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*J Immunol* 2000; 164:5961-5969; ;  
doi: 10.4049/jimmunol.164.11.5961  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Differential Ability of Exogenous Chemotactic Agents to Disrupt Transendothelial Migration of Flowing Neutrophils<sup>1</sup>

N. Thin Luu, G. Ed Rainger, and Gerard B. Nash<sup>2</sup>

Neutrophils migrate through endothelium using an ordered sequence of adhesive interactions and activating signals. To investigate the consequences of disruption of this sequence, we characterized adhesion and migration of neutrophils perfused over HUVEC that had been treated with TNF- $\alpha$  for 4 h and evaluated changes caused by exogenously added chemotactic agents. When HUVEC were treated with 2 U/ml TNF, flowing neutrophils adhered, with the majority rolling and relatively few migrating through the monolayer. If fMLP, IL-8, zymosan-activated plasma (a source of activated complement factor C5a), epithelial cell-derived neutrophil-activating peptide (ENA-78), or growth-regulating oncogene, GRO- $\alpha$ , was perfused over these neutrophils, they stopped rolling and rapidly migrated over the monolayer, but did not penetrate it. When HUVEC were treated with 100 U/ml TNF, the majority of adherent neutrophils transmigrated. If neutrophils were treated with fMLP, IL-8, C5a, ENA-78, or GRO- $\alpha$  just before perfusion over this HUVEC, transmigration, but not adhesion, was abolished. However, when platelet-activating factor was used to activate neutrophils, migration through HUVEC treated with 100 U/ml TNF was not impaired, and migration through HUVEC treated with 2 U/ml TNF was actually increased. Transmigration required ligation of CXC chemokine receptor-2 on neutrophils, and differential desensitization of this receptor (e.g., by fMLP but not platelet-activating factor) may explain the pattern of disruption of migration. Thus, transmigration may require presentation of the correct activators in the correct sequence, and inappropriate activation (e.g., by systemic activators) could cause pathological accumulation of neutrophils in the vessel lumen. *The Journal of Immunology*, 2000, 164: 5961–5969.

Neutrophils must adhere to microvascular endothelium and migrate through it to carry out their function in host protection. Transendothelial migration occurs via an ordered sequence of adhesive interactions and activating steps, primarily regulated by the endothelial cells. Cytokines such as TNF- $\alpha$  and IL-1 induce the endothelial cells to express E-selectin and P-selectin, which mediate capture and rolling adhesion of flowing neutrophils (see Refs. 1–3 for review). Rolling adhesion allows interaction of neutrophils with surface-presented chemotactic agents such as platelet-activating factor (PAF)<sup>3</sup> and IL-8, which induce activation of  $\beta_2$  integrin receptors. The  $\beta_2$  integrins bind to endothelial members of the Ig gene superfamily (IgSF; e.g., intercellular adhesion molecule-1) to immobilize the rolling cells and also support onward migration (4). It remains uncertain how neutrophils are guided to move through the endothelial monolayer and into tissue, although it seems that chemotactic agents work in concert with the IgSF member CD31 to regulate migration (5–7).

Uncontrolled adhesion and migration of neutrophils may be pathogenic (8–10). Two main scenarios can be envisaged: continuous or untimely entry of neutrophils into tissue may lead to deg-

radative damage, or failure of transmigration of neutrophils bound to the endothelial surface could cause vascular occlusion and damage if the cells released toxic oxidants and granule contents. The second of these may occur in conditions such as vasculitis and endotoxic shock, where systemic activation of neutrophils may occur along with deposition in the lumen of microvessels (11, 12). This concept gains support from findings that infusion of IL-8 or fMLP into rabbits actually impaired neutrophil transmigration induced by separate localized application of chemotactic agents (13, 14), although these effects were linked to the ability of these agents to inhibit adhesion to stimulated endothelium (15, 16) rather than to disruption of migration per se. IL-8 is generated by endothelial cells exposed to cytokines or to hypoxia and reoxygenation, and in these cases it has been shown to promote neutrophil activation, adhesion, and migration (17–19). Thus, it may be hypothesized that for efficient migration, activating signals must have a correct sequence or mode of presentation (e.g., on the endothelial surface rather than in the fluid phase), and that exposure of neutrophils to activating agents in the blood might cause them to fail to cross the endothelial barrier.

We set out to investigate these possibilities by characterizing the migration of neutrophils through endothelial monolayers stimulated with TNF, with and without exposing the neutrophils to exogenously added activating peptides (IL-8, fMLP, activated complement fragment (C5a), epithelial neutrophil-activating peptide (ENA-78), growth-regulating oncogene protein  $\alpha$  (GRO- $\alpha$ )) or phospholipid-derived PAF. Judging from measurements of changes in intracellular Ca<sup>2+</sup>, the peptides IL-8, fMLP, and C5a have been shown previously to cause homologous desensitization of responses through their own receptors, and heterologous desensitization of responses through each other's receptors and through the PAF receptor (20, 21). Effectiveness was in the order fMLP > C5a > IL-8, and recent studies suggest that these peptides can also inhibit adhesion and migration induced by each other with a similar hierarchy (22, 23). Desensitization of the response to IL-8 may

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Received for publication April 30, 1999. Accepted for publication March 21, 2000.

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<sup>1</sup> This work was supported by a grants from the Leverhulme Trust and the British Heart Foundation.

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<sup>3</sup> Abbreviations used in this paper: PAF, platelet-activating factor; IgSF, Ig gene superfamily; C5a, activated complement fragment 5; CXCR1, CXC-chemokine receptor type 1; ENA-78, epithelial neutrophil-activating peptide-78; GRO- $\alpha$ , growth-regulating oncogene protein  $\alpha$ ; PBS/BSA, PBS with albumin; ZAP, zymosan-activated plasma.

arise from down-regulation of its two CXC chemokine receptors, CXCR1 and CXCR2 (24–26). However, the effects of such agents on responses to endogenous surface-presented activators, expressed by cytokine-treated endothelial cells, and on the different steps during transendothelial migration have not been described. Here, we used a flow-based model (19, 27) to allow separate investigation of the steps in neutrophil migration, to distinguish between the effects of fluid phase and surface-presented activating agents, and to avoid the possibility of build-up of agents released by endothelial cells or neutrophils that might influence responses.

## Materials and Methods

### Preparation of neutrophils and activating agents

Blood was collected from healthy volunteers into EDTA tubes (Sarstedt, Numbrecht, Germany), and neutrophils were isolated using two-step density gradients, as previously described (19, 27). The neutrophils were washed twice in PBS containing 1 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Mg}^{2+}$  (Sigma, St. Louis, MO), 0.1% BSA (Sigma), and 5 mM glucose (PBS/BSA) and adjusted to  $1 \times 10^6/\text{ml}$  in the same medium. In chosen experiments, neutrophils were treated with mAb against CXCR1 or CXCR2 (9H1 and 10H2, respectively; 10  $\mu\text{g}/\text{ml}$ ; gift from Dr. K. Jim Kim, Genentech, San Francisco, CA) for 20 min before assay.

To prepare zymosan-activated plasma (ZAP) as a source of activated complement fragment C5a, blood was collected from healthy volunteers into sodium heparin (CP Pharmaceuticals, Wrexham, U.K.). Platelet-poor plasma was separated by centrifugation at  $1000 \times g$  for 5 min. The plasma was then incubated with zymosan (8 mg/ml) for 30 min at 37°C, followed by centrifugation at  $10,000 \times g$  for 5 min and filtration through a sterile 0.2- $\mu\text{m}$  pore size filter. ZAP was used at 1% in PBS/BSA to activate neutrophils. Other activating agents were PAF (Sigma), fMLP (Sigma), and recombinant human ENA-78, GRO- $\alpha$ , and monocyte-derived IL-8 (R&D Systems, Abingdon, U.K.; see *Results* for concentrations).

### Endothelial cell culture

Endothelial cells were isolated from the veins of human umbilical cords as previously described (28) and cultured in medium 199 (ICN Flow Laboratories, Thame, U.K.) containing 20% heat-inactivated normal human serum (National Transfusion Service, Birmingham, U.K.), 50 U/ml sodium heparin (CP Pharmaceuticals, Wrexham, U.K.), 25  $\mu\text{g}/\text{ml}$  gentamicin (David Bull Laboratories, Warwick, U.K.), and 1 mg/ml glutamine (Sigma) until confluent (~4 days). Adhesion assays were conducted using second-passage endothelial cell cultures grown on one inner surface of glass capillary tubes (microslides) as previously described (27, 28). Microslides (Camlab, Cambridge, U.K.) have a rectangular cross-section of 0.3  $\times$  3 mm, a length of 5 cm, and good optical qualities. They were treated with 3-aminopropyltriethoxysilane (Sigma) and coated with a mixture of gelatin (1% type B from bovine skin; Sigma) and collagen ( $2 \times 10^{-4}$  M human type IV; Sigma) before seeding with HUVEC at a level that gave a confluent monolayer within 24 h (27). Confluent HUVEC in microslides were stimulated with 2 or 100 U/ml of recombinant human TNF- $\alpha$  (Genzyme, West Malling, U.K.) for 4 h before assays (27). Each experiment was conducted using HUVEC from a single primary culture from a single donor.

### Adhesion and migration of neutrophils under conditions of flow

The adhesion and migration assay was recently described (27). Microslides containing confluent HUVEC were mounted on the stage of a phase-contrast, video microscope enclosed in a Perspex chamber (Wolfson Applied Technology Laboratory, Birmingham, U.K.) at 37°C. They were attached at one end, via flexible silicon tubing, to a Harvard syringe pump (Harvard Apparatus, Natick, MA), and washing buffer or a suspension of neutrophils was selectively drawn through the microslides via an electronic switching valve (Lee Products, Gerards Cross, U.K.) attached to the other end. A constant wall shear stress of 0.1 Pascal unit was maintained in the microslide by choice of the appropriate flow rate. This stress is comparable to that found in postcapillary venules (see Ref. 29 for review) and does not allow direct adhesion of neutrophils to endothelial cells in the absence of selectins (19).

HUVEC was perfused with PBS/BSA to remove excess TNF. Neutrophil suspension was flowed over the HUVEC for 4 min, followed by a period of washout with cell-free PBS/BSA. A video record of adhesive behavior was made during inflow of neutrophils and at intervals during washout for a series of fields along the center line of the microslide. After 1 min of washout, the number of adherent cells was counted in at least four

complete microscopic fields of known dimension and converted to cells per square millimeter per  $10^6$  perfused.

Video microscopic recordings of neutrophils adherent to the endothelial cell layer were analyzed off-line. Three populations of adherent neutrophils could be distinguished: 1) rolling adherent cells, which were spherical, and rotated slowly over the surface at a velocity (~10  $\mu\text{m}/\text{s}$ ) far below that of free flowing cells (~700  $\mu\text{m}/\text{s}$ ); 2) activated neutrophils, which were phase bright with distorted shape, either stopping or migrating on the top of the endothelial monolayer; and 3) transmigrated neutrophils, which were phase dark and migrated underneath the monolayer. The percentage of adherent cells in each category was determined, and the velocity of rolling cells was measured from digitized images and custom software (30). The velocity of migrating cells was also measured (7). The outlines of neutrophils were traced onto acetate sheets overlying the video monitor. This process was repeated as the video record was moved forward in 1-min intervals, and the distance advanced by the leading cellular margin was measured. The screen size was calibrated using a stage micrometer, and velocity (microns per minute) was averaged over 3–5 min of movement.

### Disruption of neutrophil adhesion and migration by activating agents

**Experiments with 2 U/ml TNF.** To examine the effect of activating agents on neutrophils already adherent to HUVEC but predominantly rolling, a low concentration of TNF (2 U/ml) was used to activate endothelial cells. Neutrophils were perfused over HUVEC for 4 min. After 1 min of washout, activating agents (fMLP, IL-8, C5a, PAF, ENA-78, or GRO- $\alpha$ ) in PBS/BSA were perfused continuously over the already adherent neutrophils, and their behavior was recorded. To test whether activation was reversible, fMLP was perfused for 1 min over adherent neutrophils and then the microslides were perfused with PBS/BSA alone for up to 60 min. In some experiments, IL-8 was perfused over the adherent neutrophils for 1 min, and then IL-8 and PAF were perfused together for 10 min.

**Experiments with 100 U/ml TNF.** To examine the effect of preactivation of neutrophils on subsequent migration, HUVEC were stimulated with 100 U/ml TNF, which induced a high proportion of adherent neutrophils to migrate. Activating agents (fMLP, IL-8, C5a, PAF, ENA-78, or GRO- $\alpha$ ) were added to neutrophils 30 s before perfusion over the endothelial cell monolayer and were perfused along with the neutrophils. fMLP, IL-8, C5a, and PAF were also included in the PBS/BSA used to washout neutrophils for 10 min after the bolus. Because of the scarcity of ENA-78 and GRO- $\alpha$  and the need to use relatively high concentrations (see *Results*), these agents were not included in the washout PBS/BSA. To specifically test reversibility, fMLP was perfused with the 4-min bolus of neutrophils, but then PBS/BSA was perfused alone for up to 60 min. In some experiments where neutrophils were treated with the mAb 10H2 against CXCR2 before perfusion, PAF was added to the washout PBS/BSA after completion of the 4-min bolus.

### Surface expression of CD11b and CXC chemokine receptors

The effect of activation of neutrophils on their surface expression of CD11b was analyzed by direct immunofluorescence and flow cytometry using an R-PE-conjugated mAb against CD11b (R841, Dako, High Wycombe, U.K.) as previously described (31). The expression of CXCR1 and CXCR2 was analyzed by indirect immunofluorescence, using 9H1 and 10H2 as primary Abs and FITC-conjugated goat anti-mouse (Dako) as secondary Ab. In every case, the intensity of fluorescence labeling and the percentage of cells positively labeled were measured relative to those of cells labeled using a nonspecific conjugated or unconjugated mouse Ab (Dako) as appropriate. Binding of IL-8 to the surface of neutrophils was quantified by flow cytometry using biotin-conjugated IL-8 and avidin-FITC, supplied as a kit (Fluorokine, R&D Systems), which was used according to the manufacturer's instructions.

### Statistical analysis

Effects of time and treatments were tested using two-way ANOVA. Comparison of individual treatments at single time points were made using paired *t* test when appropriate. All tests were performed using the computer program Minitab (Minitab, State College, PA).

## Results

We have described previously how adhesion of flowing neutrophils to HUVEC varies with the stimulatory concentration of TNF and with time (27, 32). Here, neutrophils bound in similar numbers to HUVEC treated with 2 or 100 U/ml TNF (Table I), but did not attach to untreated HUVEC (<1% of binding to TNF-treated

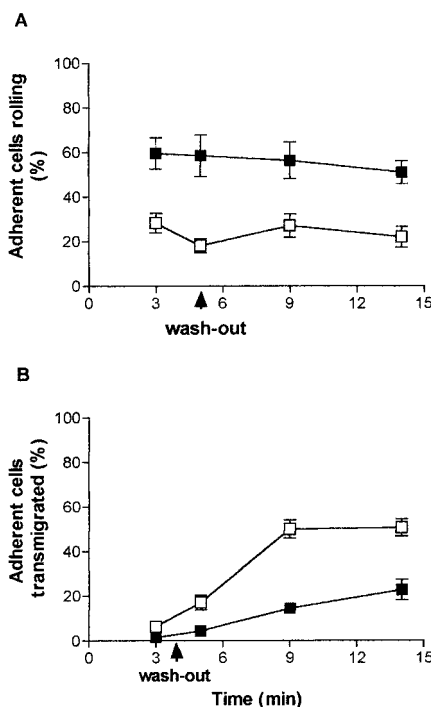
Table I. Effect of exogenous activating agents on the number of neutrophils adherent to HUVEC treated with 2 or 100 U/ml TNF<sup>a</sup>

Activating Agent	No. of Neutrophils Adherent (per mm <sup>2</sup> /10 <sup>6</sup> perfused)	
	HUVEC treated with 2 U/ml TNF	HUVEC treated with 100 U/ml TNF
None	356 ± 18	373 ± 13
fMLP (10 <sup>-7</sup> M)	317 ± 32	241 ± 37*
IL-8 (10 <sup>-7</sup> M)	491 ± 98	388 ± 54
ZAP (1% v/v)	361 ± 21	382 ± 30
PAF (10 <sup>-7</sup> M)	358 ± 26	388 ± 54

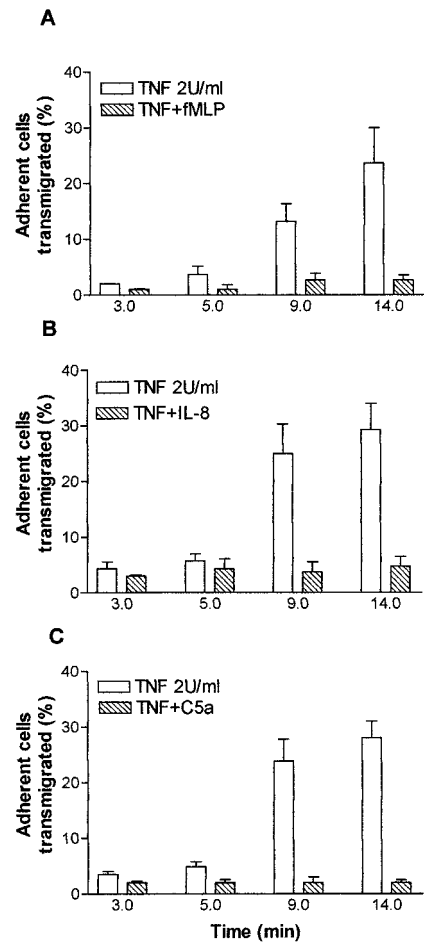
<sup>a</sup> A 4-min bolus of neutrophils was perfused over HUVEC. For 2 U/ml TNF, agents were perfused over adherent neutrophils at the end of the bolus, and counts were made 2 min later. Further video recordings of these surfaces did not show detachment of neutrophils. For 100 U/ml TNF, neutrophils were treated with agents immediately before perfusion over the HUVEC, and counts were made 1 min after the completion of the neutrophil bolus. Data are mean ± SEM of three experiments. \*, *p* < 0.05 for fMLP-treated vs untreated neutrophils by paired *t* test.

cells). At 2 U/ml TNF, the majority of adherent neutrophils were rolling, and about 20% gradually migrated through the monolayer (Fig. 1). At 100 U/ml TNF, relatively few adherent neutrophils rolled, and 50–60% typically transmigrated within about 5 min of attachment (Fig. 1). These results agree with our previous reports (27, 32).

The abilities of fMLP, IL-8, C5a, and PAF to modify neutrophil adhesion have been described. We have shown, for instance, that each can cause rolling neutrophils to become immobilized through activation of β<sub>2</sub> integrins (7, 33). Here, addition of any one of these exogenous activating agents had a marked effect on the behavior of the adherent neutrophils, although the effects were not identical for



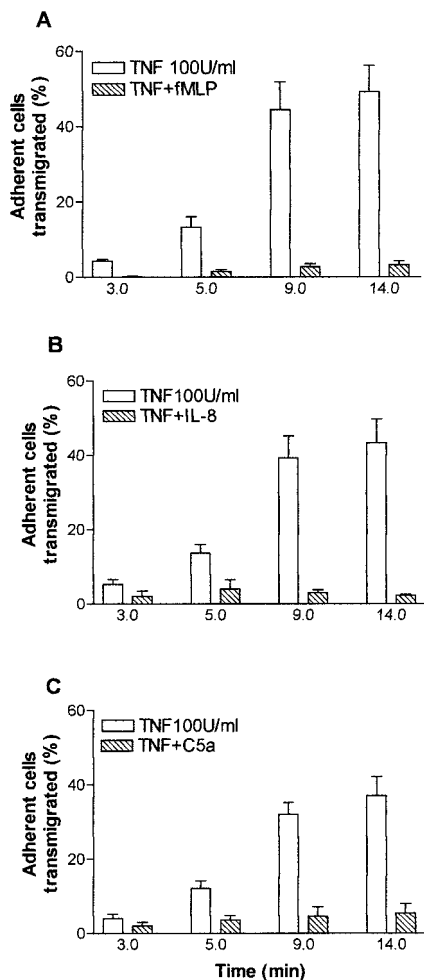
**FIGURE 1.** Comparison of percentage of adherent neutrophils rolling (A) and percentage of adherent neutrophils transmigrated (B) as a function of time after binding to HUVEC treated with 2 (■) or 100 (□) U/ml TNF. Neutrophils were perfused for 4 min at a wall shear stress of 0.1 Pascal, followed by washout with PBS/BSA; the time scale origin coincides with the start of their perfusion. Data are the mean ± SEM from three experiments.



**FIGURE 2.** Effect of exogenous activating agents on the percentage of adherent neutrophils transmigrated with time when HUVEC was stimulated with 2 U/ml TNF. Agents were perfused over neutrophils already adherent to the HUVEC and remained present throughout measurement; the time scale origin coincides with the start of perfusion of neutrophils. A, B, and C, Effects of perfusion of 10<sup>-7</sup> M fMLP, 10<sup>-8</sup> M IL-8, and 1% ZAP (C5a), respectively. Data are the mean ± SEM of three or four experiments. In each case, ANOVA showed a significant inhibitory effect of the activating agent on the percentage of migrated neutrophils (*p* < 0.01).

the different types of agent. If fMLP (10<sup>-7</sup> M), IL-8 (10 ng/ml), or C5a (1% ZAP) was perfused over neutrophils that were already adherent to HUVEC that had been treated with 2 U/ml TNF, cells stopped rolling in seconds. Essentially all adherent cells then activated and migrated over the luminal surface of the endothelial cell monolayer, but very few migrated through it (Fig. 2). Transendothelial migration was thus markedly reduced. If neutrophils were treated with fMLP, IL-8, or C5a immediately before perfusion over HUVEC that had been treated with 100 U/ml TNF, the cells immobilized immediately on landing, and rolling was absent. The activated cells migrated over the surface, but again, transmigration was almost abolished (Fig. 3). When fMLP was washed out from neutrophils adherent to HUVEC treated with either 2 or 100 U/ml TNF, during the first 10 min the neutrophils still failed to transmigrate (Fig. 4) but remained activated and did not recommence rolling. If washout was prolonged for 30–60 min, transmigration still failed to occur with either 2 or 100 U/ml TNF, but an increasing number of cells tended to round up in shape and recommence rolling (data not shown).

Examining the dose dependence of the effects, we found that 10<sup>-11</sup> M fMLP did not affect the percentage of adherent cells

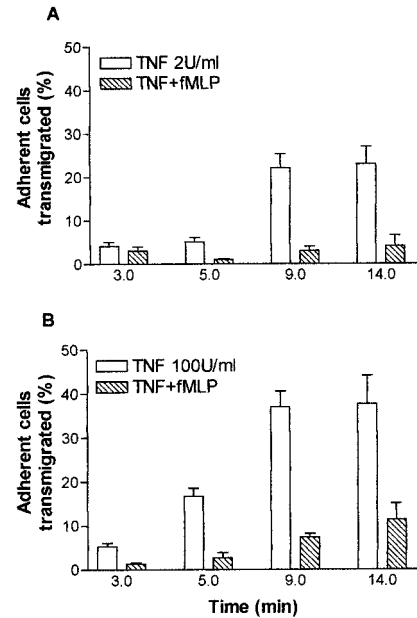


**FIGURE 3.** Effects of exogenous activating agents on the percentage of adherent neutrophils transmigrated with time when HUVEC was stimulated with 100 U/ml TNF. Neutrophils were treated with agents immediately before perfusion over the HUVEC, and agents were present throughout measurements; the time scale origin coincides with the start of perfusion of neutrophils. *A*, *B*, and *C*, Effects of perfusion of  $10^{-7}$  M fMLP,  $10^{-8}$  M IL-8, and 1% ZAP (C5a), respectively. Data are the mean  $\pm$  SEM of three or four experiments. In each case, ANOVA showed a significant inhibitory effect of the activating agent on the percentage of migrated neutrophils ( $p < 0.01$ ).

rolling or transmigrating on HUVEC treated with 100 U/ml TNF, while  $10^{-9}$  M fMLP caused rolling cells to become stationary but had little effect on the proportion transmigrating (Fig. 5). When IL-8 was perfused over neutrophils adherent to HUVEC treated with 2 U/ml, 1 ng/ml had no evident effect on the behavior of adherent neutrophils, while 100 ng/ml inhibited transmigration in a similar manner as 10 ng/ml (data not shown).

Interestingly, if PAF ( $10^{-7}$  M) was used to treat neutrophils, they became activated (i.e., there was no rolling adhesion after treatment), but transmigration was not inhibited. When PAF was perfused over neutrophils bound to HUVEC treated with 2 U/ml TNF, transmigration was actually increased compared with that in untreated neutrophils (Fig. 6A). When neutrophils were treated with PAF just before perfusion over HUVEC treated with 100 U/ml TNF, transmigration was not significantly affected (Fig. 6B).

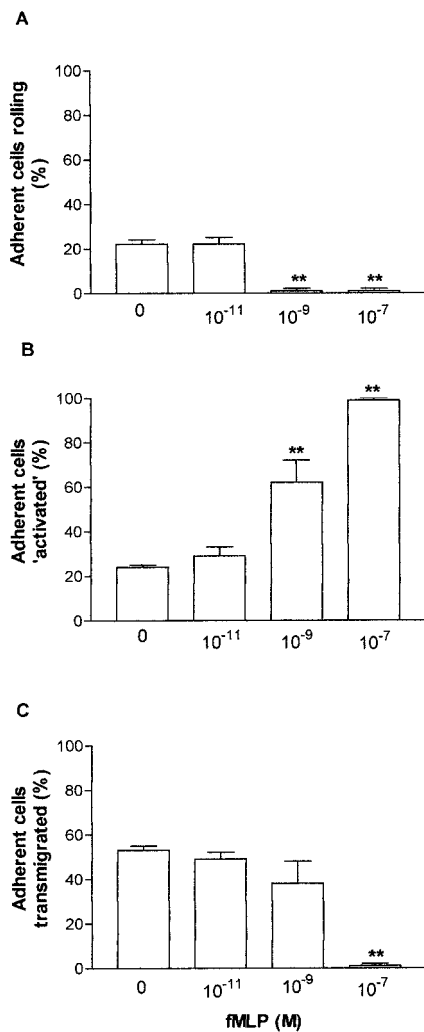
It was evident that none of these exogenous agents markedly impaired the adhesiveness or motility of neutrophils, but that effects were specific to transmigration. Thus, only fMLP tended to decrease binding when neutrophils were treated just before perfu-



**FIGURE 4.** Effect of washout of fMLP on its ability to inhibit transmigration of neutrophils through the HUVEC treated with 2 (*A*) or 100 (*B*) U/ml of TNF for 4 h. *A*, fMLP was perfused over adherent cells for 1 min and then washed out for 10 min. *B*, Neutrophils were pretreated and were perfused in the presence of fMLP for 4 min, and fMLP was then washed out for 10 min. The time scale origin coincides with the start of perfusion of neutrophils. Data are the mean  $\pm$  SEM of four experiments. In each case, ANOVA showed that the effect of fMLP was significant ( $p < 0.01$ ) even though it had been washed out.

sion, and no agent caused loss of binding of neutrophils already adherent to HUVEC (Table I). Moreover, the agents actually increased the speed of migration of neutrophils on top of the HUVEC treated with either dose of TNF (Fig. 7).

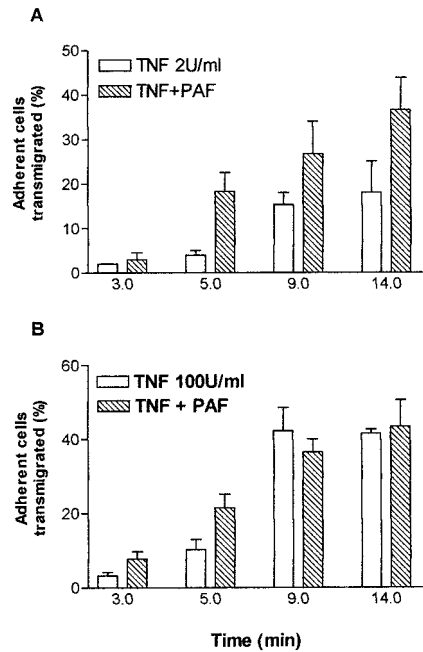
The identity of the endogenous chemotactic agent(s) responsible for activation of neutrophils rolling on the surface of TNF-treated HUVEC is uncertain. In our previous studies (27), blockade of PAF receptors on neutrophils or exposure of endothelial cells to neutralizing mAb against IL-8 had no effect on activation or transmigration. We thus investigated whether mAb 9H1 against CXCR1 or mAb 10H2 against CXCR2 could inhibit neutrophil activation and migration on HUVEC. Treatment of neutrophils with mAb 9H1 had no effect on patterns of adhesion and migration on HUVEC treated with 100 U/ml TNF. However, mAb 10H2 caused most neutrophils to continue rolling, allowed relatively few to become activated, and nearly abolished transmigration (Fig. 8). A combination of the Abs had no additional effect (data not shown). mAb 9H1 and 10H2 have previously been shown to block approximately 90% of the binding of IL-8 to cells transfected with CXCR1 or CXCR2 individually and blocked 20 and 50% of IL-8 binding to neutrophils, respectively (34, 35). We achieved similar levels of inhibition of binding of biotinylated IL-8 with the individual mAbs (24 and 44%; mean from three experiments), and maximal inhibition of binding was 67% when they were used in combination. By immunofluorescence, we verified that 98 and 86% of neutrophils were positive for binding of 9H1 and 10H2, respectively (means from three experiments). We also examined their ability to block activation by IL-8 itself, judged by an increase in the expression of CD11b. IL-8 (10 ng/ml for 20 min) increased the expression of CD11b by  $102 \pm 26\%$ , while after treatment with a combination of both Abs the increase was  $77 \pm$



**FIGURE 5.** Effects of different concentrations of fMLP on the percentage of adherent cells rolling (A), the percentage of adherent cells activated (B; i.e., migrating on the HUVEC), the percentage of adherent cells transmigrated through HUVEC treated with 100 U/ml TNF (C). Neutrophils were treated with fMLP immediately before perfusion over the HUVEC, and fMLP was present throughout; data are shown for measurements made 9 min after the start of perfusion and are the mean  $\pm$  SEM from three experiments. ANOVA showed significant effects of concentration of fMLP on each parameter ( $p < 0.01$ ). \*\*,  $p < 0.01$  compared with samples without fMLP, by paired  $t$  test.

19% (mean  $\pm$  SEM from four experiments;  $p > 0.05$ ). The individual Abs did not inhibit the increase in expression of CD11b in response to IL-8. Finally, we checked whether the mAbs themselves caused neutrophil activation. Neither mAb caused an increase in CD11b expression (expression was 94 or 97% of the untreated control value for 9H1 or 10H2 respectively; means from two experiments with each mAb).

The above results suggested that neutrophil activation occurred through a chemokine(s) that binds to CXCR2, other than IL-8. ENA-78 and GRO- $\alpha$  are known to be able to act through this receptor, with a  $K_d$  lower than that for CXCR1, and to be chemotactic for neutrophils (36–38). Their effects on adhesion of flowing neutrophils have not been described. First, we tested their ability to up-regulate the expression of CD11b. At 1  $\mu$ g/ml ( $\sim$ 100 nM), each caused an increase in CD11b similar to that found with 10 ng/ml IL-8 (99, 94, and 96% increase with IL-8, ENA-78, and GRO- $\alpha$ , respectively; means after 20-min treatment in three com-

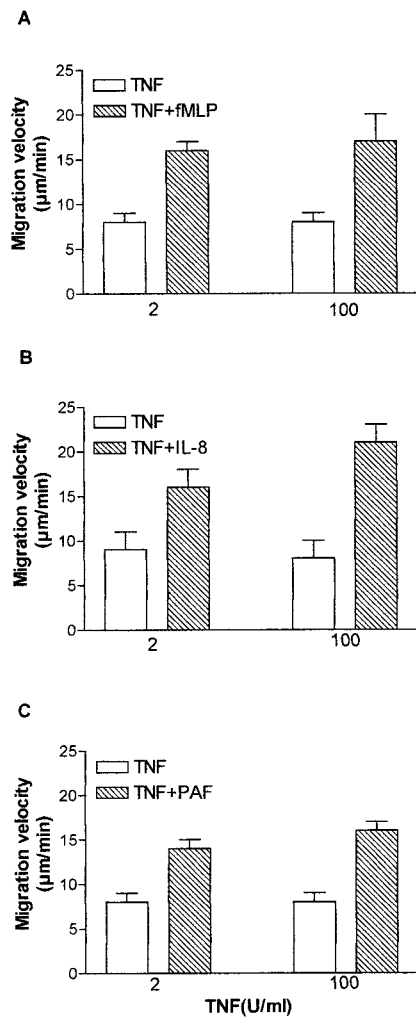


**FIGURE 6.** Effect of PAF on the percentage of adherent neutrophils transmigrated with time when HUVEC was stimulated with 2 (A) or 100 (B) U/ml TNF. A, PAF was perfused over neutrophils already adherent to the HUVEC and remained present throughout measurement. B, Neutrophils were treated with PAF immediately before perfusion over the HUVEC, and PAF was present throughout the measurements. The time scale origin coincides with the start of perfusion of neutrophils. A, ANOVA showed a significant increase in transmigration after treatment with PAF ( $p < 0.05$ ). There was no significant effect of treatment in B.

parative experiments). We also checked whether mAb 9H1 or 10H2 inhibited this up-regulation. Perhaps surprisingly, either Ab reduced up-regulation by about 50% for each chemokine (pooling data for two tests with each Ab on each chemokine; inhibition average, 53%). However, it should be noted that at 100 nM, each chemokine is at a concentration greater than its  $K_d$  for CXCR1 or CXCR2 (36), so receptor selectivity may not be expected. A combination of Abs reduced up-regulation of CD11b by 80% for ENA-78 and 56% for GRO- $\alpha$  (means from two experiments each).

When either ENA-78 or GRO- $\alpha$  was perfused at 1  $\mu$ g/ml over neutrophils already adherent to HUVEC that had been treated with 2 U/ml TNF, rolling rapidly converted to stationary adhesion, and cells changed shape and migrated over, but not through, the monolayer (Table II). When neutrophils were pretreated and perfused with these agents over HUVEC that had been treated with 100 U/ml TNF, they adhered without rolling and were motile, but failed to transmigrate efficiently (Table II). It was notable in the experiments with HUVEC treated with 100 U/ml TNF that when the agents were washed out after the neutrophil bolus, some rolling and transmigration was re-established within 10 min, especially for ENA-78 (Table II).

Of all the activators tested, PAF was the only one that allowed and possibly promoted transmigration. We therefore tested whether this agent could reconstitute transmigration after it had been blocked through other treatments. Neutrophils adherent to HUVEC that had been treated with 2 U/ml TNF were superfused with IL-8 for 1 min and then with IL-8 plus PAF for 10 min. Transmigration was not reintroduced by PAF (only 5% transmigration with PAF plus IL-8 vs 39% without added activators, means from two experiments). When transmigration on HUVEC

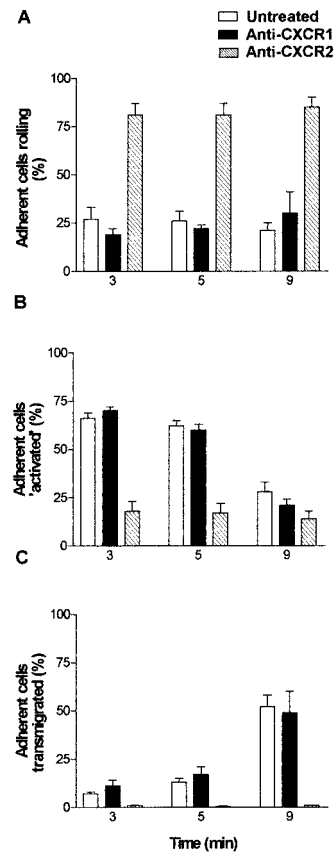


**FIGURE 7.** Effects of exogenous activating agents on the velocity of migration of neutrophils on the surface of HUVEC stimulated with 2 or 100 U/ml TNF. For 2 U/ml TNF, agents were perfused over neutrophils already adherent to the HUVEC and remained present throughout measurement. For 100 U/ml TNF, neutrophils were treated with agents immediately before perfusion over the HUVEC, and agents were present throughout the measurements. A, B, and C, Effects of perfusion of  $10^{-7}$  M fMLP,  $10^{-8}$  M IL-8, and PAF, respectively. Data are the mean  $\pm$  SEM of three or four experiments. In each case, ANOVA showed a significant effect of activating agent on migration ( $p < 0.05$ ), but no difference in velocity between 2 and 100 U/ml TNF

treated with 100 U/ml TNF was inhibited by treatment of neutrophils with mAb 10H2, perfusion of PAF over the neutrophils caused immobilization of rolling neutrophils but no increase in transmigration ( $9 \pm 4\%$  of adherent neutrophils migrated over 5 min with PAF vs  $13 \pm 3\%$  without PAF; means from three experiments).

## Discussion

Previous studies have shown that when neutrophils are perfused over an endothelial monolayer that has been treated with TNF or IL-1, they bind efficiently and migrate through it in minutes (27, 32, 39). Here, however, it was apparent that if the neutrophils were exposed to exogenous activators immediately before or during attachment to HUVEC treated with TNF, then the migration process could be disrupted so that the cells bound to the monolayer but were unable to penetrate it. While the chemotactic agents fMLP,



**FIGURE 8.** Effect of treatment of neutrophils with mAb against CXCR1 or CXCR2 on the percentage of adherent cells rolling (A), the percentage of adherent cells activated (B; i.e., migrating on the HUVEC), and the percentage of adherent cells transmigrated through HUVEC treated with 100 U/ml TNF (C). Neutrophils were treated with mAb for 20 min before perfusion over the HUVEC. The time scale origin coincides with the start of perfusion of neutrophils. ANOVA showed a significant effect of treatment on each parameter and specifically that neutrophils treated with mAb against CXCR2 differed from untreated cells ( $p < 0.01$  in each case).

IL-8, C5a, ENA-78, and GRO- $\alpha$  were each capable of causing such disruption, PAF was not. The results imply that successful transmigration may depend on the order or mode of presentation of chemotactic agents and on the specific agent presented, and that incorrect presentation in vivo could cause neutrophils to be trapped in vessels.

The adhesion molecules by which neutrophils bind to cytokine-treated endothelial cells have been well described (2, 3), but the signals that regulate migration over and through the monolayer are less clear. In the present model using TNF-stimulated HUVEC, initial attachment from flow and subsequent rolling adhesion were supported by endothelial E- and P-selectin, and immobilization and migration were dependent on neutrophil  $\beta_2$  integrin (27). Migration on endothelial cells has previously been shown to be supported by interaction between  $\beta_2$  integrins and ICAM-1, ICAM-2, and possibly additional receptors on endothelium (4, 40). In general, neutrophils can migrate along a concentration gradient of chemotactic agent (41) at a rate that depends on the steepness of the gradient (42), although a gradient of a soluble agent(s) can hardly exist in the lumen of a perfused blood vessel. In flow models, the rate and direction of migration driven by a uniform concentration of chemotactic agents can be modulated by ligation of neutrophil CD31 and  $\alpha_v\beta_3$  integrin, respectively (7, 43). Others have shown that endothelial transmigration is regulated through ligation of

Table II. Effect of ENA-78 and GRO- $\alpha$  on the proportion of adherent neutrophils transmigrated through HUVEC treated with 2 or 100 U/ml TNF<sup>a</sup>

	Percentage of Adherent Neutrophils Transmigrated After			
	3 min	5 min	9 min	14 min
2 U/ml TNF				
Activating agent				
None	3 $\pm$ 1	9 $\pm$ 3	22 $\pm$ 7	30 $\pm$ 9
ENA-78	3 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 2
GRO- $\alpha$	2 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 2
100 U/ml TNF				
Activating agent				
None	14 $\pm$ 5	38 $\pm$ 10	79 $\pm$ 8	84 $\pm$ 2
ENA-78	1 $\pm$ 1	4 $\pm$ 2	13 $\pm$ 2	36 $\pm$ 6
GRO- $\alpha$	1 $\pm$ 1	2 $\pm$ 1	12 $\pm$ 6	18 $\pm$ 7

<sup>a</sup> For 2 U/ml TNF, agents were perfused over adherent neutrophils. For 100 U/ml TNF, neutrophils were treated with agents immediately before perfusion over the HUVEC. Time is measured from the start of the neutrophil bolus; washout commenced at 4 min. Data are mean  $\pm$  SEM of three experiments. ANOVA showed a significant inhibitory effect of each activating agent on percentage of migrated neutrophils at 2 or 100 U/ml TNF ( $p < 0.01$ ).

CD31 both in vitro and in vivo (5, 6). Thus migration over and through endothelium may require adhesive signals acting in concert with chemotactic or chemokinetic stimulus. Moreover, it is clear that activation per se is not adequate to cause transmigration, because exogenous agents used here caused immobilization of rolling neutrophils and increased the speed of subsequent migration, but disallowed diapedesis. Our results raise questions about the specificity, sequence, mode of presentation, and concentration of agent(s) required for this step.

Considering undisturbed migration through TNF-treated HUVEC first, others have implicated IL-8 and PAF as neutrophil activators (17, 44, 45). However, in our previous flow-based studies using TNF-stimulated HUVEC, treatment of neutrophils with PAF receptor antagonists or of HUVEC with neutralizing Ab against IL-8 had no effect on migration (27). Here, we found that blockade of CXCR2 (IL8RB), but not CXCR1 (IL8RA), greatly reduced both transmigration and conversion to stationary adhesion. CXCR-2 is the higher affinity IL-8R (36) and has been specifically implicated in the promotion of cell migration (46). ENA-78 and GRO- $\alpha$  can also activate neutrophils through CXCR2 and induce migration, and have higher affinity for CXCR2 than CXCR1 (36–38). Both ENA-78 and GRO- $\alpha$  can be produced by activated endothelial cells (47, 48). We found that either could cause up-regulation of CD11b and immobilization of rolling neutrophils as efficiently as IL-8, albeit at a much higher concentration (1  $\mu$ g/ml). At this concentration their up-regulation of CD11b did not appear to be specifically through CXCR2, judged from blockade with Abs against CXCR1 and CXCR2. However, if presented on HUVEC at low concentration, ENA-78 or GRO- $\alpha$  should selectively act through CXCR2 and might then induce neutrophil activation and migration. To date, we have not been able to determine whether they are present or active on TNF-treated HUVEC. We conclude that when endothelial cells are exposed to TNF, neutrophils are captured by selectins and then use CXCR2 to ligate a surface-bound, unidentified CXC-chemokine(s) that induces integrin activation. We cannot rule out the possibility that separate agents promoted immobilization and transmigration. It was notable, however, that of those neutrophils that did become activated after blockade of CXCR2, few went on to transmigrate.

Considering disruption of migration by exogenous peptide agents next, this may be explained by their desensitization of the

response of neutrophils through CXCR2. Each of fMLP, IL-8, and C5a has been shown to desensitize the response of neutrophils to IL-8 (20, 21), which implies loss of function of both CXCR1 and CXCR2. GRO- $\alpha$  has been shown to reduce surface expression of CXCR2, but not CXCR1 (24). The concentration of fMLP or IL-8 that we found to be required for disruption of transmigration ( $\geq 10^{-9}$  M) is close to that previously found to induce effective homologous and heterologous desensitization (21). PAF does not desensitize the response of neutrophils through any of the peptide receptors (21), and this would explain its failure to inhibit transmigration. All the exogenous activators themselves clearly enabled immobilization and migration of neutrophils on the endothelial surface, while only transmigration failed. Thus, if desensitization is the cause of disrupted transmigration, CXCR2 must have been required for this later step as well as for immobilization.

While desensitization may explain why exogenous agents inhibited response to the endogenous inducer(s) of transmigration, it does not explain why added agents did not themselves cause transmigration. This also raises the question of how migration through the endothelium is guided, as opposed to migration over it. Each of the disrupting agents tested here can induce neutrophil motility and chemotaxis (7, 37, 38, 41). While the ability to induce transmigration through a monolayer might be specific to certain agents, several tested here are able to act through CXCR2, which appears to be the endogenous inducer of transmigration. An additional degree of specificity must exist, arising, for instance, from the ability to bind to the endothelial surface. This might explain why fMLP or C5a failed to induce transmigration. However, this ability alone may not be sufficient. We previously found that IL-8 could bind to HUVEC when added exogenously and subsequently induce activation of rolling neutrophils (49), but this agent did not allow transmigration here. The order of the signals might also be critical in the sense that early exposure to an agent may desensitize the response to others or even to the same agent required for transmigration. However, since transmigration was disrupted even when agents were added to neutrophils already adherent, this cannot be the full explanation. It seems therefore that an agent presented from solution is unable to cause transmigration, even if that agent has the potential to induce migration, and can bind to the surface. One possibility is that presentation of an endogenous agent(s) is localized, for instance to endothelial cell margins, and that this guides transmigration. In this scenario, uniform ambient or surface presentation of any chemotactic agents would not be able to induce transmigration.

The concentration of agents may also be critical. For example, at  $10^{-9}$  M, fMLP could activate most neutrophils, as judged by cessation of rolling and shape change, but did not affect the percentage transmigrated. At  $10^{-7}$  M fMLP, transmigration was lost. With increasing concentrations of agents, different thresholds may be reached where cells first become activated but not refractory to other stimuli, and then become refractory at a higher concentration. The loss of response might be through concentration-dependent desensitization of receptors, but it is also possible to envisage that exposure to a high level of ambient chemoattractant simply over-rides a directional stimulus that is still in existence. A linked possibility is that a guidance system into the wall would be disrupted because the presence of soluble agents in the lumen would effectively set up a chemotactic gradient in the opposite direction. However, inhibition of transmigration was still evident when fMLP was washed away and indeed appeared unrecoverable. Moreover, recent studies show that at least in the case of IL-8, neutrophils can move down a concentration gradient when exposed to a positive gradient of a second chemotactic agent such as leukotriene B<sub>4</sub> (50). fMLP did, however, inhibit migration toward



agents such as IL-8 in that (50) and other (22) studies. Thus, disruption of migration may depend on the position of competing agents in a hierarchy of potency of heterologous desensitization, to date defined as fMLP > C5a > IL-8 >> PAF (21, 22, 50), on the concentrations of the agents, and on the order in which they are presented.

The failure of PAF to disrupt migration may be explained by its position in this hierarchy of desensitization. In fact, there is reason to believe that PAF can positively promote transmigration. For instance, when generated and presented by endothelium, PAF appears to induce transmigration as opposed to immobilization of flowing neutrophils (19, 51). In the present studies exogenous PAF actually augmented diapedesis through HUVEC treated with 2 U/ml TNF, but had little effect with 100 U/ml TNF. If added PAF can itself induce transmigration, its failure to do so at 100 U/ml TNF may have because 1) a strong migratory stimulus was already present, and PAF could not add to this; 2) the endogenous activator actually desensitized the migratory ability of PAF; 3) PAF in the soluble phase activated neutrophils but could not induce transmigration (i.e., mode of presentation was crucial); or 4) PAF was already present, as suggested by some studies (44, 45). The last possibility is not consistent with failure of the residual immobilized cells to migrate after Ab blockade of CXCR2 or of PAF receptor antagonist to affect migration in our hands. To investigate the potency of PAF further, we treated neutrophils with anti-CXCR2 to block their activation and transmigration on HUVEC treated with 100 U/ml TNF, but added PAF to the adherent cells. We were surprised to find that PAF did not induce transmigration. This is not consistent with explanations 1 and 2 above and strongly suggests that exogenous PAF added in solution cannot induce transmigration. We also added PAF to neutrophils adherent to HUVEC treated with 2 U/ml TNF shortly after IL-8 had been added to inhibit transmigration. Here, however, the inability of PAF to cause transmigration may be explained by the previously described ability of IL-8 to desensitize the PAF receptor (21). The finding that exogenous PAF did promote transmigration through HUVEC treated with 2 U/ml TNF may be explained if the PAF did not itself drive the migration but added to the effectiveness of an existing agent. Thus, PAF caused immobilization that may have aided the action of the endogenous inducer of transmigration (i.e., the percentage of cells becoming immobilized was increased by PAF, but the percentage of immobilized cells that then transmigrated was not). Hence, even in the case of PAF, which can induce transmigration under the correct circumstances (19, 51), a specific surface distribution might be important.

Others have previously shown that infusion of IL-8 or fMLP into rabbits can cause a loss of ability of neutrophils to migrate into tissue (13, 14), although interpretation of these results is made difficult because inhibition occurred at a stage after the agent itself, and the original circulating neutrophils had been cleared from the blood. Results from stationary adhesion assays indicated that added IL-8 actually inhibited the adhesion of neutrophils to cytokine-treated endothelial cells (15, 16), although various studies using stationary and flow assays indicate that endothelial-generated IL-8 promotes adhesion (17–19). In the present study capture of neutrophils on TNF-treated HUVEC was selectin mediated (27), and pretreatment of neutrophils with IL-8, fMLP, C5a, or PAF had little effect on this process. In addition, already adherent cells treated with these agents remained attached. In either case, neutrophils actually migrated more rapidly on the endothelial monolayer after addition of the agents. Thus, disruption of transmigration cannot be attributed to loss of adhesive or motile capability. The apparent discrepancy between studies of the effects of IL-8 on adhesion may be explained by variations in time course. Activated

neutrophils gradually shed L-selectin and also down-regulate adhesion through P-selectin glycoprotein ligand 1 after activation (52, 53) and may thus decrease their capture by endothelium with time. We detected relatively little loss of adhesion, presumably because cells either contacted HUVEC within a short period of exposure to exogenous activators or were already bound at the time of exposure. In either case neutrophils could use integrins to become firmly bound. We did not use prolonged pre-exposure in our studies, as we considered a short time scale more appropriate to the circulation time of the blood in vivo of about 1 min.

Disordered migration may be pathogenic. Neutrophils adherent to the surface of endothelial cells but unable to transmigrate out of microvessels will increase resistance to flow (54) and also have the potential to release lytic enzymes and reactive oxygen species. We did not test whether these potentially damaging compounds were released from cells migrating over HUVEC, but our recent studies in a flow model did show that elastase was released from adherent cells exposed to fMLP, C5a, and IL-8, but not PAF (33). Agents such as fMLP and C5a should not normally be encountered in the vascular lumen, and their immunological roles may reside in their ability to cause extravascular chemotaxis and to encourage bacterial killing and tissue remodeling. However, local or systemic release into the blood may occur in situations such as septic shock or autoimmune disease. High levels of IL-8 have been associated with a range of vascular pathologies (55, 56), while anti-neutrophil cytoplasm Abs associated with vasculitis may cause trapping of neutrophils in microvessels (12) and can inhibit chemotaxis of neutrophils by causing them to release IL-8 (57). Thus, a range of immune mediators required for host protection may actually cause tissue damage if they are released at times, concentrations, or sites that are inappropriate and then cause intravascular trapping of neutrophils.

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