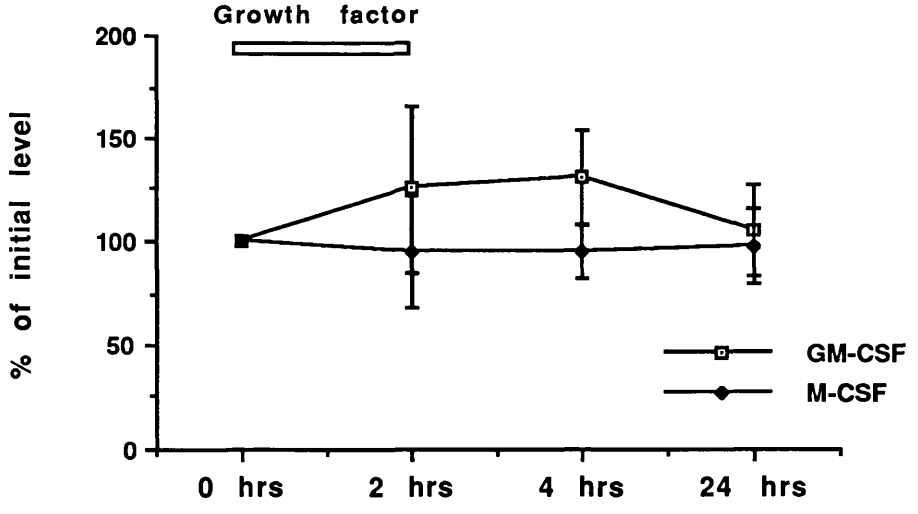


Figure 10.6. Plasma u-PA antigen levels in 4 patients each who received GM-CSF and M-CSF, expressed as mean ± SD percentage of initial levels.

DISCUSSION

These results show that GM-CSF, but not M-CSF, has effects on the fibrinolytic activity of plasma, when administered *in vivo*. This is manifested as a rise in t-PA activity, and is associated with a similar, but smaller rise in tPA antigen levels, and also a fall in PAI-1 activity. The level of t-PA activity in the plasma varies with the level of its inhibitor, PAI-1; high PAI-1 levels resulting in a greater percentage of t-PA being complexed as t-PA/PAI-1, and hence a smaller percentage is present as active t-PA, and vice versa (Chandler et al, 1990). The data presented here suggests that the rise in t-PA activity following GM-CSF administration results, both from an increased secretion of t-PA, as well as a decrease in PAI-1 activity. The changes are not simply attributable to circadian variations, in which both total t-PA , as well as t-PA/PAI-1 complex decrease in the evening, but to a lesser extent than active PAI-1, thus resulting in a net increse in t-PA activity, of about 78% in normal adults (Chandler et al, 1990). In patients receiving GM-CSF, the rise in t-PA activity is much greater than this (245%) and appears to result from GM-CSF induced secretion of t-PA antigen, combined with the diurnal fall in PAI-1 activity.

The fibrinolytic activity in plasma is almost all accounted for by t-PA, as there is very little active u-PA (Collen, 1980, Blasi et al, 1987). Vascular endothelium is the main source of t-PA in the circulation (Binder et al, 1987, Rijken et al, 1980), and plasma levels are low (3-5 ng/mL), but the concentration rises markedly in response to physiological stresses, such as exercise or venous occlusion, due to release from the vascular endothelium (Cash, 1978). Plasma u-PA (2 ng/mL) circulates primarily in its single chain form, which is inactive, and the role of this plasma u-PA in normal physiology is not yet clearly defined. In order to rule out the possibility that any u-PA present in plasma samples might have cross-reacted in the assay employed for t-PA (unlikely because the t-PA assay employs a specific t-PA stimulator), plasma u-PA antigen levels were also measured in these patients. Plasma u-PA levels did not change following either GM-CSF or M-CSF infusions.

GM-CSF has been reported to induce plasminogen activator activity in murine peritoneal macrophages (Hamilton et al, 1980), and, more recently, in purified human monocytes *in vitro* (Hart et al, 1991). In the latter study, synthesis and expression of cell-associated u-PA was increased following 18 hours incubation with GM-CSF alone. This was not associated with an increase in secreted u-PA in cell supernatants, which is consistent with the results in this chapter. In the same study by Hart et al (1991), monocytes secreted t-PA in response to GM-CSF, but

only when simultaneously incubated with IFN-γ; GM-CSF was also able to augment LPS induced tPA secretion in these cells. In view of these reports, it is possible that systemically infused GM-CSF might act in concert with local inflammatory mediators present in infective foci to stimulate local monocyte/macrophage production of plasminogen activators. However, the rise in plasma t-PA antigen and activity in response to GM-CSF occurs more rapidly (within 2 hours), than the *in vitro* responses demonstrated by Hart et al (1991), which suggests a different mechanism of release and/or cellular source. Finally, the lack of effect of M-CSF (which is a specific activator of monocytes/macrophages) on plasma t-PA activity would support the hypothesis that the response seen with GM-CSF is endothelial in orgin.

The rise in t-PA activity following infusions of GM-CSF may relate to endothelial activation resulting from the transient increase in the number of adherent phagocytes in the microcirculation. However, there was no change in plasma levels of vWf antigen, which might be expected to be released upon activation of endothelial cells. In this study, there was also no evidence of activation of the coagulation pathway, in that plasma levels of D-dimers were unchanged. The physiological significance of GM-CSF induced t-PA activity seen here *in vivo* is unclear, but may relate to activation of the thrombotic/fibrinolytic system, hence accounting for the side effects of thrombosis around central venous catheters which have been reported with GM-CSF at high doses (Antman et al, 1988, Nissen et al, 1988). In situations where GM-CSF is present in supraphysiological concentrations, there is also evidence of disturbances in the thrombosis/fibrinolysis balance, and the chronic bleeding seen in GM-CSF transgenic mice (Metcalf & Moore, 1988) may relate to increased t-PA activity.

The results presented here further emphasize the varied proinflammatory effects of GM-CSF, which exerts not only direct effects on phagocytic cells, but also indirect effects, probably mediated by the release of secondary signalling molecules, as well as by increased cell adhesion.

CHAPTER ELEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

Effects of the myeloid growth factors on the adhesive interactions of phagocytes with endothelium.

This work examines the way in which the myeloid growth factors, in particular GM-CSF, modulate the adhesive interactions between neutrophils and endothelium. Both GM-CSF and G-CSF have effects on the adhesive properties of mature phagocytic cells. *In vitro*, both growth factors increase surface expression of the β2 integrins, CD11/CD18, and downregulate surface levels of the selectin receptor, L-selectin (LECAM-1, LAM-1). Administration of GM-CSFand G-CSF leads to an *in vivo* rise in surface levels of CD11/CD18 on circulating cells, but, in contrast to the results of *in vitro* studies, there is no effect on L-selectin expression of peripheral cells. GM-CSF enhances the adhesion of neutrophils to cultured human endothelial cells *in vitro*, while G-CSF has no effect. Following infusions of GM-CSF or G-CSF, there is an immediate transient leucopenia, associated with the retention of phagocytic cells in the lungs, a phenomenon which may reflect changes in neutrophil-endothelial adhesion *in vivo*. In support of this hypothesis, histological examination of lung sections from *cynomolgus* monkeys following GM-CSF administration shows leucocytes, not only contained in capillaries, but also adherent to the endothelium of large vessels. Hence both GM-CSFand G-CSF are able to modulate the adhesive properties and functions of mature cells, but there are important differences between the two growth factors. Both are equally effective in upregulating CD11/CD18 receptors, but G-CSF is less effective in downregulating L-selectin expression. G-CSF has no effect on the adherence of neutrophils to HUVEC, while GM-CSF produces a consistent and significant enhancement of neutrophil adhesion to HUVEC. The kinetics of the margination responses seen *in vivo* also differ; following G-CSF administration, full recovery of peripheral counts occurs by 60 minutes; following GM-CSF infusions, however, peripheral cell counts are still only 50% of initial levels at 120 minutes. These observations suggest that GM-CSF has a greater proadhesive effect on neutrophils, and this may relate to an effect on surface adhesion receptors.

Role of the β2 integrin receptors in neutrophil-endothelium interactions

Functional inhibition studies using anti-CD18 MoAb suggest that GM-CSF induced adhesion of neutrophils to endothelium *in vitro* is dependent on the β2 integrin

(CD11/CD18) adhesion receptors. However, prior administration of this antibody to nonhuman primates has only a small effect on GM-CSF induced margination *in vivo*. In addition, when GM-CSF was administered to a patient with partial leucocyte adhesion deficiency (LAD), whose neutrophils expressed only 10% of normal levels of CD11b/CD18, the margination response was comparable to those seen in haematologically normal subjects.

These *in vivo* observations, together with those of other workers (Lundberg & Wright, 1990) studying leucocyte margination in response to the chemotactic peptide, FMLP, in rabbits, suggest that quantitative changes in the surface levels of CD11/CD18 are not sufficient to account for the enhanced phagocyte-endothelium interactions which follow the systemic administration of these growth factors. Some *in vitro* observations support this notion. Despite producing equivalent upregulation of the CD11/CD18 receptors, GM-CSF and G-CSF differ in their effects on neutrophil adhesion to human umbilical vein endothelial cells *in vitro*, suggesting that the *in vitro* adhesion response to GM-CSF is not directly related to the upregulation of CD11/CD18. Despite this, however, anti-CD18 MoAb was able to inhibit GM-CSF stimulated adhesion to HUVEC by 80%. It is possible that the quantitative upregulation of CD11/CD18 receptors on neutrophils is necessary, but not sufficient, for growth factor induced neutrophil-endothelial adhesion, and that cell stimulation with GM-CSF, but not G-CSF, activates another, crucial, adhesive mechanism. One hypothesis is that, while both growth factors increase the actual numbers of surface receptors, only GM-CSF is able to induce a functional change in the binding activity of these receptors, which is sufficient to produce the enhanced adhesion to endothelium.

On the other hand, qualitative changes in CD11/CD18 are unlikely to be the major mechanism underlying phagocyte margination induced by growth factors *in vivo* because LAD cells expressing only 10% of normal levels demonstrate a margination response to GM-CSF. It is becoming increasingly clear that the β2 integrin-dependent pathway is only one of several different adhesion pathways which mediate the interaction of circulating phagocytes with vascular endothelium.

Different stages of neutrophil-endothelium interactions are mediated by distinct families of adhesion receptors

Recent studies using *in vitro* assays conducted under dynamic conditions of flow, as well as intravital video microscopy on exteriorized vessels, suggest that CD11/CD18 and L-selectin receptors cooperate in mediating different forms of neutrophil-endothelium interactions, at distinct stages of the process of neutrophil recruitment

into inflammatory areas (Smith et al, 1991, von Andrian et al, 1991). About 20 years ago, Atherton and Born (1973) quantitated, in postcapillary venules of externalised mouse mesentery, the proportion of granulocytes that were seen to be rolling (marginating pool) at a much lower velocity than the mean velocity of blood flow in the vessels. The proportion of rolling granulocytes was greatly increased by local application of chemoattractants, and under these conditions, some granulocytes were seen to arrest on the endothelial cell surface, and then to transmigrate. The recent characterisation of the various families of adhesion receptors, has enabled workers to explore the different molecular mechanisms underlying these separate processes. In the initial step of neutrophil recruitment, circulating neutrophils roll along the walls of inflamed postcapillary venules. This adhesive interaction, which takes place under shear stress, is reversible and is mediated by L-selectin on neutrophils, together with E-selectin, and perhaps P-selectin, on the surface of activated endothelium (Lawrence & Springer, 1991, Ley et al, 1991). Activation of rolling neutrophils, for example, by chemoattractants, leads to the upregulation of the β2 integrins, CD11/CD18, which bind to ligands on the surface of endothelial cells. Adhesion via the β2 integrin receptors can only occur in cells which are rolling, is resistant to high shear stresses and leads to the immobilisation of neutrophils on endothelium, with subsequent spreading and polarisation of cells. Some of these cells then, presumably, go on to transmigrate into the extravascular space. Hence both selectins and β2 integrins play a crucial part in the localisation of phagocytic cells in infective foci. This would explain the inhibitory effect of MoAbs to both L-selectin and CD18 on neutrophil recruitment into inflammatory areas (Arfors et al, 1987, Lindbom et al, 1990).

Nonadhesive mechanisms of leucocyte retention in capillaries

The interaction of neutrophils with endothelium *in vivo* is not simply influenced by cell surface adhesive mechanisms, but may also involve changes in the haemodynamic properties of the system. Neutrophil activation leads to changes in cytoskeletal organisation, cells become stiffer and less deformable, and this results in an increased transit time in capillaries (Downey & Worthen, 1988, Worthen et al, 1989). Changes in mechanical properties of neutrophils, for example, have been implicated in the sequestration of neutrophils in pulmonary capillaries seen in endotoxaemia (Erzurum et al, 1992). Decreased flow rates would allow cell surface adhesive mechanisms to come into play, such as the engagement of CD11/CD18 receptors with ligands on the surface of endothelial cells. While the initiation of pulmonary leucostasis could relate to a decrease in deformablity of cells, the kinetics of the rebound in cell counts may be determined by the strength of the resultant adhesive interactions between leucocytes and endothelium. Such adhesive

mechanisms may not be fully activated in cells stimulated by G-CSF, and hence marginated cells would return more quickly into the peripheral circulation. This would explain the different kinetics of the demargination response between G-CSF and GM-CSF.

GM-CSF regulates neutrophil migration across endothelium

In the development of the inflammatory response, neutrophil adherence to endothelium is followed by the migration of adhered cells into the extravascular tissues. Following on from these studies on adhesion, an *in vitro* model was developed, in which to study transendothelial cell migration. GM-CSF stimulation increases neutrophil migration across unstimulated endothelium, but inhibits migration of neutrophils across IL-1 activated endothelium. These effects are independent of concentration gradients. While GM-CSF induced neutrophil migration across unstimulated endothelium may be mediated by effects on adhesion, the inhibition of migration across cytokine activated endothelium appears not to be related to changes in adhesion. In this situation, GM-CSF may act to downregulate some molecular mechanism which is involved in the migration of neutrophils across cytokine activated endothelium. The differential effect of GM-CSF on neutrophil migration, depending on the conditions of endothelial activation, has important implications for the way in which this growth factor regulates neutrophil emigration in inflammation, and can explain the decreased neutrophil migration into inflammatory sites seen following GM-CSF administration *in vivo* (Peters et al, 1988, Addison et al, 1989). These studies also confirmed the role of CD18 in the transendothelial migration of cells, and extended the *in vivo* observations of Kishimoto et al (1989), that migrated neutrophils express lower L-selectin levels than nonmigrating cells.

Lack of effect of GM-CSF and G-CSF on endothelial cell proliferation and function

GM-CSF and G-CSF do not have direct effects on endothelial cells, as assessed *in vitro* in the culture system employing HUVEC. Furthermore, HUVEC do not express receptors for GM-CSF. However, the transient increase in the numbers of cells interacting with endothelium may have indirect effects on endothelial cell function. The finding that GM-CSF infusions lead to an increase in plasma tissue-plasminogen activator activity is suggestive of some degree of endothelial activation, whether mediated by secondary cytokine release, or by direct contact with activated and adherent phagocytic cells in the microvasculature.

Different biological roles for GM-CSF and G-CSF in inflammation and haemopoiesis

The results of this work highlight the fact that GM-CSF and G-CSF, quite apart from their effects on haemopoiesis, belong to a group of cytokines, including tumour necrosis factor (TNF), γ-IFN and IL-8, which activate mature phagocytic cells (Klebanoff et al, 1986, Baggiolini et al, 1989). Such proinflammatory cytokines augment the adherence dependent responses of mature cells, including homotypic and heterotypic cell-cell adhesion, phagocytosis, and ADCC, stimulate degranulation, prime the oxidative response, and modulate the surface expression of adhesion molecules. These cytokines are highly synergistic in many of these effects on neutrophil function (Khwaja et al, 1992, Yuo et al, 1991). In addition, these mediators regulate the emigration of phagocytic cells into local inflammatory areas, acting, not only as chemoattractants, but also to specifically activate leucocytes, altering their adhesive and migratory properties (Huber et al, 1991).

The differences between GM-CSF and G-CSF in their proadhesive effects on neutrophils is in accord with other work on the neutrophil respiratory burst, where G-CSF was found to be a much weaker priming agent than GM-CSF (Khwaja et al, 1991). The difference in their range of activities and relative potencies suggests that GM-CSF may be a more important neutrophil activator *in vivo*, while G-CSF may be more important in regulating neutrophil production in response to stress. Circulating levels of G-CSF are undetectable in normal subjects, but are elevated in conditions such as infection, congenital or cyclical neutropenia, or following high dose cytotoxic therapy (Watari et al, 1989, Kawakami et al, 1990, Mempel et al, 1991), where G-CSF levels varied inversely with the neutrophil count. In patients with AML, there was a clear correlation between elevated serum G-CSF levels and documented infections (Sallerfors & Olofsson, 1991). Dogs which develop anti-human G-CSF antibodies which can cross react with canine G-CSF develop a profound neutropenia (Hammond et al, 1991). These observations suggest that G-CSF may function as a systemic regulator of neutrophil production under stress. GM-CSF, on the other hand, is undetectable in the systemic circulation, even under conditions of stress (Sallerfors & Olofsson, 1991). In contrast to G-CSF, therefore, GM-CSF have a more important role in exerting its proinflammatory effects on phagocytic cells locally where, following production by activated macrophages and stromal cells, it is anchored to stromal elements and extracellular matrix, (Gordon et al, 1987, Roberts et al, 1987).

Studies on transgenic animals further support the hypothesis that G-CSF and GM-CSF have different biological roles. Transgenic mice expressing very high levels of

G-CSF displayed a sustained neutrophilia in the peripheral blood, as well as an infiltration of neutrophils in various organs, but this granulocytic hyperplasia did not result in tissue damage, or malignant transformation (Chang et al, 1989). On the other hand, GM-CSF transgenic animals suffer fatal multi-system organ damage, with evidence of macrophage infiltration and activation (Lang et al, 1987).

Future directions

Several issues are outstanding. This work has shown that growth factors, paritcularly GM-CSF, have significant effects on neutrophil binding to endothelium. The relationship of these effects to the normal physiological control of neutrophil emigration remains to be clarified. It is not clear from these studies whether phagocyte margination in response to growth factor infusions represents one stage in a series of neutrophil-endothelium interactions which lead eventually to leucocyte extravasation into inflammatory sites. If so, one would predict that this margination response would be inhibited, at least in part, by antibodies against the neutrophil L-selectin receptor. A further, and related question, is whether the increase in marginating cells means that more neutrophils are avaible for transmigration into extravascular tissues, in response to appropriate local stimuli. The effect of GM-CSF to increase neutrophil migration across resting endothelium could simply be due to an increase in the number of adherent cells. On the other hand, the demonstration that GM-CSF inhibits neutrophil migration across cytokine activated endothelium emphasizes that vascular endothelium plays an active role in regulating neutrophil extravasation. The range and relative concentrations of the various local inflammatory mediators and cell-associated signalling molecules to which the cells are exposed will determine the net movement of cells into inflammatory sites. The demonstration of synergism between the CSFs and other inflammatory cytokines such as TNF and IL-8 (Khwaja et al, 1992) in the priming of the neutrophil respiratory burst raises the question of how GM-CSF interacts with these cytokines, and other mediators, in the regulation of neutrophil adhesion to, and migration across, endothelium. The role of G-CSF in neutrophil migration has yet to be addressed.

Finally, a careful examination of the interactions of various factors which influence neutrophil binding and migration will further our understanding of the molecular basis for neutrophil emigration. More specifically, the demonstration that GM-CSF inhibits neutrophil migration across cytokine activated endothelium suggests that it may be possible to study the molecular mechanisms underlying cell migration across, as distinct from adhesion to, endothelium. The way in which the shedding of the neutrophil L-selectin receptor is involved in cell movement across endothelium has yet to be clearly defined. It would be interesting to study the migratory capacity

of cells in which L-selectin shedding has been blocked, for, example, by protease inhibitors.

Clinical implications of these studies

The clinical relevance of this work can be divided into two broad areas. Firstly, G-CSF and GM-CSF have now been licensed for use in the treatment of marrow hypoplastic states. A full understanding of the biological effects of these cytokines on mature cells is essential in order to optimise their clinical value in the management of these, and other disorders. The inhibition of neutrophil migration across inflamed endothelium by GM-CSF raises the possibility that administration of this growth factor *in vivo* may impair phagocyte movement into infective areas. On the other hand, it has been shown that GM-CSF, in its direct actions on neutrophils, is highly synergistic with other inflammatory cytokines such as TNF-α and IL-8, such that, when present together in local inflammatory areas, only picomolar concentrations of each are required for maximal cell activation (Khwaja et al, 1992); that is, much lower concentrations than those which would produce migration inhibition. A careful comparison of the dose response profiles of these different actions of GM-CSF on phagocyte functions is required in order to determine the optimal dose of the growth factor which would activate cells in inflammatory areas without inhibiting neutrophil migration.

Secondly, the adhesive and immune responses of phagocytic cells can produce clinical benefit in infective situations, but can also result in pathological states of tissue destruction. The increasing recognition of the role of GM-CSF and G-CSF as inflammatory mediators, which control the immune responses and adhesive interactions of phagocytes opens up the possibility of clinical manipulation of these processes.

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APPENDIX 1 : EFFECT OF TPA AND GM-CSF ON NEUTROPHIL ALKALINE PHOSPHATASE STANDARD CURVES

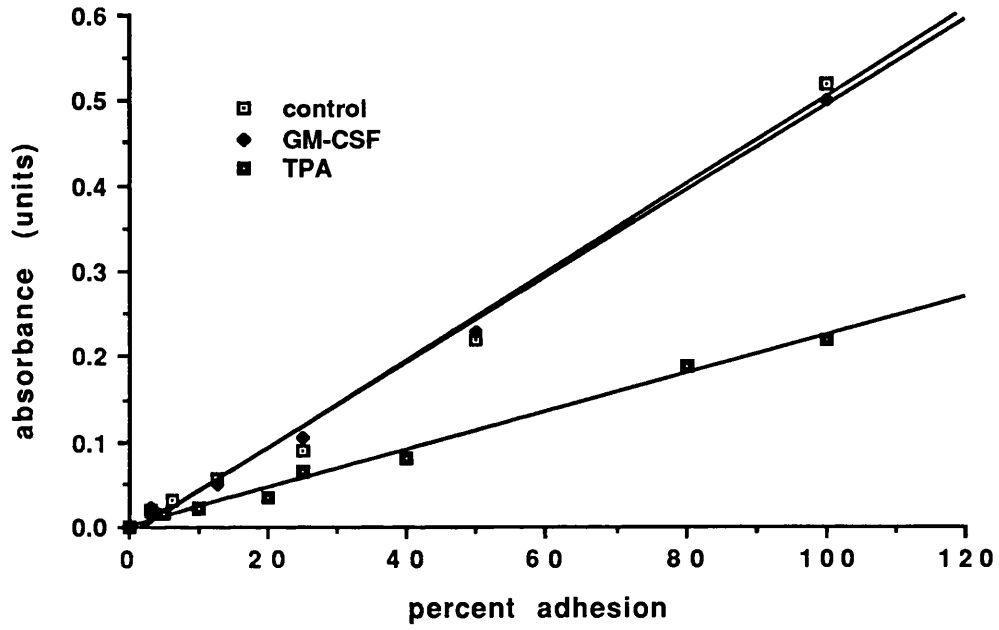

Figure 11.1. Standard curves in neutrophil adhesion assay. Cell suspensions, incubated separately with TPA (1 μg/mL), GM-CSF (100 ng/mL), or medium as control in parallel with adhesion plates. Cells were washed and cell counts readjusted before placing in microtitre wells for the alkaline phosphatase assay.

APPENDIX 2: TITRATION OF PLASMA ANTIBODY LEVELS

1. PLASMA LEVELS OF MOAB 44 IN 1 ANIMAL

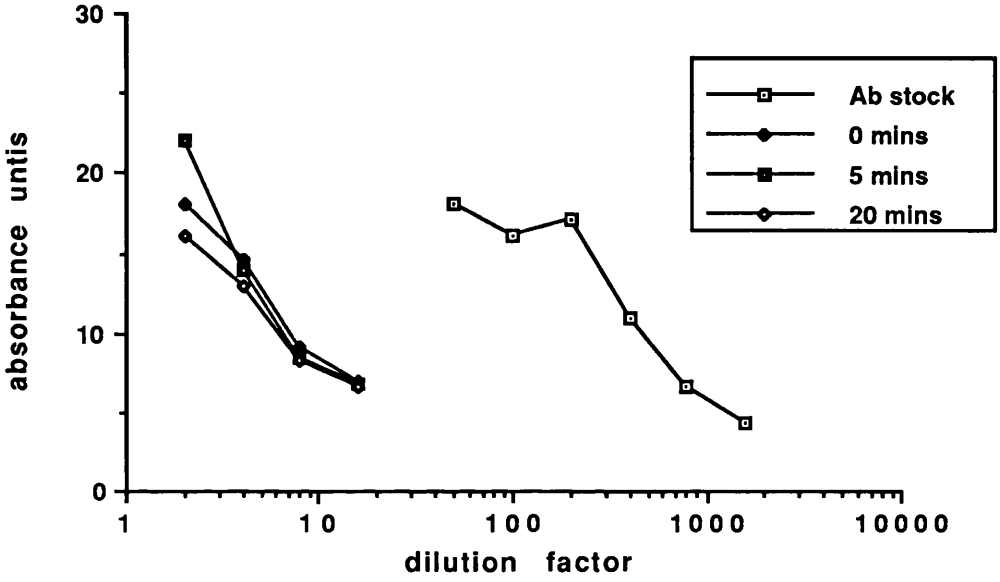

Figure 11.2. Titration curves of plasma from 1 animal which received 0.35 mg/kg of MoAb 44, and the antibody stock (0.66 mg/mL). Plasma concentrations of the antibody at 0, 5 and 20 minutes after GM-CSF administration were 10, 10, and 8.7 μg/mL respectively.

2. PLASMA LEVELS OF MOAB MHM23 IN 1 ANIMAL

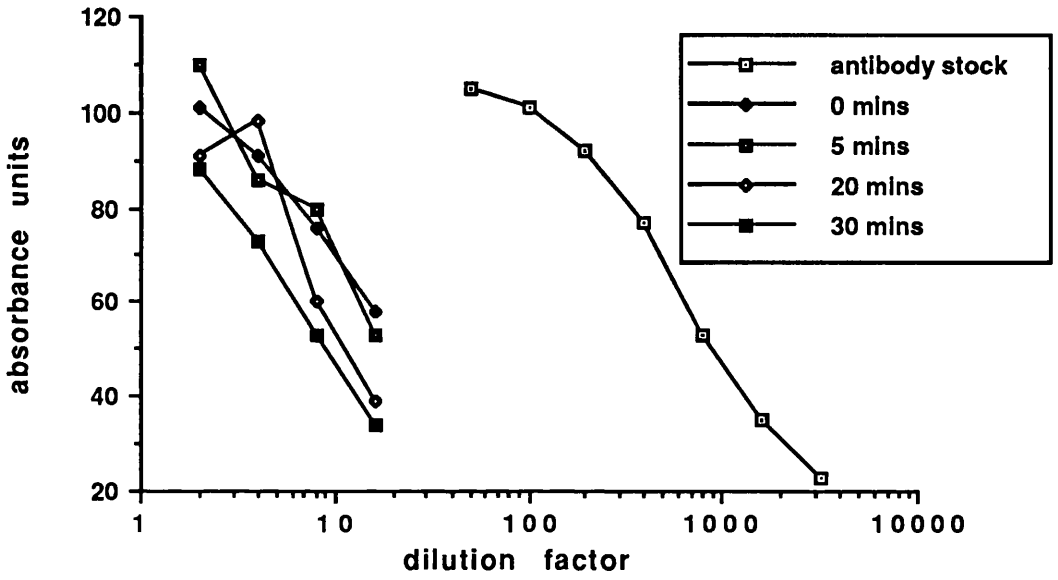

Figure 11.3. Titration curves of plasma from 1 animal receiving 1 mg/kg of MoAb MHM23, and the antibody stock (1 mg/mL). Calculated plasma concentrations of MHM23 at 0, 5, 20 and 30 minutes after GM-CSF was administered are 20, 16, 13.2 and 11 μg/mL respectively.

APPENDIX 3: CALIBRATION OF MTT ASSAY OF CELL PROLIFERATION

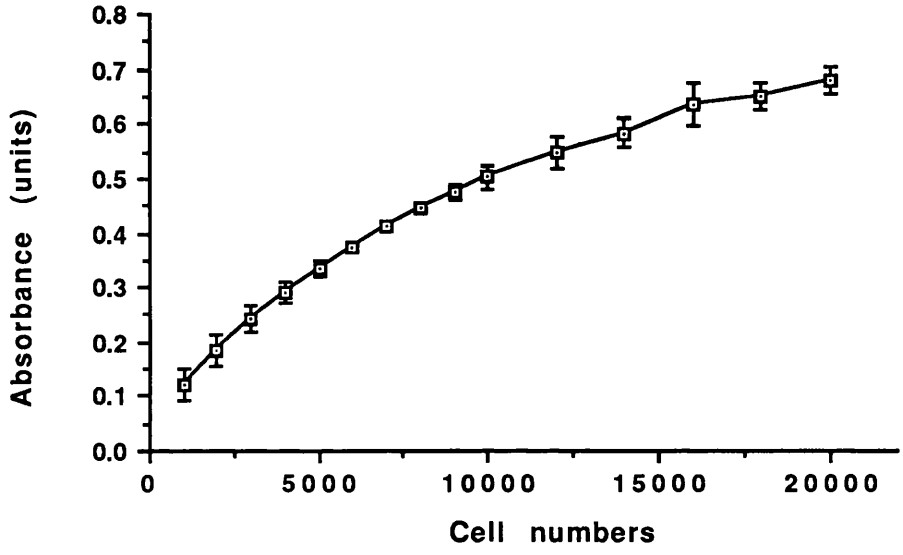

Figure 11.4. Calibration of absorbance units against cell numbers in the MTT assay. Known cell numbers were seeded in 96-well microtitre plates, and left for 2 hours for cells to adhere. Wells were rinsed, and on half of each plate, cells were detached by trypsin-EDTA, and quantitated. The number of cells recovered was within 5-10% of the initial number seeded. On the other half of the plate, an MTT assay was carried out as detailed in Chapter 9. Mean ± SD absorbance units of 3 experiments are plotted against cell numbers.

APPENDIX 4 : BINDING CHARACTERISTICS AND BIOLOGICAL ACTIVITY OF ^{125}I-GM-CSF

METHODS

Binding of ^{125}I-GM-CSF to neutrophils

A self-displacement technique was used to determine the binding capacity and characteristics of iodinated GM-CSF (Calvo et al, 1983). Briefly, the maximal binding capacity of ^{125}I-GM-CSF was initially determined by equilibrating separate aliquots of freshly purified neutrophils (1.5 X 10^6 cells in 200 µL) with approximately 200 000 cpm of labelled GM-CSF for 30 minutes at 37°C. Cells were pelleted by centrifugation and the supernatant was used for the next cycle of binding. 4 cycles were performed, and the radioactivity determined in the pellets and supernatant at each cycle. Nonspecific binding was obtained from the radioactivity associated with the cell pellet in the presence of excess cold GM-CSF. Percentage cpm left in the supernatant at each cycle (100% at the first cycle) was plotted against percentage specific binding, and the reciprocal of the ordinate gives the maximal binding capacity (Figure 11.5).

For self displacement analysis, 2 sets of experiments were performed. In 1 set, separate aliquots of purified neutrophils were equilibrated with 5 µL of ^{125}I-GM-CSF and different quantities of cold GM-CSF (50-1000 pg) in a final volume of 200 µL, for 2 hours at room temperature. In a second set of experiments, neutrophils were equilibrated with different concentrations of ^{125}I-GM-CSF. For both sets of experiments, non-specific binding was assayed in parallel incubations containing 5 µg of cold GM-CSF. At the end of the reaction, the incubation mixture was layered onto foetal calf serum, centrifuged at 7000g for 60 seconds, and snap frozen in liquid nitrogen. The associated radioactivity of the cell pellet was measured in an automated gamma counter (LKB, Milton Keynes, U.K.). The ratio of bound/free radioactivity was calculated for each set of experiments, and plotted against concentration of cold GM-CSF added (for the first set of experiments) or cpm added (for the second set) (Figure 11.3). The activity of the iodinated product can then be determined by comparing the 2 displacement curves.

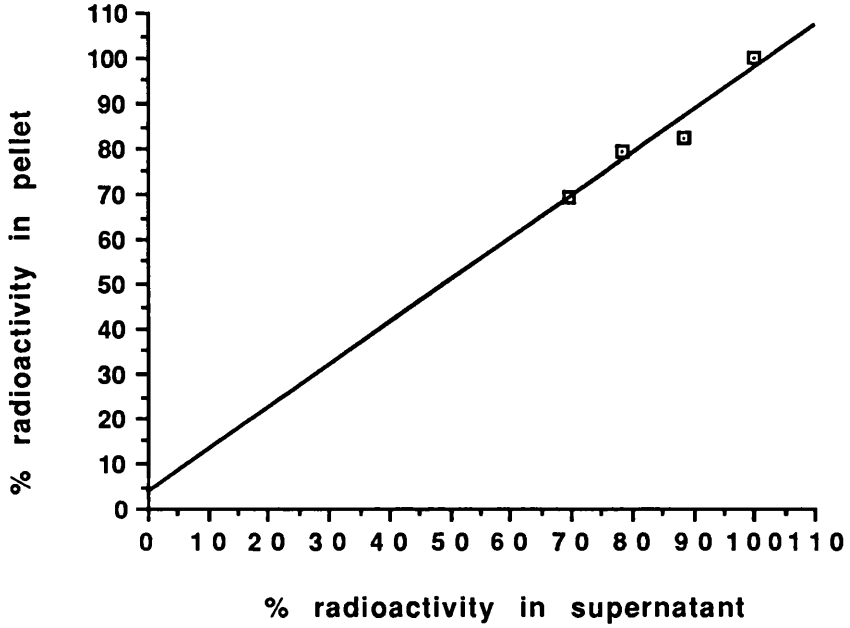

Figure 11.5. Maximal binding capacity of ^{125}I-GM-CSF, as assessed in 1 experiment, where it approximated 100% (reciprocal of the ordinate).

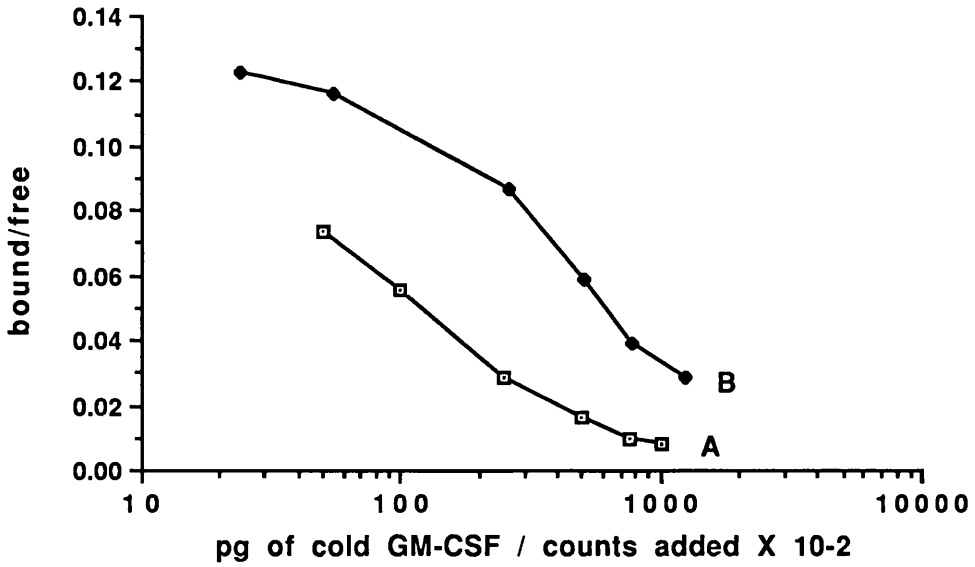

Figure 11.6. Self-displacment curves showing the effect of adding increasing amounts of cold GM-CSF (**A**) or ^{125}I-GM-CSF (**B**) on the specific binding of the iodinated product.

Respiratory burst assay.

Neutrophil H_2O_2 production was measured in a whole blood assay using 2'7'dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR) as previously described (Jaswon et al 1990). Heparinised venous blood was incubated with 100 μmol/L of DCF-DA for 15 minutes at 37°C, varying concentrations of unlabelled GM-CSF, ^{125}I-GM-CSF, or diluent as control was added, and incubation continued for a further 45 minutes. Following this, FMLP, or diluent as control, was added and incubation continued for 10 minutes at 37°C. After stimulation, samples were placed on ice, and processed using the Coulter Immunoprep Workstation, as detailed in Chapter 2 (section 2.2.3 Leucocyte Phenotyping). Analysis was performed by flow cytometry using the Coulter EPICS-C flow cytometer, the neutrophils being selectively gated on their light scattering properties. The percentage of responding cells was assessed by setting the diluent control to approximately 5% positive. An estimate of total H_2O_2 production was made for each sample by taking the product of the percentage of responding cells and their mean cell fluorescence (measured on a linear scale) and is described in arbitrary units.

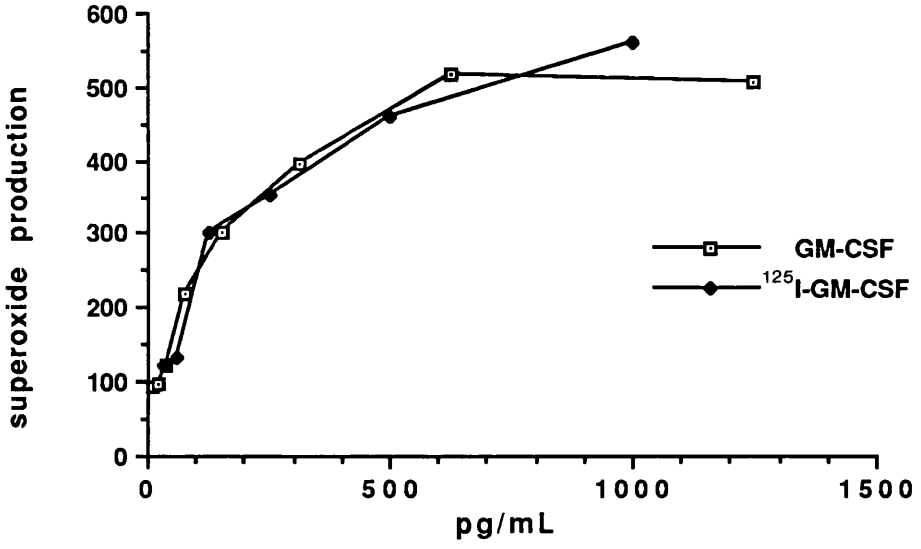

Figure 11.7. The dose response curves of unlabelled GM-CSF and the iodinated product, ^{125}I-GM-CSF, on the priming of FMLP-stimulated neutrophil respiratory burst are virtually identical.

PUBLICATIONS AND SUBMISSSIONS ARISING FROM WORK PRESENTED IN THIS THESIS

1. **K Yong**, IE Addison, B Johnson, ADB Webster, DC LInch (1991) Role of leucocyte integrins in phagocyte responses to granulocyte-macrophage colony-stimulating factor (GM-CSF): In vitro and in vivo studies on leucocyte adhesion deficiency neutrophils. British Journal of Haematology 77:150-157

2. **K Yong**, H Cohen, A Khwaja, HM Jones, DC Linch (1991) Lack of effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) on cultured endothelial cells. Blood 77:1675-1680

3. A Khwaja, B Johnson, IE Addison, **K Yong**, K Ruthven, S Abramson, DC Linch (1991) In vivo effects of macrophage colony-stimulating factor on human monocyte function. British Journal of Haematology 77:25-31.

4. **K Yong**, PM Rowles, K Patterson, DC Linch (1992) Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces neutrophil adhesion to pulmonary vascular endothelium in vivo: Role of leucocyte integrins. Blood (in press)

5. **K Yong**, DC Linch (1992) Differential effects of granulocyte and granulocyte-macrophage colony-stimulating factor (G- and GM-CSF) on neutrophil adhesion in vitro and in vivo. European Journal of Haematology (in press)

6. **K Yong**, DC Linch. GM-CSF differentially regulates neutrophil migration across IL-1 activated, and nonactivated, human endothelium (submitted for publication)

7. **K Yong**, PM Rowles, DC LInch. Neutrophils migrating across cultured human endothelium in vitro express low levels of L-selectin (submitted for publication)

PUBICATIONS AND SUBMISSIONS ARISING FROM OTHER WORK DURING PERIOD OF STUDY

1. **K Yong**, A Khwaja (1990) Leucocyte Adhesion Molecules. Blood Reviews 4:211-225

2. A Khwaja, PJ Roberts, HM Jones, **K Yong**, MS Jaswon, DC Linch (1990) Isoquinoline-sulphonamide protein kinase inhibitors H7 and H8 enhance the effects of GM-CSF on neutrophil function and inhibit GM-CSF receptor internalisation. Blood 75: 996-1003.

3. A Khwaja, **K Yong**, HM Jones, R Chopra, AK McMillan, AH Goldstone, K Patterson, C Matheson, K Ruthven, SB Abramson, DC Linch (1991) The effects of macrophage colony-stimulating factor on haemopoietic recovery after autologous bone marrow transplantation. British Journal of Haematology 81:288-295

4. AP Jewell, **K Yong**, CP Worman, FJ Giles, AH Goldstone, PD Lydyard (1992) Cytokine induction of LAM-1 on CLL cells. Leukaemia 6:400-404

5. **K Yong**, N Salooja, UM Hegde, RE Donahue, DC Linch (1992) Human macrophage colony-stimulating factor (M-CSF) levels are raised in pregnancy and in immune thrombocytopenia. Blood (in press)

6. PM Roberts, **K Yong**, A Khwaja, B Johnson, A Pizzey, JE Carver, IE Addison, DC Linch (1992) Pentoxyfilline, at clinically achievable levels inhibits FMLP-induced neutrophil responses, but not priming, upregulation of cell adhesion molecules, or migration induced by GM-CSF. European Journal of Haematology (in press)

7. AP Jewell, **K Yong**, CP Worman, AH Goldstone, PD Lydyard. Differential adhesion of lymphocytes to human high endothelium and umbilical vein endothelium in vitro (submitted for publication)

8. AP Jewell, **K Yong**, CP Worman, FJ Giles, AH Goldstone, PD Lydyard. Interaction of B-CLL cells with cultured human endothelium: Effects of cytokines and the role of L-selectin. (submitted for publcation)