The Role of Neutrophil Membrane Glycoprotein GP-150 in Neutrophil Adherence to Endothelium In Vitro


We have previously described two patients with a congenital defect in neutrophil function characterized by an inability to form pus. The patients' neutrophils lack a membrane glycoprotein of mol wt 150,000 daltons (GP-150) on analysis by SDS-PAGE. This glycoprotein is part of a membrane antigen complex recognized by the murine monoclonal antibody (MoAb) 60.3. Addition of MoAb 60.3 to normal neutrophils produces defects in chemotaxis and phagocytosis in vitro similar to those observed in the patients. Since neutrophil adherence to vascular endothelium is prerequisite to neutrophil emigration in vivo, we examined the interaction of the patients' neutrophils and normal neutrophils treated with MoAb 60.3 with cultured endothelium. Adherence was determined as the percentage of 51Cr-labeled purified peripheral blood neutrophils which remained adherent to plastic wells or endothelial monolayers after a 45-minute incubation at 37°C. The percentage of neutrophils from patient 1 remaining adherent to uncoated, fibronectin-coated, or laminin-coated plastic was similar to that observed in normal neutrophils (55% to 84% adherence with normal neutrophils vs 73% to 78% adherence with the patient's neutrophils and 63% to 82% adherence with MoAb 60.3-treated normal neutrophils). The adherence of the neutrophils from patient 1 and MoAb 60.3-treated normal neutrophils to human or bovine endothelium in serum-free medium was also not significantly different from that observed in normal neutrophils (<10% adherence with normal, MoAb 60.3-treated, and patient neutrophils). In medium containing 10% autologous or heterologous human plasma, however, the adherence of neutrophils from patient 1 or MoAb 60.3-treated normal neutrophils to endothelial monolayers was significantly reduced (35% ± 7% of normal neutrophils in seven experiments). Although phorbol myristate acetate (PMA) (10 ng/mL) and calcium ionophore A23187 (10^-8 mol/L) markedly increased the adherence of normal neutrophils to endothelial monolayers in serum-free medium (40% to 85% adherence), neither agent increased the adherence of the neutrophils from patient 1 or normal neutrophils treated with MoAb 60.3 (<5% adherence). The adherence of PMA-activated neutrophils from patient 2 to endothelial monolayers was also markedly decreased when compared with that of normal neutrophils. Postsecretory cell-free supernatants from PMA-activated normal neutrophils failed to augment adherence of neutrophils from patient 1 (<5% adherence). However, when intact normal neutrophils were added to the patient's neutrophils, the patient's neutrophils adhered normally in the presence of PMA (70% to 80% adherence). The migration of neutrophils from patient 1 and normal neutrophils treated with MoAb 60.3 across endothelial monolayers on polycarbonate filters in response to formylmethionyl-leucyl-phenylalanine (FMLP) (10^-7 mol/L) was also markedly decreased compared to normal neutrophils (<30% of control neutrophils at 90 minutes). GP-150 was not detected in the postsecretory medium of PMA-activated normal neutrophils on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, activation of normal neutrophils with PMA increased binding of MoAb 60.3 and the inhibition of PMA-induced neutrophil adherence by MoAb 60.3 required continued presence of the MoAb during the incubation. These observations suggest that GP-150 is not a secreted protein but remains membrane-associated and that the new antigen expressed during activation is critical to increased adheriveness. We conclude that the neutrophil membrane glycoprotein GP-150 is required for augmented neutrophil adherence to and chemotaxis across endothelial monolayers in vitro. The inability of the patients' neutrophils to augment adherence to endothelium when activated may account for the failure of the patients' neutrophils to migrate to sites of inflammation in vivo.  

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recognized by the murine monoclonal antibody (MoAb) 60.3. Moreover, addition of MoAb 60.3 to normal neutrophils induces defects in neutrophil spreading and chemotaxis in vitro similar to those observed in the patients.\textsuperscript{4}

The in vitro defects in adherence and chemotaxis observed in the patients' neutrophils and normal neutrophils treated with MoAb 60.3 suggested that the membrane glycoprotein complex recognized by MoAb 60.3 may be necessary for normal neutrophil interaction with the endothelium and thereby account for the failure of the glycoprotein-deficient neutrophils to migrate from the vascular space into infected tissue. In order to investigate this possibility, we have examined the interaction of the patients' neutrophils and MoAb 60.3-treated normal neutrophils with cultured human and bovine endothelial cells.

MATERIALS AND METHODS

Endothelial cells. Bovine aortic and pulmonary artery and human umbilical vein endothelial cells were prepared by collagenase treatment of vessels as previously described\textsuperscript{16} and maintained in 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, Calif) in Waymouth's 752/1 medium (GIBCO, Grand Island, NY). Bovine endothelial cells were used in the sixth through 15th passages. Individual experiments were performed with bovine endothelial cells of the same strain and passage number. Human umbilical vein endothelial cells were used in the first passage only.

Preparation of neutrophils and postsecretory supernatants. Peripheral blood was obtained from normal healthy volunteers or the patients with informed consent in accordance with an institutionally approved protocol. The peripheral blood was obtained by venipuncture in syringes containing heparin (20 U/mL), layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 400 g for 40 minutes at 25 °C. Plasma and mononuclear cells were separated. The red blood cells were sedimented by 37 °C and the purified neutrophils were resuspended in 0.5% NP-40 in PBS with 2 mmol/L PMSF and incubated with cytochalasin B 5 μg/mL for five minutes. PMA (10 ng/mL final) or PBS were then added for a 30-minute incubation at 37 °C. The cells were pelleted by centrifugation at 11,000 g for three minutes, and the cell-free supernatant medium was decanted and frozen at −80 °C. Equal aliquots of the cell-free, postsecretory supernatant medium and detergent extracts of the cell pellet were analyzed by SDS-PAGE as described above.

Monoclonal antibodies. The MoAb designated 60.3 is an IgG\textsubscript{\textalpha} antibody, which recognizes a multimeric cell-surface antigen complex which on radioimmunoprecipitation of detergent extracts of [\textsuperscript{125}I]-lactoperoxidase surface-labeled neutrophils consists of major polypeptides of apparent mol wt of 95,000, 130,000, and 150,000 daltons under reducing conditions.\textsuperscript{11} This antigen is found on all human peripheral blood B and T lymphocytes, monocytes, and polymorphonuclear leukocytes but not on red blood cells, platelets, or cultured endothelial cells (J.M.H., unpublished observations, June 1984). MoAb 60.1 is an IgG antibody that recognizes a cell surface antigen complex consisting of polypeptides of mol wt 95,000 and 150,000 daltons (M. Braun and P.G.B, unpublished observations, March 1984).

The following control murine monoclonal antibodies were provided by Dr John Hansen, Fred Hutchinson Cancer Center and Puget Sound Blood Bank, Seattle. MoAb 9E8 recognizes a mouse viral coat protein not present on human leukocytes.\textsuperscript{12} MoAb 60.5 recognizes an HLA class I framework antigen present on all peripheral blood leukocytes. MoAb 1G10 was provided as ascites fluid by Dr Irwin Bernstein, Fred Hutchinson Cancer Center and Department of Pediatrics, University of Washington, Seattle. This MoAb is an IgM antibody that recognizes a differentiation antigen consisting of a membrane glycosphingolipid bearing the "X determinant." This antigen is present in high density on human peripheral blood granulocytes.\textsuperscript{13,14} MoAbs 9E8, 60.3, 60.1, and 60.5 were purified by solid-phase absorption on a staphylococcal protein A column\textsuperscript{14} and the concentration of purified antibody determined by Bradford assay.\textsuperscript{15} The saturating concentration of each MoAb for purified peripheral blood neutrophils was determined by fluorescence-activated cell sorter as previously described.\textsuperscript{12}

Solid-phase binding assay. A solid-phase binding assay of 0.5% NP-40 extracts of patient and control neutrophils was performed in a modification of the procedure described by Skubitz et al.\textsuperscript{16} Twenty-five-microliter aliquots of NP-40 extracts from patient and control neutrophils containing equal amounts of protein (40 μg) diluted in PBS containing 0.02% NaN\textsubscript{4} were dried overnight at 37 °C in 96-well microtiter dishes (Linbro, Flow Laboratories, Inc, Hamden, Conn). The wells were then incubated with 200 μL of 5% bovine serum albumin (BSA) in PBS with 0.02% NaN\textsubscript{4} for two hours at 37 °C. The supernatant medium was decanted, and the monoclonal antibodies diluted in PBS were added at concentrations previously determined to be saturating in normal neutrophil extracts. After a one-hour incubation at 4 °C, the wells were washed four times with 1% BSA in PBS containing 0.1% Triton X-100 (New England
Nuclear, Boston). The wells were then incubated with 100 μL of PBS containing 50 nCi of ¹²⁵I-labeled F(ab')₂, sheep anti-mouse Ig (2 to 10 μCi/μg, New England Nuclear) for one hour at 4 °C. The wells were then washed four times with 1% BSA in PBS with 0.1% Triton X-100 and incubated with 100 μL of 2 mol/L NaOH for 60 minutes at 37 °C. The solution was then decanted and counted in a gamma spectrophotometer. Specific binding of the test MoAb was determined by subtracting nonspecific binding observed in wells incubated with a saturating concentration of the irrelevant MoAb (9E8).

Suspension binding assay and immunofluorescence analysis. Neutrophils were suspended in cold HBSS without calcium or magnesium (GIBCO) at 2 x 10⁹ per microliter. Then 50 μL of this suspension was transferred to individual polypropylene microfuge tubes, and 50 μL of a saturating concentration of MoAb in HBSS without calcium and magnesium was added. One hundred microliters of HBSS containing 0.006 mol/L calcium and 0.002 mol/L magnesium with or without PMA (200 ng/mL) or A23187 (2 μmol/L) were then added, and the suspension was incubated at 37 °C for ten minutes. The cell suspension was then fixed with 200 μL of cold 0.5% paraformaldehyde in PBS.

For analysis of MoAb binding by radiolabeled second antibody, the cells were then washed twice in 0.15 mol/L NaCl with 0.1 mol/L Tris and 1 mmol/L PMSF pH 7.4. After washing, the cell pellet was resuspended in 50 μL of 0.15 mol/L NaCl-Tris buffer containing a saturating concentration of ¹²⁵I-labeled F(ab')₂, sheep anti-mouse Ig (2 to 10 μCi/μg, New England Nuclear). After a one-hour incubation at 4 °C, the cells were washed five times in buffer to remove unbound ¹²⁵I-labeled F(ab')₂, pelleted, and the microfuge tube placed in a tube for counting in a gamma spectrophotometer.

Analysis of MoAb binding in resting and stimulated cells by immunofluorescent flow cytometry was performed as previously described. Preparation of plasma. Autologous plasma was obtained from each neutrophil donor by centrifugation of whole blood containing heparin (20 U/mL) at 150 g for ten minutes. The supernatant platelet-rich plasma was then centrifuged for ten minutes at 11,000 g at 4 °C to remove any residual platelets or cells.

Preparation of chemotaxis chambers. Twenty-five–millimeter diameter polycarbonate membranes with 2-μm pores (Nucleopore Corporation, Pleasanton, Calif) were used. The filters were glued with methacrylate (Woodhill Permatex, Cleveland) to polycarbonate rings (13-mm diameter, 9.5-mm inside diameter, 7-mm height), which had been cut from polycarbonate tubing (Universal Plastics, Seattle) by the Scientific Instruments Division of the University of Washington.

The filter and attached ring formed a well with an inner diameter of 9.5 mm. The wells were processed for cell culture by treatment with 6 N H₂SO₄ at 56 °C for 15 minutes followed by repeated rinses with distilled water. Sterilization was achieved by immersion in 70% ethanol for five minutes. Wells placed in multiwell plates (Costar, Cambridge, Mass), formed chambers with two compartments with a 9.5-mm inner well and 24-mm outer diameter. The filters were then placed on stainless steel pins (Clay Adams, Parsippany, NY) to facilitate mixing of solutes.

Endothelial cells were plated in the inner well at confluent density (2 x 10⁶ cells per cubic centimeter) and were refed every two to three days.

After three to five days, the endothelial monolayers on polycarbonate filters were tested for their ability to exclude macromolecules by adding horseradish peroxidase to the inner well and quantitating its appearance in the outer well. One microgram of horseradish peroxidase in 150 μL of medium (total volume) was placed in the inner well. One hundred-μL aliquots were removed from the outer compartment at half-hour intervals for determination of peroxidase activity. The medium in the outer compartment was replaced at each sampling by an equal volume of control medium. The chambers were mixed during testing on an Ames aliquot mixer (Division of Miles Laboratories, Elkhart, Ind). Similarly, prepared filters without endothelial cells were used to determine maximum passage of the tracer. Peroxidase activity was measured by determination of optical density after incubation with orthiodianisidine dihydrochloride (0.12 mg/mL) and hydrogen peroxide (0.003%) in 0.05 mol/L sodium phosphate buffer, pH 5.0. The reaction was stopped after five minutes by the addition of HCl to a final concentration of 0.6 N. Those monolayers excluding >90% of the peroxidase added to the inner well from reaching the outer well by one hour were selected for further use. Approximately three fourths of the endothelial monolayers on filters were usable by these criteria.

Preparation of coated wells. Sixteen-millimeter plastic wells (Costar Cluster 3524) were pretreated for two hours at 37 °C with Waymouth’s medium alone or medium containing 100 μg/mL of purified human plasma fibronectin (Collaborative Research, Waltham, Mass) or 20 μg/mL of purified laminin (Bethesda Research Laboratories, Bethesda, Md). The wells were then washed once with medium alone and used immediately in the adherence assay.

Microscopy. Filters for light and scanning electron microscopy were fixed with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer.

For scanning electron microscopy the filters were pinned onto Teflon sheets (Universal Plastics), dehydrated through increasing concentrations of ethyl alcohol, and critical point dried using liquid carbon dioxide. Segments of the filters were then adhered to scanning electron microscope stubs using sputum paste, coated with a layer of conductive heavy metal, and viewed in a JEOL (Tokyo) 35 C scanning electron microscope.

For light microscopy, the filters were embedded in Epon and 1-μm sections prepared.

Preparations of ⁵¹Cr-labeled neutrophils. Radiolabeled neutrophils were prepared according to the method of Gallin et al. Purified PMNs in PBS were incubated with ⁵¹Cr (as sodium chromate, 200 to 500 Ci/g, New England Nuclear) at 37 °C for one hour with frequent gentle agitation. Following incubation, unbound ⁵¹Cr was removed in a series of washes with PBS. Labeled neutrophils were resuspended in medium 199 (M199) (GIBCO) or Waymouth’s medium at a final concentration of 2 x 10⁶ per milliliter for use in the adherence and chemotaxis assays. Cell-associated ⁵¹Cr-cpm were consistently >90% of the total ⁵¹Cr-cpm.

Adherence assay. For the adherence assay, bovine or human endothelial cells were plated in 16-mm wells in 24-well plates (Costar Cluster 3524) in a 10% FCS in Waymouth’s medium at 10⁵ cells per well and grown to confluence. The plates were washed twice with serum-free M199 immediately before use in the adherence assay.

Neutrophil adherence was measured by adding 0.45 mL of the purified ⁵¹Cr-labeled neutrophil suspension (approximately 1 x 10⁶ neutrophils) to each well of the 24-well plate. Then 50 μL of plasma or activating agent was added to the endothelial monolayer immediately before plasma (or ten minutes after PMA, A23187, FMLP) the addition of the neutrophils. The wells were then gently agitated to allow adequate mixing and incubated at 37 °C for 45 minutes. After incubation, the supernatant medium and nonadherent ⁵¹Cr-labeled neutrophils were aspirated and the plate washed once in serum-free M199. Aspirated cells, supernatant medium, and the wash medium from individual wells were collected in counting vials and set aside. The endothelial monolayer and adherent ⁵¹Cr-labeled neutrophils were then lysed by addition of 0.5 mL of 1 N NH₄OH. After overnight incubation, the lyase from each well was aspirated and the wells were washed once with 0.5 mL of 1 N NH₄OH. The
pooled lysate and wash were then counted in a gamma spectrophotometer. Adherence was determined as the percentage of the total $^{51}$Cr-cpm added, as follows: % adherence = ($^{51}$Cr-cpm in lysate/total $^{51}$Cr-cpm added). Total $^{51}$Cr-cpm added represented $^{51}$Cr-cpm in medium, washes, and lysate and was consistently $\geq$95% of the total $^{51}$Cr-cpm added. Total $^{51}$Cr-cpm recovered varied between wells by $\pm$10%.

**Chemotaxis assay.** For the migration studies, the chemotaxis chambers were placed in 24-mm diameter wells in 12-well plates (Costar Cluster 3512) containing a second nitrocellulose filter (Nuclepore Corporation, Pleasanton, Calif) to trap the $^{3}H$-labeled neutrophils that migrated across the endothelial monolayer and polycarbonate filter (Fig 1). $^{3}H$-labeled neutrophils ($1 \times 10^6$ per milliliter) in Serumless (Neuman-Tytell medium, GIBCO) were added to the inner well of the chemotaxis chamber. The outer well contained medium alone or medium with FMLP $10^{-7}$ mol/L. After 90-minute incubation at 37 °C the nitrocellulose filter was removed and counted in a gamma spectrophotometer (Searle Analytic Inc, Division of GP Searle and Co, Des Plaines, Ill). Results are expressed as percent migration calculated as follows: % migration = ($^{51}$Cr-cpm recovered in lower filter/$^{51}$Cr-cpm added to inner well) × 100%.

**Reagents.** Horseradish peroxidase, orthoanisididine dihydrochloride, PMA, PMSF, FMLP, Nonidet P-40, cytochalasin B, and A23187 were obtained from Sigma Chemical Corp. St Louis. Molecular wt standards were obtained from Sigma Chemical Corp, La Jolla, Calif.

**RESULTS**

**SDS-PAGE and solid-phase binding assay of neutrophil extracts.** On analysis by SDS-PAGE NP-40 extracts of both patients' neutrophils lack a protein migrating with mol wt of 150,000 daltons (Fig 2). This protein was present in all normal neutrophils examined by SDS-PAGE on multiple determinations.

Detergent extracts of the patients' neutrophils also lack the antigen recognized by MoAb 60.3 when analyzed by solid-phase binding assay (Table 1). Again, this antigen was detectable by the solid-phase binding assay in all normal neutrophil extracts on multiple determinations.

**Neutrophil adherence to artificial surfaces.** The adherence of neutrophils from patient 1 to uncoated, fibronectin-coated, and laminin-coated tissue culture plastic (polystyrene) was similar to control neutrophils (Table 2). The adherence of normal neutrophils treated with MoAb 60.3 to uncoated plastic was only slightly reduced compared with normal neutrophils alone or normal neutrophils treated with MoAb 60.5 (Table 2). Although a significant fraction of the patient's neutrophils or MoAb 60.3-treated normal neutrophils adhered to the coated- and uncoated-plastic substrate, they failed to flatten and spread, as previously described.14

**Neutrophil adherence to endothelium.** The adherence of the neutrophils from patient 1 to bovine or human endothelial monolayers was similar to control when the incubation was performed in serum-free medium (Table 3). In previous experiments,20 we had observed that normal human plasma produced a somewhat variable, but always significant, increase in neutrophil adherence to endothelial monolayers when compared to serum-free medium (20% to 50% adherence in medium containing 10% autologous plasma $\leq$10% adherence in serum-free medium) (R.F.T. and J.M.H., unpublished observations, November 1983). In medium containing 10% heterologous or autologous plasma, the adherence of neutrophils from patient 1 to the endothelial monolayers was significantly reduced compared with simultaneously prepared normal neutrophils when examined on multiple occasions (Table 3). Similarly, the adherence of MoAb 60.3-treated normal neutrophils in 10% autologous plasma was significantly reduced compared with untreated normal neutrophils or with normal neutrophils treated with the control MoAb 60.5 (Table 3). Of note, addition of the plasma from patient 1 to normal neutrophils produced an increase in the adherence of normal neutrophils similar to
was the most striking. Normal neutrophils activated that PMA-augmented neutrophil adherence was also demonstrated and A23187 did not augment the adherence of neutrophils in serum-free medium (Table 4). In striking contrast, PMA in the presence of normal neutrophils to endothelial monolayers was observed with plasma prepared simultaneously from a patient 1 and normal donors to uncoated, fibronectin-coated, and laminin-coated tissue culture plastic was determined after a 45-minute incubation in serum-free medium at 37 °C. Each line represents a separate experiment with a different normal donor as control. Neutrophils were pretreated with MoAbs (40 μg/mL) for ten minutes, and MoAbs were present throughout the incubation. Values represent the means ± SE of three replicates.

that observed with plasma prepared simultaneously from a normal donor (data not shown).

PMA, A23187, and FMLP markedly increased the adherence of normal neutrophils to endothelial monolayers in serum-free medium (Table 4). In striking contrast, PMA and A23187 did not augment the adherence of neutrophils from patient 1. On one occasion, we were able to examine both patients' neutrophils simultaneously. Figure 3 demonstrates that PMA-augmented neutrophil adherence was also markedly decreased in patient 2.

The failure of neutrophils from the patients to adhere to endothelial monolayers when activated by PMA or A23187 was confirmed in each case by light microscopic examination of the monolayers. The difference between normal and patient neutrophil adherence observed following PMA activation was the most striking. Normal neutrophils activated by PMA formed small aggregates tightly adherent to the endothelial monolayer (Fig 4A). In addition, PMA-activated normal neutrophils produced focal disruption of the endothelial monolayer at sites of neutrophil adherence. The patients' neutrophils when stimulated by PMA failed to aggregate or to adhere to the endothelial monolayer and also did not disrupt the monolayer (Fig 4B).

Addition of MoAb 60.3 to normal neutrophils also prevented the increase in adherence induced by PMA and A23187 and, in addition, blocked FMLP-augmented adherence, whereas the control MoAb 60.5 was without effect (Table 4). Figure 5 demonstrates the dose-dependent inhibition of PMA- and A23187-augmented neutrophil adherence by MoAb 60.3. Of note, if neutrophils were preincubated with MoAb 60.3 and then washed to remove unbound MoAb, the inhibition of PMA-augmented adherence was markedly attenuated unless MoAb 60.3 were again added to the medium (Table 5). Addition of MoAb 60.5 to cells pretreated with MoAb 60.3 failed to inhibit PMA-augmented adherence (63% ± 2% adherence in cells pretreated with MoAb 60.3 and then coincubated with MoAb 60.5 compared to 1% ± 1% adherence in cells pretreated with MoAb 60.3 and then coincubated with MoAb 60.3). Pretreatment of the endothelial monolayers with MoAb 60.3

Table 2. Adherence of Neutrophils to Uncoated and Coated Plastic

<table>
<thead>
<tr>
<th>Polystyrene Well</th>
<th>Control Adherence (% of Control)</th>
<th>Patient Adherence (% of Control)</th>
<th>Control + MoAb 60.5 Adherence (% of Control)</th>
<th>Control + MoAb 60.3 Adherence (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated</td>
<td>67 ± 1</td>
<td>78 ± 1</td>
<td>69 ± 1</td>
<td>63 ± 1</td>
</tr>
<tr>
<td></td>
<td>55 ± 3</td>
<td>73 ± 2</td>
<td>83 ± 1</td>
<td>82 ± 1</td>
</tr>
<tr>
<td></td>
<td>71 ± 2</td>
<td>69 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>84 ± 1</td>
<td>83 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin-coated</td>
<td>65 ± 4</td>
<td>75 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminin-coated</td>
<td>65 ± 2</td>
<td>75 ± 1</td>
<td></td>
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</tbody>
</table>

The percentage of adherence of 51Cr-labeled neutrophils from patient 1 and normal donors to uncoated, fibronectin-coated, and laminin-coated tissue culture plastic was determined after a 45-minute incubation in serum-free medium at 37 °C. Each line represents a separate experiment with a different normal donor as control. Neutrophils were pretreated with MoAbs (40 μg/mL) for ten minutes, and MoAbs were present throughout the incubation. Each line represents a separate experiment with a different normal donor as control. Values represent the means ± SE of three replicate wells.

Table 3. Adherence of Neutrophils to Cultured Endothelium

<table>
<thead>
<tr>
<th>Addition to Endothelial Monolayer</th>
<th>Control Adherence (% of Control)</th>
<th>Patient Adherence (% of Control)</th>
<th>Control + MoAb 60.5 Adherence (% of Control)</th>
<th>Control + MoAb 60.3 Adherence (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine endothelial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum-free</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>10% heterologous plasma</td>
<td>53 ± 3</td>
<td>35 ± 2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% autologous plasma</td>
<td>19 ± 2</td>
<td>4 ± 1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% autologous plasma</td>
<td>19 ± 3</td>
<td>7 ± 1*</td>
<td>50 ± 1</td>
<td>20 ± 3*</td>
</tr>
<tr>
<td>Human endothelial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum-free</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>14 ± 2*</td>
<td></td>
</tr>
<tr>
<td>10% heterologous plasma</td>
<td>33 ± 6</td>
<td>4 ± 1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% autologous plasma</td>
<td>34 ± 8</td>
<td>8 ± 1*</td>
<td></td>
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</tbody>
</table>

The percentage of adherence of 51Cr-labeled neutrophils from patient 1 and normal donors to bovine or human endothelial monolayers was determined after a 45-minute incubation in serum-free medium or medium containing 10% autologous or heterologous plasma. Neutrophils were preincubated with the MoAbs (40 μg/mL) for ten minutes, and MoAbs were present throughout the incubation. Each line represents a separate experiment with a different normal donor as control. Values represent the means ± SE of three replicate wells.

*Significantly different from control at P < .01.
Table 4. Augmented Neutrophil Adherence to Endothelium

<table>
<thead>
<tr>
<th>Addition to Endothelial Monolayer</th>
<th>Adherence of 51Cr-Labeled Neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PMA</td>
<td>80 ± 1</td>
</tr>
<tr>
<td></td>
<td>72 ± 1</td>
</tr>
<tr>
<td>A23187</td>
<td>82 ± 3</td>
</tr>
<tr>
<td></td>
<td>85 ± 1</td>
</tr>
<tr>
<td>FMLP</td>
<td>52 ± 3</td>
</tr>
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<td></td>
<td>40 ± 3</td>
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</tbody>
</table>

The percentage of adherence of 51Cr-labeled neutrophils from patient 1 or normal donors to human or bovine endothelial monolayers was determined after a 45-minute incubation in serum-free medium with PMA (10 ng/mL), A23187 (10⁻⁸ mol/L), or FMLP (10⁻⁹ mol/L). Neutrophils were preincubated with MoAbs (40 μg/mL) for ten minutes, and MoAbs were present throughout the incubation. Each line represents a separate experiment with a different normal donor as control. In each experiment adherence in the absence of the activating agent was <10%. Values represent the mean ± 1 SE of three or four replicate wells.

*Significantly different from control at $P < .01$.

Also failed to inhibit subsequent PMA-augmented neutrophil adherence. The loss of the inhibitory effect of MoAb 60.3 with washing was not due to removal of bound MoAb, since washing does not remove MoAb 60.3 from neutrophils as assessed by fluorescence-activated cell sorter. Thus, these studies demonstrate that neutrophil adherence was inhibited only when unbound MoAb 60.3 was present in the incubation medium at the time the neutrophils were activated by PMA.

Additional studies were also performed with MoAbs 60.1 and 1G10. MoAb 1G10 recognizes a neutrophil membrane antigen consisting of a glycosphingolipid bearing the "X determinant. MoAb 60.1, like MoAb 60.3, recognizes a neutrophil membrane complex with major polypeptides of mol wt 95,000 and 150,000. On analysis by fluorescence-activated cell sorter the binding of MoAb 1G10 to neutrophils from patient 1 was similar to normal neutrophils, whereas the binding of MoAb 60.1 to patient cells was markedly decreased compared to normal cells and not significantly different from the binding of the irrelevant MoAb 9E8 (T.H.P. and P.G.B., unpublished observations). The adherence of PMA-activated neutrophils to endothelial monolayers was significantly inhibited by MoAb 60.1 (10 μg/mL) but not MoAb 1G10 (1:100 dilution) (84% ± 1% adherence in control neutrophils, 83% ± 1% in MoAb 1G10-treated neutrophils, and 10% ± 1% in MoAb 60.1-treated neutrophils).

Effect of normal neutrophils on the adherence of patient neutrophils to endothelium. In order to determine whether the increase in neutrophil adhesiveness following activation was due to a stable secretory product, we examined the effect of postsecretory medium from PMA-activated normal neutrophils on the adherence of glycoprotein-deficient cells.
addition of cell-free postsecretory supernatant medium from PMA-activated normal neutrophils did not increase adherence of the patient's neutrophils to endothelial monolayers. The percent adherence of patient cells was 2% ± 1% with PMA alone vs 2% ± 1% with PMA and a 1 to 10 dilution of the postsecretory supernatant medium from PMA-activated normal neutrophils (10⁹ per milliliter).

We next determined whether the addition of intact normal cells to a suspension of glycoprotein-deficient cells could promote the adherence of glycoprotein-deficient cells to endothelial monolayers. For these studies only patient cells were ⁵¹Cr-labeled so that an increase in ⁵¹Cr-cpm in the lysate represented an increase in adherent patient cells. In contrast to postsecretory supernatant, the addition of intact normal neutrophils to PMA-treated patient neutrophils markedly increased the adherence of the glycoprotein-deficient neutrophils to endothelial monolayers (Table 6). The 70% increase in adherent ⁵¹Cr-cpm was not likely a result of ⁵¹Cr transfer from patient to normal cells since we have found that <10% of the total neutrophil ⁵¹Cr-labeled is lost into the supernatant medium during a 45-minute incubation with PMA. Moreover, when the monolayers of mixed ⁵¹Cr-labeled patient and unlabeled normal cells were examined by phase contrast microscopy, we observed the usual pattern of tightly adherent neutrophil aggregates without a significant number of nonadherent cells.

SDS-PAGE of postsecretory medium. GP-150 was not detected in the postsecretory medium of PMA-activated normal neutrophils on analysis by SDS-PAGE, although increases in other proteins were readily apparent (Fig 6). The antigen recognized by MoAb 60.3 was also not detected in postsecretory medium on analysis by solid-phase binding assay (data not shown).

Chemotaxis of neutrophils across endothelial monolayers. The percentage of ⁵¹Cr-labeled neutrophils migrating across endothelial monolayers in the absence of a chemotactic stimulus was similar with control, patient 1, and MoAb 60.3-treated normal neutrophils (Table 7). However, both MoAb 60.3-treated normal neutrophils and neutrophils from patient 1 failed to increase migration across the endothelial monolayer in response to FMLP (Table 7). FMLP was selected as the chemotactic stimulus because the patient’s neutrophils have been shown to have a normal number of FMLP receptors.³ Light microscopic examination of thick sections of the polycarbonate filters showed only occasional patient neutrophils adherent to the endothelial monolayers after the 90-minute incubation. Neutrophils from the patient were not seen between the endothelial monolayer and the polycarbonate filter and were only occasionally observed in the polycarbonate filter pores.

Effect of neutrophil activation on MoAb binding. Activation of neutrophils by PMA significantly increased binding of MoAb 60.3, but not MoAbs 9E8 or 60.5 (Fig 7). Similar augmentation of MoAb 60.3 binding was observed when binding was quantitated by fluorescence-activated cell sorter (160% of resting control following PMA and 208% of resting control following A23187).

Table 6. Effect of Normal Neutrophils on PMA-Mediated Adherence of Patient Neutrophils to Endothelium

<table>
<thead>
<tr>
<th>Addition to Endothelial Monolayer</th>
<th>⁵¹Cr-Labeled Neutrophils</th>
<th>Neutrophil Adherence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>Patient</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>PMA</td>
<td>70 ± 3</td>
<td>82 ± 1</td>
</tr>
</tbody>
</table>

The percentage of adherence of ⁵¹Cr-labeled neutrophils from patient 1 to human endothelial monolayers was determined after a 45-minute incubation at 37 °C in medium with PMA (10 ng/mL final) with and without equal numbers of unlabeled normal control or patient neutrophils. Each line represents a separate experiment with a different normal donor as control. Values represent the mean ± 1 SE of three replicates.

Fig 5. Dose-dependent inhibition of augmented neutrophil adherence by MoAb 60.3. ⁵¹Cr-labeled neutrophil adherence to bovine aortic endothelial cell monolayers was determined after a 45-minute incubation at 37 °C with (A) PMA (10 ng/mL final) or (B) A23187 (10⁻⁵ mol/L final) with and without MoAb 60.3. Values represent the mean ± 1 SE of four replicates.

Table 5. Effect of Preincubation or Coincubation on the Inhibition of PMA-Augmented Neutrophil Adherence to MoAb 60.3

<table>
<thead>
<tr>
<th>Addition to Endothelial Monolayer</th>
<th>Adherence of ⁵¹Cr-Labeled Neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Control</td>
</tr>
<tr>
<td></td>
<td>(B) Preincubation</td>
</tr>
<tr>
<td></td>
<td>With MoAb 60.3</td>
</tr>
<tr>
<td></td>
<td>(C) Coincubation</td>
</tr>
<tr>
<td></td>
<td>With MoAb 60.3</td>
</tr>
<tr>
<td>Medium alone</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>PMA</td>
<td>67 ± 5</td>
</tr>
<tr>
<td></td>
<td>41 ± 4</td>
</tr>
<tr>
<td></td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

⁵¹Cr-labeled human peripheral blood neutrophils were incubated on ice for 15 minutes in medium alone (A) or medium containing MoAb 60.3 (40 μg/mL) (B and C). The neutrophils were then washed with medium and resuspended in medium alone (A and B) or medium containing MoAb 60.3 (40 μg/mL) (C). The percentage of adherence of these ⁵¹Cr-labeled neutrophils to bovine aortic endothelial cell monolayers was then determined after a 45-minute incubation at 37 °C in control medium (A) or medium containing PMA (10 ng/mL) (A, B, and C). Values represent means of three replicate wells ± 1 SE.
defect in neutrophil function characterized by delayed migration of glycoproteins on SDS-PAGE has long been recognized. The anomalous migration of glycoproteins on SDS-PAGE has long been recognized. This may in part be due to charged sugar residues, as well as to differences in binding of SDS per unit dry weight. Post-translational processing therefore may account for substantial variability in mol wt when compared with unmodified protein mol wt standards. The mol wt of 150,000 daltons determined in these experiments was derived by interpolation from protein standards on gels which varied from 5% to 8%. Higher concentrations of acrylamide did not allow the high mol wt standard adequate penetration of the gels to result in a linear plot of migration and log mol wt.

Neutrophils from these patients have a major defect in adherence characterized by a failure to flatten and spread on artificial substrates or to adhere to nylon wool. This observation suggests that the defect in chemotaxis observed in these patients' neutrophils in vivo could result from a primary defect in neutrophil adherence to endothelium or to subendothelial substrate. Buchanan and co-workers recently examined this possibility in their patient whose neutrophils fail to migrate to extravascular sites of infection. They found normal, unstimulated neutrophil adherence to and migration under cultured endothelial monolayers on coverslips, whereas neutrophil adherence to fibronectin-coated plastic was decreased compared to normal neutrophils. In addition, their patient had normal demargination of neutrophils in response to epinephrine. These observations suggested to them that the defect was not due to abnormal interaction of the patients' neutrophils with endothelium, but rather to abnormal interaction with subendothelial connective tissue.

Several patients have been reported with a congenital defect in neutrophil function characterized by delayed umbilical cord separation and recurrent severe infection without pus formation. Crowley et al first reported the absence of a neutrophil membrane glycoprotein in one of these patients. The mol wt of the missing glycoprotein in their patient was initially reported as 110,000 daltons but subsequently reported as 180,000 daltons. More recently, others have reported the mol wt of the missing polypeptide in several other patients to be 150, 110, and 138 kD. Although we now report that the mol wt of the missing polypeptide in our two patients is approximately 150, 110, 138, and 138 kD, these patients' neutrophils in vivo could result from a primary defect in neutrophil adherence to endothelium or to subendothelial substrate. Buchanan and co-workers recently examined this possibility in their patient whose neutrophils fail to migrate to extravascular sites of infection. They found normal, unstimulated neutrophil adherence to and migration under cultured endothelial monolayers on coverslips, whereas neutrophil adherence to fibronectin-coated plastic was decreased compared to normal neutrophils. In addition, their patient had normal demargination of neutrophils in response to epinephrine. These observations suggested to them that the defect was not due to abnormal interaction of the patients' neutrophils with endothelium, but rather to abnormal interaction with subendothelial connective tissue.

<table>
<thead>
<tr>
<th>Table 7. Neutrophil Migration Across Endothelial Monolayers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Addition to Outer Well</strong></td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Medium alone</td>
</tr>
<tr>
<td>FMLP</td>
</tr>
<tr>
<td></td>
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<td></td>
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</tbody>
</table>

The percentage of migration of 51Cr-labeled neutrophils from patient 1 or normal donors across the endothelial monolayers on the polycarbonate filters was determined after a 90-minute incubation in serum-free medium at 37 °C. The outer well contained control medium alone or medium with FMLP (10 ng/mL). Neutrophils were treated with the MoAbs (40 μg/mL) for ten minutes, and MoAbs were present throughout the incubation. Each line represents a separate experiment with a different normal donor as control. Values represent the mean ± 1 SE of three replicates.

*Significantly different from control at P < .01.
In this report, we also have investigated the role of the membrane glycoprotein in neutrophil-endothelial interactions. In addition to the patients’ neutrophils, we have examined normal neutrophils treated with MoAb 60.3. This MoAb recognizes a multimeric cell surface antigen complex with major polypeptides of mol wt 95,000, 150,000, and 150,000 daltons. Previous analysis by fluorescence-activated cell sorter demonstrated that neutrophils from patient 1 failed to bind MoAb 60.3, although they bound the control MoAb 60.5. Recent studies demonstrate that neutrophils from patient 2 also fail to bind MoAb 60.3 on analysis by fluorescence-activated cell sorter (P.G.B. and T.H.P., unpublished observation, February 1984). We have confirmed the absence of the antigen recognized by MoAb 60.3 in detergent extracts of neutrophils from both patients by solid-phase binding assay.

Our results demonstrate that unstimulated neutrophils from patient 1 adhere normally to endothelium in serum-free medium and migrate normally across endothelial monolayers in the absence of a chemotactic stimulus. We did not, however, observe a decrease in our patient’s neutrophil adherence to fibronectin-coated plastic as reported by Buchanan et al. In contrast, as noted by Brown and Lackie, we have observed that increasing concentrations of fibronectin (>100 μg/mL) decrease neutrophil adherence to tissue culture plastic. Because Terranova et al have reported that laminin is important in neutrophil adherence and chemotaxis in vitro, we also examined the role of the membrane glycoprotein complex in neutrophil attachment to laminin. The adherence of neutrophils from patient 1 to laminin-coated plastic was similar to normal neutrophils.

Although the adherence of neutrophils from patient 1 to endothelial monolayers in serum-free medium was not different from that of normal neutrophils, neutrophils from our patient and normal neutrophils treated with MoAb 60.3 had significantly reduced adherence in medium containing heterologous or autologous plasma. The most striking defect of the glycoprotein-deficient or MoAb 60.3-treated neutrophils, however, was their failure to augment adherence when stimulated by PMA, FMLP, or A23187. Each of these agonists produced a marked increase in normal neutrophil adherence that was completely absent in neutrophils from patient 1 and was totally prevented by addition of MoAb 60.3 to normal neutrophils. Of note, PMA produced a slight but significant augmentation of neutrophil adherence in patient 2 who has had a less severe clinical course.

Neutrophils from patient 1 and normal neutrophils treated with MoAb 60.3 also failed to increase their migration across endothelial monolayers in response to FMLP. The failure of the patient’s neutrophils or MoAb 60.3-treated normal neutrophils to migrate across the endothelial monolayers on the polycarbonate filter appeared to be due to an inability of the neutrophils to adhere to and to penetrate the endothelial monolayers rather than an inability to adhere to the polycarbonate filter, since light microscopic examination of thick sections of the filters revealed only a rare patient neutrophil on the endothelial surface or between the endothelial monolayer and the polycarbonate filter (or in the filter pores).

In order to determine whether increased neutrophil adherence following activation resulted from a secreted product, we examined the effect of postsecretory, cell-free supernatant medium from PMA-activated normal neutrophils on adherence of glycoprotein-deficient cells. The failure of postsecretory supernatant from PMA-activated normal neutrophils to correct the adherence defect in the patient’s cells suggests that the glycoprotein complex mediating augmented adherence in the PMA-stimulated neutrophils remains membrane-associated and is not secreted into the medium. In support of this, we were not able to detect the 150-kd glycoprotein in the postsecretory supernatant medium of PMA-activated normal neutrophils on analysis by SDS-PAGE.

In contrast to postsecretory medium, the addition of intact normal neutrophils and PMA allowed the glycoprotein-deficient neutrophils to adhere to endothelial monolayers. By phase microscopy it appeared that the patient’s neutrophils adhered to the endothelial monolayers by forming small aggregates with normal neutrophils. Because of limited opportunities to examine the patient’s neutrophils, it was not possible to determine a dose-response for the normal neutrophils, ie, the minimum number of normal neutrophils required to correct the adherence defect of the patient’s neutrophils. Of note, similar results were reported by Anderson et al, who reported that their patient’s cells failed to aggregate with each other but aggregated with normal cells. The adherence-promoting effect of intact neutrophils on the glycoprotein-deficient cells could result from the release of a very labile or extremely short-lived mediator that was not present in immediate postsecretory medium. An alternative, and perhaps more likely, explanation of the results of the mixing experiments is that when normal neutrophils are activated by PMA (or other agents) they become more adherent, sticking to endothelium and to other neutrophils including, in this case, those of the patient.

These in vitro studies suggest that the neutrophil membrane antigen complex recognized by MoAb 60.3, which is absent in our patients, is a membrane-associated moiety required for neutrophils to augment adherence to or migration across endothelium. The critical role of the 95- and 150-kd polypeptides of this antigen complex is evidenced by the observation that MoAb 60.1, which precipitates polypeptides of mol wt 95,000 and 150,000 but not 130,000 daltons, produces similar inhibition of PMA-augmented adherence as MoAb 60.3.

It is not clear from these studies how this membrane glycoprotein complex mediates augmented neutrophil adherence to endothelium. Although the glycoprotein complex is expressed on the external cell membrane of neutrophils isolated by Ficoll-Hypaque gradient and dextran sedimentation (based on the binding of MoAb 60.3 to paraformaldehyde-fixed, unstimulated neutrophil suspensions), these cells do not avidly adhere to cultured endothelium when unstimulated. Also, if normal neutrophils are pretreated with MoAb 60.3 and then washed to remove unbound MoAb, there is a marked reduction in the inhibitory effect of the MoAb. These observations suggest that there is a functionally important qualitative or quantitative change in the antigen during activation that promotes adherence and requires the
continued presence of MoAb 60.3 for complete inhibition. A functional alteration in the glycoprotein could occur as a result of a number of mechanisms during activation such as protease cleavage or phosphorylation. Alternatively, activation may lead to increased surface membrane expression of additional, functionally active, glycoprotein. In keeping with this latter possibility, we observed a significant increase in the surface-binding of MoAb 60.3 as assessed by both a suspension binding assay and fluorescence-activated cell sorter following activation of normal neutrophils by PMA and A23187. These studies suggest that increased expression of the antigen complex recognized by MoAbs 60.3 and MoAb 60.1 is functionally correlated with the increase in neutrophil adherence. Additional studies are required to determine the precise mechanism by which new antigen expression increases membrane adhesiveness.

Although increased surface expression of 60.3 antigen could result from a conformational change in plasma membrane, one possible source for new membrane antigen is the specific granule. Specific granule contents may be translocated to the plasma membrane following activation,32,33 and the release of specific granule contents is associated with an increase in adhesiveness.34-37 In this respect Todd et al38 recently demonstrated that the large subunit of Mol-1, a 155-kd membrane glycoprotein, is also located in the specific granules of neutrophils. During degranulation it is translocated to the plasma membrane resulting in increased expression of Mol-1-antigen.

Sanchez-Madrid et al have recently summarized evidence that a number of monoclonal antibodies recognize a leukocyte antigen family with three α subunits of 150,000 to 177,000 mol wt and a common β subunit of 95,000 mol wt.39 These include MoAbs to LFA-1 antigen, which contains subunits of mol wt 177,000 and 95,00040 and Mol antigen with subunits of 165,000 and 95,000 mol wt.41 The epitopes recognized by these MoAbs are on the α subunits. The precise epitope recognized by MoAb 60.3 has not yet been determined. Several of these glycoprotein-deficient patients,27,42 including our two patients (P.G.B., unpublished observation, March 1984), fail to bind the MoAbs to LFA-1 and Mol antigens. One additional MoAb (TS1/18) is directed to an epitope on the β subunit, but additionally precipitates a distinct α subunit of mol wt 150,000.39 Two of the glycoprotein-deficient patients also fail to bind this MoAb.4,27,42 Although we are unable to detect an absent polypeptide at mol wt 95,000 on analysis of detergent extracts of the patients' neutrophils by SDS-PAGE and protein staining, we cannot exclude that this component of the membrane antigen complex is also absent in our patients.

Anti-LFA-1 and anti-Mol-1, like MoAb 60.3,4,12,43 have been reported to be important in cytolytic T cell functions44 and phagocytosis of C3bi-coated particles.45 The effects of these MoAbs on neutrophil adherence and chemotaxis have not been reported, although our studies indicate that one MoAb to the Mol-1-antigen (OKM1) does not affect neutrophil adherence to endothelium or neutrophil spreading on plastic (J.M.H. and W.J.W., unpublished observation, April 1984). Comparative studies with a panel of monoclonal antibodies that recognize different epitopes in this leukocyte antigen family may provide a means of characterizing these membrane-associated glycoproteins and define a molecular basis for several neutrophil functions.

Diseases of nature such as the deficiency of leukocyte membrane glycoprotein(s) in our patients provide unique opportunities to establish the functional significance of cellular proteins. Although previous studies have implicated lactoferrin as the major regulator of the adherent properties of neutrophils,46,47 the in vitro studies reported herein indicate that the membrane-associated glycoprotein complex defined by MoAbs 60.3 and 60.1 is the critical neutrophil constituent promoting the adherence of activated neutrophils to vascular endothelium. We postulate that the absence of the membrane-associated glycoprotein(s) observed in these patients prevents neutrophil adherence to endothelium following neutrophil activation and accounts for the failure of their neutrophils to migrate to sites of inflammation in vivo, thereby permitting recurrent infections without pus formation.

Acknowledgment

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The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro

JM Harlan, PD Killen, FM Senecal, BR Schwartz, EK Yee, RF Taylor, PG Beatty, TH Price and HD Ochs