Granulocyte-Macrophage Colony-Stimulating Factor Induces Neutrophil Adhesion to Pulmonary Vascular Endothelium In Vivo: Role of β2 Integrins

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) causes upregulation of neutrophil surface CD11b/CD18 expression, and enhances the adhesion of neutrophils to cultured human endothelial cells in vitro. Systemic administration of GM-CSF results in a rapid, transient decrease in circulating phagocyte numbers. Using a nonhuman primate model (Cynomolgus), we provide histologic evidence that this transient leukopenia is associated with the margination of neutrophils in the pulmonary microcirculation. In four animals receiving 2 to 15 μg/kg recombinant human GM-CSF (rHGM-CSF), light microscopic sections of lung contained 38 ± 8, 17 ± 7, 21 ± 6, and 15 ± 8 (mean ± SD, n = 20) neutrophils within a graticule grid, as compared with two control animals receiving saline injections whose lung sections contained 2.1 ± 1.6 and 3.1 ± 2.1 (mean ± SD, n = 20) neutrophils within the same grid. Scanning electron microscopy shows activated leukocytes adherent to pulmonary vascular endothelium, but no morphologic evidence of endothelial damage, and no migration of cells into the extravascular space. Margination is associated with an increase in surface expression of CD11b/CD18 on circulating phagocytes, which could contribute to the adhesion to capillary endothelial cells, but CD11b/CD18 levels remain elevated even when margination is complete. In vitro, monoclonal antibodies (MoAbs) to CD18 and CD11b were able to inhibit neutrophil aggregation and adhesion to endothelium. FMLP-induced neutrophil aggregation was inhibited by 39.8% ± 11.5% and 48.8% ± 12.3%, respectively, by MoAbs to CD18 and CD11b (P < .0005, n = 4 for both); a similar effect was demonstrated on TPA-induced aggregation. MoAb CD18 reduced the adhesion of unstimulated neutrophils to endothelium by 44% (P < .01, n = 7), and inhibited the amount of GM-CSF-stimulated adhesion by 74% (P < .001, n = 7), while MoAb to CD11b produced a reduction of unstimulated neutrophil adhesion by 30%, and of GM-CSF–stimulated adhesion by 40% (P < .01, n = 5, for both). However, when administered in vivo, MoAb CD18 produced only a small, albeit significant, amelioration of GM-CSF–induced margination in vivo, while MoAb CD11b was without effect. These results show that GM-CSF–induced transient leukopenia is associated with enhanced neutrophil adherence to pulmonary vascular endothelium, but suggest that the β2 leukocyte integrins CD11/CD18 play only a minor role in this process.

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neutrophil activation by a variety of agonists, including GM-CSF, leads to the release of intracellular stores of CD11b/CD11c, resulting in rapid upregulation of surface expression of these molecules. Similarly, surface expression of CD11b on circulating neutrophils increases rapidly during GM-CSF administration, and this may be related to the phagocyte margination. However, levels of this molecule remain high on cells that have demarginated and returned into the peripheral circulation. Hence, the relevance of changes in surface expression of these molecules to the observed margination of neutrophils is unclear.

We therefore set out to define the effects of GM-CSF on the adhesion of neutrophils to endothelium in vivo, using a nonhuman primate model so as to allow tissue analysis. We have used this primate model, as well as an in vitro system using cultured human endothelial cells, to study the mechanisms underlying the adhesive responses of neutrophils to GM-CSF, focusing on the role played by the β2 leukocyte integrins.

**MATERIALS AND METHODS**

**Materials**

Recombinant human GM-CSF (rhGM-CSF) was provided by Hoechst (Hounslow, Middlesex, UK).

Antibodies. Antibody 44 (anti-CD11b) was a gift from Dr N Hogg (ICRF, London, UK). MHH 23 ascites (anti-CD18) was a gift from Dr A McMichael (Oxford, UK). Fluorescein-conjugated rabbit antimouse Ig (FITC-RAM) was obtained from Dako Ltd (Highwickham, UK). Antibodies were purified from ascites fluid using a protein A column, and concentrated by dialyzing against phosphate-buffered saline (PBS). The protein concentration of the final stock was determined by measuring the absorbance of light at 280 nm, and various titrations of the antibody were incubated with purified neutrophils to determine the saturating concentrations.

Others. Formyl-methionyl-leucyl-phenylalanine (FMLP), 12-0-tetradecanoylphorbol 13-acetate (TPA), and endothelial cell growth supplement (ECGS) were obtained from Sigma (Poole, Dorset, UK). PBS, RPMI, Iscove's Modified Dulbecco's Medium (Iscove’s MDM), penicillin/streptomycin, and fetal calf serum (FCS) were obtained from Gibco (Paisley, UK). Trypsin/ethylenediaminetetraacetic acid (EDTA), fibroconnectin, and collagenase were obtained from Boehringer Mannheim (Mannheim, Germany).

**Cell Culture and Purification**

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cords by collagenase treatment (0.1% in PBS), and grown to confluence in fibronectin (2 μg/cm²)-coated Falcon tissue culture flasks in Iscove’s MDM with 20% FCS, ECGS (50 μg/mL), and heparin (50 U/mL). The cells were passaged using trypsin (0.05%) EDTA (0.01%) in PBS, seeded in 96-well fibronectin-coated tissue culture plates (Nunc Life Technologies, Paisley, UK) at 2 × 10^5 cells/well, and grown to confluence (5 to 7 days) for adherence experiments. Cells at passages 1 through 5 were used.

Neutrophils from normal human subjects were purified from heparinized citrated blood by Hetalast sedimentation and double density centrifugation (Histopaque 1119, Sigma Diagnostics, Lymphoprep; Nycomed, Birmingham, UK). The neutrophils obtained by this method were greater than 95% pure and greater than 99% viable by trypan blue exclusion.

**Leukocyte Deformability Studies**

Leukocyte suspensions were obtained by Hetalast sedimentation of heparinized venous blood from normal volunteers. An Erythrometre II (Lea Scientific, Milton Keynes, UK) was used to determine cell deformability. This instrument measures the pressure built up on the proximal side of a 5-μm Nucleopore filter (Costar, Highwickham, UK) across which leukocyte suspensions (1 × 10^6 leukocytes/mL in RPMI) are injected at a constant rate of 5 mL/min. This method was preferred to the measurement of flow rate under gravity as the latter is likely to be affected by cell adhesion to the membrane rather than simply reflecting changes in leukocyte deformability. Measurements were made after incubation of cell suspensions with GM-CSF or medium for 30 minutes at 37°C.

**Neutrophil Adhesion to Endothelial Monolayers**

Confluent HUVECs in microtiter plates were washed twice with RPMI at 37°C before being used in the adherence assay. One hundred microliters of purified neutrophils at 1 × 10^6/mL in RPMI with 5% FCS was added to each well with or without GM-CSF (final concentration, 100 ng/mL). In some experiments, neutrophils were incubated with varying concentrations of antibodies for 10 minutes at room temperature before addition to endothelial monolayers, and antibodies were present throughout the adhesion assay. At least 6 wells were used for each data point. After an incubation of 30 minutes at 37°C, unattached cells were removed by three washes of 300 μL exchanges of medium in the wells. At the end of the washing procedure, the plates were checked by microscopy to ensure that there was no disruption of the monolayer.

The number of adherent neutrophils was then quantified by measuring the optical density of the adherent cells with a MR 700 Microplate Reader (Dynatech, Billinghamurst, Sussex, UK). 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.

**Neutrophil Aggregation**

Neutrophil aggregation was determined using an aggregometer (Biodata Corp, PA). Three hundred microliters of neutrophil suspension (5 to 10 × 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. In some experiments, saturating amounts of antibodies were added at the beginning of the 10-minute preincubation, and were present throughout the experiment.

**Animals**

Adult Macaque fascicularis (family Cynomolgus) monkeys were used in the study. Anaesthesia was induced with Ketamine (Parke-Davis Medical, Eastleigh, Hampshire, UK) and maintained using propofol. Vascular access was achieved using a cut-down technique in the groin, and the femoral vein and artery were cannulated.
GM-CSF at 2 μg/kg or 15 μg/kg was administered by slow intravenous bolus injection over 5 minutes. Venous blood samples were drawn before starting the infusion, and at regular time intervals afterwards. Control animals received a bolus injection of saline in the same volume. In some animals, purified antibody in sterile saline was injected intravenously 10 to 20 minutes before the administration of GM-CSF.

In six animals, a technique of perfusion-fixation was used to preserve tissues for histologic 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 at 37°C, and flush-through continued as rapidly as possible. Asystole occurred within 1 to 2 minutes of commencing glutaraldehyde perfusion, which was performed for 20 to 30 minutes. Postmortems were performed on the animals and lungs and kidneys were removed and stored in glutaraldehyde.

Human Subjects

GM-CSF (30 μg/m² over 2 hours) was administered to three hematologically normal patients, two with non-Hodgkin’s lymphoma and one with Hodgkin’s disease. Venous blood was drawn at various times for blood counts and cellular adhesion molecule expression.

Peripheral Blood Counts

Blood counts were performed on an automated S-Plus STKR blood counter (Coulter Electronics, Luton, Bedfordshire, UK), and white blood differentials were performed manually on blood films stained with May-Grünwald-Giemsa.

Cellular Adhesion Molecule Expression

Heparinized venous blood from animals or normal human subjects was taken into polypropylene tubes and put immediately on ice. Staining of cell surface antigens was performed in whole blood to avoid activating cells by purification steps.34 One hundred microliters of whole blood was incubated with saturating concentrations of MoAb 44 (anti-CD11b MoAb) for 45 minutes, followed by three washes in RPMI with 2% FCS. The samples were then incubated for a further 45 minutes with FITC-RAM, and washed and again three times. All steps were performed at 4°C. The red blood cells were then lysed and the white blood cells fixed using an automated system (Coulter Q-prep Immuno Workstation) before analyzing on an EPICS CD flow cytometer (Coulter Electronics). 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 some experiments, heparinized blood samples from two animals not receiving infusions and from several normal human subjects were preincubated in vitro with GM-CSF 10 ng/mL or RPMI as control for 30 minutes at 37°C before antigen expression was measured by the release of preformed molecules from intracellular stores in secondary granules.36 Degranulation may involve changes in cytoskeletal organization and, hence, the mechanical properties of the cell. We therefore studied the effect of GM-CSF on leukocyte deformability using the Erythrometre II. Figure 2 shows that preincubation of

Effect of GM-CSF on Neutrophil CD11b Expression In Vitro

Whole blood samples from human subjects or monkeys were incubated with GM-CSF 10 ng/mL or RPMI as control for 30 minutes at 37°C before antigen expression was determined as outlined in Materials and Methods. After stimulation with GM-CSF, surface CD11b on human neutrophils increased to 238% ± 77% of control (P < .005, n = 10, Fig 1A). A similar effect of GM-CSF is seen on neutrophils from macaque monkeys, and Fig 1B shows the results of two experiments on separate animals in which, after preincubation with GM-CSF 10 ng/mL for 30 minutes at 37°C, CD11b expression increased to 184% and 140% of control, respectively.

Effect of GM-CSF on Leukocyte Deformability In Vitro

The upregulation of surface CD11/CD18 receptors occurs by the release of preformed molecules from intracellular stores in secondary granules.36 Degranulation may involve changes in cytoskeletal organization and, hence the mechanical properties of the cell. We therefore studied the effect of GM-CSF on neutrophil deformability using the Erythrometre II. Figure 2 shows that preincubation of...
leukocyte suspensions with GM-CSF increases the pressure readings in a dose-dependent manner, with maximal effect (approximately 150% of pressure in control) achieved at 1 ng/mL or greater. This effect was seen by 15 minutes of incubation with GM-CSF, and was sustained for up to 2 hours (data not shown). Control cells incubated with medium alone showed no change in pressure reading over this period.

Effect of MoAbs to the CD11b/CD18 Complex on Neutrophil Adhesion and Aggregation In Vitro

In initial experiments, the effect of GM-CSF on the adhesion of purified neutrophils to HUVECs was characterized. The adhesion of unstimulated human neutrophils to HUVECs is 13.8% ± 1.4% (mean ± SE, n = 7). GM-CSF (added to neutrophil suspensions at start of the adhesion assay) is able to increase the adherence of neutrophils in a dose-dependent manner, with maximal effects seen at concentrations of 10 ng/mL or greater (Fig 3A). This effect is evident (and often maximal) at 15 minutes and sustained for up to 2 hours (data not shown).

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, MHM23, or an isotype-matched leukocyte antibody as control for 10 minutes at room temperature before being added to endothelial monolayers with or without GM-CSF 100 ng/mL. After 30 minutes of incubation at 37°C, the percentage of adherent neutrophils was assessed as detailed in Materials and Methods. Figure 3B 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 < .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 < .01, n = 7) (by 44%) and 11.3% ± 2.6% (P < .001, n = 7) (a reduction in the amount of stimulated adherence of 74%), respectively (Fig 3B). Inhibition of GM-CSF–mediated neutrophil adhesion occurs in a dose-dependent manner, with maximal effect seen at antibody concentrations of 5 to 10 μg/mL or greater (Fig 3C). MoAb 44 (anti-CD11b, 10 μg/mL) had a similar, although lesser, effect on neutrophil adhesion, reducing unstimulated adherence to 9.7% ± 1.2% (by 30%) and decreasing GM-CSF–induced adherence to 17.9% ± 3.3% (by 40%, n = 5, P < .01 for both, Fig 3B). In view of the smaller inhibitory effect of this MoAb, two further experiments were performed on FMLP-stimulated neutrophil adherence. Figure 3D shows that MoAb 44 blocks both FMLP– and GM-CSF–mediated neutrophil adhesion in a dose-dependent way, with maximal inhibition seen at concentrations of 10 μg/mL or higher.

The effect of CD11b and CD18 MoAbs on neutrophil aggregation was studied using purified human neutrophils, stimulated with FMLP 10⁻⁶ mol/L, TPA 1 μg/mL, or GM-CSF 10 ng/mL. Both FMLP and TPA induced neutrophil aggregation, by 28.8% ± 10.6% and 47.8% ± 19.3% at 5 minutes, respectively (n = 4, Fig 4). Spontaneous aggregation in the absence of agonist was less than 5% at 3 minutes.
GM-CSF EFFECT ON NEUTROPHIL ADHESION IN VIVO

Fig 3. Effect of MoAbs to CD18 and CD11b on GM-CSF–induced neutrophil adhesion. (A) GM-CSF increases the percentage of neutrophils adhering to HUVECs in a dose-dependent manner; data are the mean ± SE of four experiments. (B) Effects of MoAbs to (■) CD11b (44) and to (□) CD18 (MHM23) on unstimulated and GM-CSF–induced neutrophil adhesion to HUVECs. Data are the mean ± SE of five experiments with MoAb 44 and seven experiments with MoAb MHM23. (■) Control. *P < .01; **P < .001. (C) MoAb MHM23 blocks GM-CSF–induced adhesion in a dose-dependent manner. Data are the mean ± SD of six replicate wells in one experiment representative of two. (D) MoAb 44 blocks GM-CSF– and FMLP–induced adhesion in a dose-dependent manner. Data are mean ± SD of six replicate wells in one experiment representative of three.

Fig 4. Effect of MoAbs 44 and MHM23 on neutrophil aggregation in response to FMLP and TPA in vitro. MoAbs were added to suspensions of human neutrophils 10 minutes before the start of an assay, and were present throughout. Data are expressed as mean ± SD for four experiments in each. (■) Control; (□) CD11b; (△) CD18. *P < .05; **P < .01.

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 (Fig 4). 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 < .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 < .001, n = 4) and of 42.8% ± 23.0% (P < .05, n = 4) by anti-CD18.

GM-CSF was found to be a very weak stimulator of neutrophil aggregation; in five of six experiments, there was no aggregation in response to GM-CSF (1 ng/mL to 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 GM-CSF on Neutrophil Counts and CD11b Expression In Vivo

Figure 5A shows these effects in humans. Within 15 minutes of starting a 2-hour infusion of GM-CSF (30
Adherence

are shown for three hematologically normal human subjects in (A), and for four animals in or 15 pg/kg) leads to a rapid (within 5 minutes) decrease in macaque monkeys, a bolus injection of GM-CSF (2 pg/kg to increase, as has been reported previo~sly. Similarly, in circulating neutrophils and an increase in neutrophil CD11b expression (Fig 5B). Figure 5A shows that neutrophil CD11b expression continues to increase as the cells gradually return into the peripheral circulation, and remains high at 2 hours, despite the fact that 50% of the neutrophils have demargined. A similar dissociation between the demargination of cells and the continued high expression of CD11b is also seen in the monkey.

Adherence of Neutrophils in the Microcirculation

Histologic 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 (Fig 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 (Fig 6B). The paucity of red blood cells in these sections suggests that the flush-through was complete, and therefore the leukocytes seen remaining in the microcirculation are adherent to endothelium. Figure 7 shows the number of neutrophils counted in lung sections taken from each of six animals, four of which received GM-CSF and the other two received control bolus injections of saline. Lung sections from animals receiving GM-CSF contained 36 ± 8, 17 ± 7, 21 ± 6, and 15 ± 8 (mean ± SD, n = 20) neutrophils, counted within a graticule grid, per microscopic field viewed under a ×40 objective, as compared with the two 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. Twenty fields, 10 from a section of each lung, were counted for each animal by each observer. Scanning electron microscopy shows leukocytes within alveolar capillaries (Fig 6C), and also adherent to the endothelial surface in larger vessels (Fig 6D). The adherent cells show membrane ruffling, but there is little morphologic evidence of endothelial cell damage. No neutrophils were seen to be migrating out of the circulation into the alveolar spaces.

Effect of GM-CSF on Fetal Heart Rate

One animal who received GM-CSF at 15 µg/kg was pregnant, and fetal 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 fetal bradycardia that lasted 5 to 10 minutes (Fig 8). Maternal heart rate showed no change.

Effect of CD11b and CD18 MoAbs on GM-CSF–Induced Neutrophil Adhesion In Vivo

To assess the effect of antibodies to the CD11b/CD18 receptor complex on GM-CSF–induced margination in vivo, purified antibody in sterile saline was injected intravenously into macaque monkeys 20 minutes before GM-CSF (2 µg/kg) administration. MoAb 44 was administered to three separate animals, at doses of 0.35, 0.46, and 1.0 mg/kg. Plasma antibody concentrations were determined as described immediately before and up to 40 minutes after GM-CSF administration. In the animal that received the lowest dose of antibody, antibody concentration in plasma was 5 µg/mL, whereas in the other two animals that received higher doses, antibody levels were between 10 and 20 µg/mL, 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 (Fig 9A). MoAb MHM23 was administered to two animals, at 1.2 and 1.0 mg/kg, doses that achieved plasma levels of 15 to 20 µg/mL. Unlike MoAb 44, which had no immediate direct effect on neutrophil numbers, MHM23 itself produced a decrease in the neutrophil count. However, although administration of the MoAb did not prevent the occurrence of a transient leukopenia after the injection of GM-CSF, in both animals the nadir count was higher than in control animals (Fig 9B).

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,
Fig 6. GM-CSF induces neutrophil retention in the pulmonary vasculature. Animals were perfused 30 minutes after receiving GM-CSF or saline as control, and tissues were fixed for histologic examination. Hematoxylin-eosin-stained lung sections (original magnification ×750) of (A) an animal receiving GM-CSF and (B) a control animal receiving saline. Scanning electron micrographs (C and D) of lung tissue from an animal which received GM-CSF show activated leukocytes adhering to endothelium.

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.

DISCUSSION

GM-CSF has been shown to increase neutrophil expression of the leukocyte integrin receptor, CD11b/CD18, both in vitro and in vivo, in humans. In these studies, we have extended these findings to a nonhuman primate model, and have used this model to define GM-CSF-mediated neutrophil adhesion in vivo, and to explore the possible mechanisms responsible. We show that the transient leukopenia after in vivo administration of GM-CSF occurs in other primates as well as in humans, and is due to the margination of cells in the pulmonary circulation. We have shown histologically that these neutrophils adhere to the pulmonary vascular endothelium, and show signs of activation, as evidenced by membrane ruffling. However, we have not found morphologic evidence of endothelial damage, or of marginated neutrophils leaving the circulation. Our histologic findings in primates receiving GM-CSF injections are similar to those reported by investigators who infused zymosan-activated plasma into sheep, who also showed, using serial lung biopsies, that the rebound in circulating counts is associated with the loss of granulocytes from lung tissue.

To define the role of the β2 leukocyte integrins in the adhesive responses of neutrophils to GM-CSF, we conducted both in vitro and in vivo studies. 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 from our laboratory using radiolabeled neutrophils. MoAb 44 (anti-CD11b) was able to block GM-CSF-induced adhesion in vitro by 40%, and reduce to the adhesion of unstimulated cells by 30%. This greater inhibitory effect of MoAb to the β2-subunit when compared with MoAb to CD11b on the adherence of activated neutrophils to endothelium in vitro is in accord with reports by other investigators. 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 shown by others.\textsuperscript{23,38,39} In our system, neutrophils did not reliably demonstrate an aggregation response to GM-CSF. Many studies of neutrophil aggregation have used cytochalasin B to enhance the response to agonists. We have not used this agent, as we were concerned to avoid nonphysiologic conditions.

The data from our in vivo studies were broadly in keeping with these in vitro results. CD18 produced a small but significant amelioration of GM-CSF–induced neutropenia, whereas CD11b was without effect. This difference between the inhibitory effects of MoAbs CD11b and CD18 on cytokine-induced leukocyte-endothelial adherence in vivo was also reported in a rabbit microcirculation model using C5a as a proadhesive agonist.\textsuperscript{40} 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 B2 integrins do not represent the major adhesive mechanism underlying GM-CSF–induced neutrophil adherence to pulmonary endothelium as shown in vivo. In keeping with these observations, Lundberg and Wright\textsuperscript{41} found that pretreatment of rabbits with anti-CD18 MoAb did not prevent neutrophil margination in lungs after systemic injection of FMLP. Other investigators have found that anti-CD18 MoAb, while inhibiting the emigration of neutrophils into inflammatory areas, did not prevent the rolling of leukocytes along vessel walls.\textsuperscript{42} The dissociation between the time course of neutrophil margination and the kinetics of the in vivo increase in CD11b/CD18 expression on the cells shown in these studies has already been commented in a previous report,\textsuperscript{29} and provides further evidence that these receptors are not solely responsible for
cytokine-induced phagocyte margination in vivo. The sur-
face levels of CD11b/CD18 remain high at 2 hours, when
50% of the cells have returned into the peripheral circula-
tion, as shown here and in previous studies. Even at 4
hours, when the neutrophil count has completely recov-
ered, circulating neutrophils still express high levels of the
adhesion molecule (data not shown). Qualitative, rather
than quantitative changes in these surface molecules may
be important in mediating GM-CSF-induced margin-
ation. However, this is unlikely as neutrophils from a
patient with partial leukocyte adhesion deficiency, which
expressed less than 10% of normal levels of CD11b,
demonstrated normal margination response to GM-CSF in
vivo.45

The contribution of the β2 integrins to the interactions of
neutrophils with endothelium also appears to be dependent
on the rate of flow in the vessel. Recent in vitro studies
have shown that, under shear stresses comparable to those
estimated to exist in postcapillary venules in vivo (1 to 10
dyne/cm²) where margination occurs, the interactions of
neutrophils with endothelium is largely β2 integrin-
independent, but is dependent on the selectin family of
adhesion receptors, member of which, leukocyte
adhesion molecule-1 (LAM-1), has been reported to partici-
pate in neutrophil-endothelial interactions, and neutrophil
evacuation in inflammation. The β2 integrins, which
mediate the binding of activated neutrophils to endothel-
ium, appear to bind only under extremely slow flow rates
(0.5 dyne/cm²) or static conditions. The sustained
enhanced adherence of GM-CSF-stimulated neutrophils
to endothelial cells in vitro (see above) contrasts with the
transient nature of the margination response seen in vivo,
and lends support to the notion 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 neutro-
phils with endothelium in lungs after GM-CSF infusion in
vivo. This would account for the greater inhibitory effect of
MoAbs to CD11b/CD18 on neutrophil-endothelial adher-
ence in vitro than that shown on margination in vivo.

These observations suggest that the pulmonary sequestra-
tion 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, after
which the β2 integrins might contribute by strengthening
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. Neutrophil stimula-
tion can produce changes in the cytoskeleton, leading to
cell stiffening, and greater retention in 5-μm pores in
vitro. A diminished ability of cells to deform during transit
through capillaries would reduce flow rates, and lead to
the retention of cells in the lungs. Our finding that GM-CSF
reduces leukocyte deformability in vitro is in agreement
with these ideas. A decrease in flow rates might allow the
engagement of adhesion receptors, thus leading to actual

neutrophil-endothelial adhesion which is shear stress resis-
tant. In support of this last theory is our finding, on
scanning electron microscopy, that leukocytes adhere to the
endothelium in large vessels within the lungs of animals
that have received GM-CSF, where such cells are not
obviously restrained by an inability to deform (Fig 6C).

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 dyspnea, hypoxemia, and decreased carbon
monoxide diffusion capacity in 7% of patients receiving 15
μg/kg of GM-CSF daily by subcutaneous injection. The
relation of these effects to the dramatic increase in the
number of neutrophils in the pulmonary microcirculation
as shown in our studies is unclear. The onset of leukopenia
preceded the decrease in oxygen saturation. Furthermore,
transient leukopenia occurred on subsequent days of GM-
CSF administration, and larger numbers of cells left the
circulation, but hypoxia did not occur. However, the de-
crease in lung diffusion capacity for carbon monoxide
paralleled the GM-CSF–induced leukopenia, and also re-
curred with subsequent leukopenic episodes.

Apart from altering gas exchange, the enhanced adher-
ence 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 pathologic states such as
adult respiratory distress syndrome (ARDS) and hemolytic
uremic syndrome (HUS). We have not found any
morphologic evidence of endothelial damage, at least at up
to 2 hours after GM-CSF administration. This contrasts
with the findings of Meyrick and Brigham, who, studying
the margination response to an injection of zymosan-
activated plasma in sheep, demonstrated transient endothe-
damage and leukocyte migration across endothelium. In
the context of GM-CSF administration, other para-
ters of endothelial perturbation/damage need to be ex-
plored, such as an alteration in hemostatic and fibrinolytic
activities, which may be relevant to the reported side effects
of central venous thrombosis and the capillary leak syn-
drome seen at high doses of this growth factor.

Finally, an unexpected clinical effect of the growth factor
was noted in a pregnant animal that received a relatively
high dose of 15 μg/kg. The transient fetal bradycardia
reported here suggests that GM-CSF should not be admin-
istered in pregnancy. The mechanisms responsible for this
effect are not clear. After maternal administration of
GM-CSF, sequestration of neutrophils may occur in the
placental circulation, in a similar manner to that occurring
in the lungs. Momentary placental insufficiency could then
give rise to the fetal distress. Alternatively, transient mater-
nal hypoxemia, although asymptomatic in the mother,
could have caused the fetal distress.

In conclusion, we have demonstrated, using a nonhuman
primate model, that GM-CSF enhances neutrophil adher-
ence to pulmonary vascular endothelium in vivo, giving rise
to a transient neutropenia. Our studies suggest that the role
of the β2 integrins in this process is minor, and that other adhesive, or nonadhesive, mechanisms are important. We have also shown that, despite the greatly increased numbers of adherent neutrophils, the pulmonary endothelium appears intact, and no leukocytes are seen to be migrating out of the vessels into the tissues.

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