

# Neutrophil Migration across Monolayers of Cytokine-prestimulated Endothelial Cells: A Role for Platelet-Activating Factor and IL-8

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**Abstract.** In a previous study we observed that neutrophils respond with a rapid rise in  $[Ca^{2+}]_i$  during adherence to cytokine-activated endothelial cells (EC), caused by EC membrane-associated platelet-activating factor (PAF). In the present study, we investigated whether this form of PAF was important in neutrophil adherence and migration across monolayers of rIL-1 $\beta$ - or rTNF $\alpha$ -prestimulated EC. PAF receptor antagonists prevented neutrophil migration across cytokine-pretreated EC by  $\sim 60\%$  ( $P < 0.005$ ) without interfering with the process of adherence. The antagonists WEB 2086 and L-652,731 had no effect on neutrophil migration across resting EC induced by formyl-methionyl-leucyl-phenylalanine (FMLP). A murine

anti-IL-8 antiserum was found to also partially inhibit the neutrophil transmigration across cytokine-activated EC. When the anti-IL-8 antiserum was used in combination with a PAF receptor antagonist, neutrophil migration across cytokine-pretreated monolayers of EC was completely prevented. During transmigration, LAM-1 and CD44 on the neutrophils were downmodulated; both WEB 2086 and anti-IL-8 antiserum partially prevented this downmodulation caused by cytokine-prestimulated EC.

Our results indicate that human neutrophils are activated and guided by EC-associated PAF and EC-derived IL-8 during the in vitro diapedesis in between cytokine-stimulated EC.

**T**RANSMIGRATION of neutrophils from the peripheral circulation into inflammatory sites requires neutrophil adherence to the endothelial cell lining of the blood vessels. The CD18 complex proteins are: lymphocyte function-associated antigen-1 (LFA-1),<sup>1</sup> complement receptor 3 (CR3) or Mac-1, and glycoprotein 150,95 (p150,95). On neutrophils they are involved in both migration and adherence (Anderson et al., 1985; Harlan, 1985; Kuijpers and Roos, 1989). In these processes, endothelial intercellular adhesion molecule-1 (ICAM-1) may function as a cellular ligand for LFA-1 and CR3 (Rothlein et al., 1986; Diamond et al., 1990; Springer, 1990). Its expression can be increased by the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Dustin et al., 1986; Springer, 1990).

Several other adhesion molecules can play an accessory role in the process of neutrophil adherence. The role of a

family of homologous lectin-like molecules in adherence is well established. The human selectin family consists of ELAM-1, GMP-140, and LAM-1 (Bevilacqua et al., 1989; Johnston et al., 1989; Tedder et al., 1989). The endothelial cell-specific adhesion molecule ELAM-1 is not expressed on resting endothelium, but can be transiently induced by IL-1 and TNF with a peak after 4–6 h of cytokine treatment (Bevilacqua et al., 1987; Leeuwenberg et al., 1989; Lusinskas et al., 1989). Upon stimulation of endothelial cells with thrombin or histamine, a momentary upregulation of GMP-140 takes place, as a result of the fusion of the intracellular Weibel-Palade bodies with the plasma membrane. GMP-140, together with enhanced expression of EC-associated platelet-activating factor (PAF), causes transient adherence of neutrophils to the endothelial cells (Gamble et al., 1990; Geng et al., 1990; Lorant et al., 1991). Recent evidence suggests a role for leukocyte adhesion molecule-1 (LAM-1) in the recruitment of leukocytes to inflammatory lesions (Smith et al., 1991; Watson et al., 1991). A role for Mel-14 (the murine equivalent of the homing receptor LAM-1; Camerini et al., 1990) to neutrophil migration was demonstrated in a murine model: neutrophils present in the tissues did not express Mel-14, in contrast to neutrophils in the circulation (Juttila et al., 1989). The polymorphic integral membrane protein CD44 (also known as the Hermes antigen, ECMRIII, Hutch-1, or murine Pgp-1) is a distinct

1. *Abbreviations used in this paper:* CR3, complement receptor 3; EC, endothelial cells; ELAM-1, endothelial leukocyte adhesion molecule-1; FMLP, formyl-leucyl-methionyl-phenylalanine; HSA, human serum albumin; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; LAM-1, leukocyte adhesion molecule-1; LFA-1, lymphocyte-function associated antigen-1; MFI, mean fluorescence intensity; PAF, platelet-activating factor; p150,95, (glyco)protein 150,95; rIL-1 $\beta$ , recombinant interleukin-1 $\beta$ ; rIL-8, recombinant interleukin-8; rTNF $\alpha$ , recombinant tumor necrosis factor- $\alpha$ ; TNF, tumor necrosis factor.

adhesion molecule. It recognizes hyaluronate (Aruffo et al., 1990; Miyake et al., 1990) and has been implicated in processes such as lymphocyte binding to high endothelial venules and matrix adhesion (Haynes et al., 1989).

We simulated the *in vivo* situation in an *in vitro* migration assay using a two-compartment system. The transmigration of neutrophils along a chemotactic gradient of formyl-leucyl-methionyl-phenylalanine (FMLP) was compared with the effect of migration through cytokine-prestimulated monolayers of endothelial cells (EC) (Furie and McHugh, 1989; Moser et al., 1989; Hakker et al., 1990). To a large extent the enhanced neutrophil adherence to cytokine-prestimulated EC can be ascribed to the expression of endothelial leukocyte adhesion molecule-1 (ELAM-1) (Bevilacqua et al., 1987, 1989; Luscinskas et al., 1989; Hakker et al., 1991), but the reason for the directed migration of neutrophils across these activated EC monolayers is unknown. We recently found endothelial cell-associated PAF (or PAF-like) molecules to be responsible for the rise of  $[Ca^{2+}]_i$  in neutrophils during their adherence to recombinant interleukin-1 $\beta$  (rIL-1 $\beta$ )-prestimulated EC. We assumed a functional role for this form of endothelial PAF in guiding the neutrophils in between the EC into the subendothelial area (Kuijpers et al., 1991a). In the present study, we describe indeed a role for this form of PAF on the surface expression of LAM-1, CD44, CD11b, and CD18 on neutrophils during the diapedesis across cytokine-prestimulated monolayers. Moreover, both this EC-associated form of PAF as well as the EC-derived chemoattractant IL-8 is shown to be of major importance in the *in vitro* neutrophil migration across cytokine-pretreated monolayers of human EC.

## Materials and Methods

### Reagents

FMLP and PAF were purchased from Sigma Chemical Co. (St. Louis, MO). The PAF receptor antagonist WEB 2086 (Dent et al., 1989) was a kind gift of Dr. H. Heuer (Boehringer Mannheim GmbH, Mannheim, Germany); L-652,731 (Hwang et al., 1985) was obtained from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ). These agents were dissolved in DMSO at 1,000 times the final concentration for cell stimulation, and were stored at  $-20^{\circ}\text{C}$ . The engineered cDNA BBG44 coding for human IL-8 ((Ser)-IL-8)<sub>72</sub> was purchased from British Biotechnology Limited (Oxford, UK). The cDNA was expressed in *Escherichia coli* strain DH5 $\alpha$  with the pMBL11 plasmid. Finally, 10  $\mu\text{g}$  of highly purified (bioactive) hrIL-8 was injected *i.p.* in a BALB/c mouse for the generation of monoclonal anti-hrIL-8 antibodies. In a standard radioimmunoassay, the (polyclonal) anti-IL-8 serum ( $\alpha$ IL-8 serum) obtained from the same animal was found to specifically recognize hrIL-8 up to a  $10^6$ -fold dilution of the antiserum (Margreet Hart, unpublished results).

The basal incubation medium for cell suspensions consisted of a 1:1 mixture of RPMI-1640 and Medium 199 (Gibco, Paisley, UK), supplemented with 0.5% (wt/vol) human serum albumin (HSA).

### Monoclonal Antibodies

The following mAbs were used: CD11b (CLB-B2.12) (Van der Reijden et al., 1983), CD18 (CLB-LFA1/1) (Miedema et al., 1984), CD32 (IV.3) (Roosenfeld et al., 1985), CD44 (PI) (Pals et al., 1989), anti-HLA class-I mAb W6/32, and anti-LAM-1 mAb Leu-8 (Kansas et al., 1985). The latter mAb was obtained from Becton Dickinson & Co. (San Jose, CA).

### Granulocyte Isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from the buffy coat of 500 ml of blood anti-coagulated with 0.4% (wt/vol)

trisodium citrate (pH 7.4) as described (Roos and De Boer, 1986). In short, blood cells were separated by density gradient centrifugation over isotonic Percoll with a specific gravity of 1.076 g/ml. The interphase, containing the mononuclear cells, was removed. The pellet fraction, containing erythrocytes and granulocytes, was treated for 10 min with ice-cold isotonic  $\text{NH}_4\text{Cl}$  solution (155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 0.1 mM EDTA, pH 7.4) to lyse the erythrocytes. The remaining granulocytes were washed twice in PBS containing HSA (0.5% wt/vol). Granulocytes were resuspended in the incubation medium at a final concentration of  $1 \times 10^6$  cells/ml. Purity of the granulocytes was  $>98\%$ , with  $>95\%$  neutrophils.

### Labeling of Neutrophils

Freshly purified neutrophils were radiolabeled with  $^{51}\text{Cr}$  according to Gallin et al. (1973). Briefly, neutrophils ( $10^7$  cells/ml) were incubated with 1  $\mu\text{Ci}$   $^{51}\text{Cr}$  per  $10^6$  cells (sodium chromate, 200 to 500 Ci/g; New England Nuclear, Boston, MA) in incubation medium (containing 0.1% HSA instead of 0.5% HSA) at  $37^{\circ}\text{C}$  for 1 h under gently shaking conditions and subsequently washed. Viability after labeling was  $>95\%$ .

### Endothelial Cell Culture

EC were isolated from human umbilical cord veins according to Jaffe et al. (1973) with minor modifications (Willems et al., 1982). Cells were first cultured in plastic flasks (80  $\text{cm}^2$ ) precoated with fibronectin. The culture medium consisted of an equal mixture of RPMI-1640 and Medium 199, supplemented with 20% (vol/vol) heat-inactivated human serum, penicillin (100 U/ml; Gibco), streptomycin (100  $\mu\text{g}/\text{ml}$ ; Gibco), fungizone (2.5  $\mu\text{g}/\text{ml}$ ; Gibco), and glutamine (2 mM). After one or two passages with trypsin/EDTA (Gibco), EC were subcultured to confluent monolayers on tissue culture-treated polycarbonate membranes (8.0- $\mu\text{m}$  pore size, 24-mm diam) of Costar Transwell cell culture chamber inserts (Costar, Cambridge, MA). The filter chambers were precoated with fibronectin before EC were added for further culturing. EC monolayers reached confluence in 3 to 4 d (as determined by May-Grünwald/Giemsa staining or silver nitrate staining; Furie et al., 1984). EC were held in culture for another 1 to 2 d to attain a real tight (post-)confluent state, and were checked once again before use. Pretreatment of EC monolayers with optimal concentrations of human recombinant IL-1 $\beta$  (10 U/ml) or TNF $\alpha$  (50 ng/ml) (see below) did not change the state of confluence.

Immunoperoxidase staining for von Willebrand Factor was homogeneous in the cultures. Volumes of the lower and upper chambers were 2.6 and 1.5 ml, respectively.

### Adherence and Migration Assay

The confluent monolayers were washed twice with basal incubation medium prewarmed to  $37^{\circ}\text{C}$ . Prewarmed incubation medium with or without an optimal amount of the chemoattractant FMLP (10 nM) was added to the lower chambers.  $^{51}\text{Cr}$ -labeled neutrophils ( $1 \times 10^6$  cells/ml), prewarmed for 10 min at  $37^{\circ}\text{C}$ , were then added to the upper compartments. In some experiments, neutrophils were preincubated for 10 min with PAF receptor antagonists WEB 2086 (10  $\mu\text{M}$ ) or L-652,731 (100  $\mu\text{M}$ ) before use. The same amount of DMSO was added to the control experiments (which had no significant effect on adherence, transmigration, or surface antigen modulation). The PAF receptor antagonists remained present during the migration assay. The EC were preincubated for 20 min with the anti-IL-8 antiserum (complement inactivated by a  $56^{\circ}\text{C}$  treatment for 45 min), before the neutrophils were added. The anti-IL-8 antiserum remained present during the assay. There was no significant effect on adherence, transmigration, or surface antigen modulation observed with preimmune, complement-inactivated serum of BALB/c mice (tested at 1:100 diluted).

The chamber plates were then incubated at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  incubator for 30 min (or other times as indicated). After 30 min, neutrophil fractions were collected, and diluted with a fixed amount of incubation medium used to wash the upper and lower chambers. Radioactivity was determined in three cell fractions: luminal, abluminal, and "adhering cell" fraction (i.e., the complete membrane, carefully cut out of its container). Recovery was always  $>92\%$ . Adhesion was measured as radioactivity found in the "membrane fraction" and migration was measured as radioactivity found in the abluminal fraction (lower compartment). The results were expressed as percentage of radioactivity added to the chambers.

In the experiments for surface antigen expression (see below), neutrophils were used that had not been radiolabeled but had undergone exactly the same procedure as radiolabeled cells (i.e., incubation for 1 h at  $37^{\circ}\text{C}$ , washing, etc.). The transmigration experiments were usually performed

simultaneously with radioactive neutrophils and with non-radioactive neutrophils, for an optimal comparison of adherence/diapedesis and the effects of migration on surface antigen expression.

EC monolayers were pretreated with optimal concentrations of human recombinant IL-1 $\beta$  (rIL-1 $\beta$ , 10 U/ml) or TNF $\alpha$  (rTNF $\alpha$ , 50 ng/ml). The cytokine was left for 4 h with the cells followed by extensive washing of the monolayer with prewarmed incubation medium before use. Human rIL-1 $\beta$  was a kind gift of Dr. P. T. Lomedico (Hoffmann-La Roche, Nutley, NJ). Human rTNF $\alpha$  was a gift of Dr. A. Creasy (Cetus, Oakland, CA).

### PAF Measurement: Extraction and Bioassay

Confluent monolayers of human EC cultured in Petri dishes were maximally stimulated either with thrombin (2 U/ml) for 7 min, or with rIL-1 $\beta$  for 4 and 24 h. Lipid extraction was performed according to Bligh and Dyer (1959). After rapid removal of the supernatant, PAF biosynthesis in the monolayers was blocked by addition of 1 ml of methanol containing 50 mM acetic acid. EC were harvested with a rubber scraper. The Petri dishes were rinsed with 2 ml of acidified methanol, and this was added to the cells. Then 2.5 ml of chloroform was added to the cell suspension and the mixture was vortexed for 3 min. Another 2.5 ml of chloroform was added and the mixture was again vortexed, followed by the addition of 2.5 ml of distilled H<sub>2</sub>O. This mixture was again vortexed (30 s), the organic phase was collected and evaporated under nitrogen. HPLC purification was performed essentially as described by Remy et al. (1989). PAF volume and retention time were determined with standard [<sup>3</sup>H]-PAF. The fractions corresponding to PAF were subsequently collected and evaporated under nitrogen.

The quantification of biologically active PAF was performed according to Benveniste et al. (1972). In short, rabbit blood anti-coagulated with 0.4% trisodium citrate (wt/vol) was centrifuged at 200 g for 15 min. The platelet-rich plasma was collected. Then platelets were centrifuged at 1,500 g for 15 min, washed twice and resuspended in a buffer containing 137 mM NaCl, 2.6 mM KCl, 1 mM MgCl<sub>2</sub>, 1.3 mM CaCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 0.1% (wt/vol) glucose and 0.25% (wt/vol) gelatine (pH 7.4). Aggregation was performed in a standard aggregometer under continuous stirring at 37°C. In a cuvette, 1.6 ml of the buffer, 25  $\mu$ l of an indomethacin solution (1.5 mg/ml ethanol), and 400  $\mu$ l of the platelet suspension were mixed and prewarmed for 5 min. Then 50  $\mu$ l of sample PAF was added and compared with the effect of standard PAF. The amount of endothelial PAF was calculated from the PAF standard aggregation curve.

WEB 2086 and L-652,731 specifically inhibited the standard PAF activity and the EC-derived PAF activity on rabbit platelets (not shown). These PAF receptor antagonists also inhibited other biological effects of PAF, such as neutrophil aggregation, elevation of neutrophil [Ca<sup>2+</sup>], and priming of neutrophil oxidase activity. In contrast, these antagonists did not influence similar effects of FMLP, recombinant C5a or IL-8 on neutrophils. WEB 2086 was found to bind only to membranes of COS-7 cells specifically

transfected with the cloned PAF receptor (Honda et al., 1991). Thus, WEB 2086 and L652,731 appear to be both effective and specific PAF receptor antagonists (see also Kuijpers et al., 1991a). In addition, the neutrophil-activating lipid associated with the EC plasma membrane after treatment of the EC with thrombin (Lorant et al., 1991), or rIL-1 $\beta$  (Kuijpers et al., 1991a) appears to be PAF.

### Determination of Surface Antigen Expression

Neutrophils in suspension or collected from either the upper or lower compartment were used for indirect immunofluorescence and flowcytometric analysis on a FACScan (a registered trademark of Becton Dickinson & Co., Mountain View, CA). The effects of FMLP (10 nM)-dependent transmigration were compared with the influence of FMLP (10 nM) on neutrophils held in suspension at 37°C during the same time needed for the transmigration assay (i.e., 30 min, see above). The collected neutrophils were washed with ice-cold PBS and subsequently incubated with primary antibody for 30 min at 4°C. Thereafter, the cells were washed twice in excess of ice-cold PBS, and the procedure was repeated with FITC-labeled goat-anti-mouse-Ig for another 30 min at 4°C. Neutrophils were distinguished by forward-sideward scatter pattern. Data were collected from 10,000 cells and are represented as Mean Fluorescence Intensity (MFI). No subpopulations were observed.

### Statistical Analysis

For statistical analysis *t* tests were performed. *P* values >0.05 were considered NS.

## Results

### Adhesion and Migration

Previous studies have already shown a transmigration of neutrophils across EC monolayers prestimulated for 4 to 8 h with IL-1 (Furie and McHugh, 1989; Moser et al., 1989; Hakkert et al., 1990). However, the mechanism underlying this process remained unexplained. We adopted a chamber system with confluent monolayers of EC cultured on top of microporous membranes to simultaneously study the process of adherence and migration. In the presence of a chemotactic amount of FMLP (10<sup>-8</sup> M) in the lower compartment, the optimal adherence of neutrophils to the resting

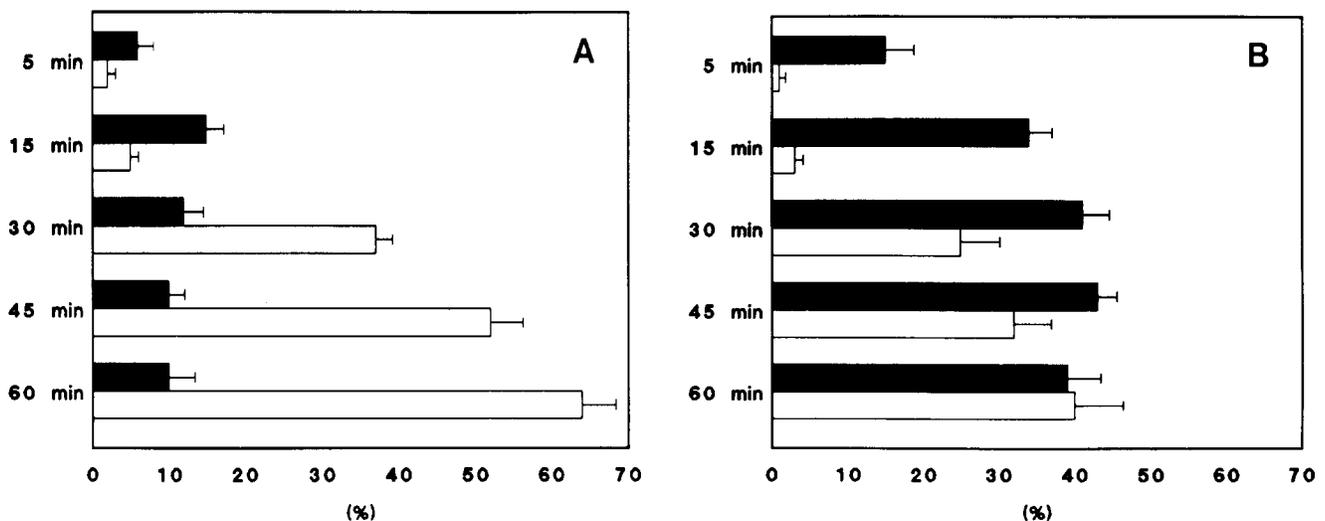


Figure 1. Neutrophil adherence to and migration across confluent monolayers of EC. At various times both adherence and migration were determined. (A) Neutrophils were attracted to the lower chamber by a chemotactic amount of FMLP (10<sup>-8</sup> M). (B) The EC were pre-stimulated for 4 h with rIL-1 $\beta$ . Results expressed as the mean  $\pm$  SEM for three experiments on different occasions. (■) Adherence; (□) migration.

EC monolayers was observed at 10–15 min after the neutrophils had been added to the upper compartment (Fig. 1 A). On the other hand, FMLP-induced migration continued to increase (Fig. 1 A). Experiments with rIL-1 $\beta$ -pretreated monolayers showed optimal adherence between 15 and 30 min and considerable neutrophil migration at about 30 min (Fig. 1 B). Adherence of neutrophils to recombinant tumor necrosis factor- $\alpha$  (rTNF $\alpha$ )-pretreated EC also reached its optimum at 30 min (not shown). In all further experiments, we therefore allowed neutrophils to adhere and migrate for 30 min in either the FMLP- or cytokine-dependent assays. Lysis of cells during the migration assay was negligible (LDH release <5%, not shown).

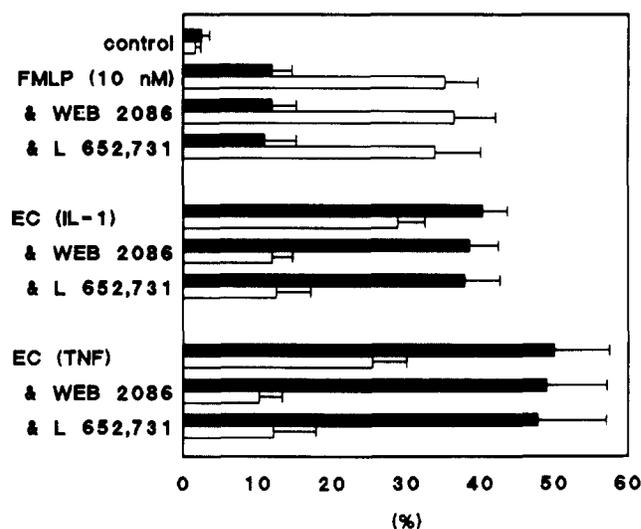
Our previous experiments (Kuijpers et al., 1991a) had disclosed that IL-1 treatment of EC monolayers led to the generation of an EC-associated factor able to induce a rise of [Ca<sup>2+</sup>]<sub>i</sub> in adhering neutrophils. For the detection of [Ca<sup>2+</sup>]<sub>i</sub> changes we used suspended EC, non-enzymatically detached with EDTA-containing medium. This rise of [Ca<sup>2+</sup>]<sub>i</sub> in adhering neutrophils was caused by endothelial PAF, as demonstrated by the fact that: (a) the use of PAF receptor antagonists; (b) neutrophilic PAF receptor desensitization; or (c) PLA<sub>2</sub> inhibitors (mepacrine and *p*-bromophenacylbromide) prevented the rise of [Ca<sup>2+</sup>]<sub>i</sub> in adhering neutrophils. Adherence was insignificantly affected under these conditions. Washing of the cytokine-activated EC did not diminish their capacity to invoke the [Ca<sup>2+</sup>]<sub>i</sub> changes, unless the EC were washed in medium containing defatted albumin, known to remove polar substances such as PAF. The endothelium-derived PAF was not released into the medium, either in the absence or the presence of adhering neutrophils (Kuijpers et al., 1991a). As shown here for rIL-1 $\beta$ , PAF was newly synthesized upon cytokine treatment, although less than observed upon optimal activation of EC monolayers with thrombin (Table I). PAF expression upon EC treatment with rIL-1 $\beta$  (or rTNF $\alpha$ ) followed the same kinetics as ELAM-1 expression, i.e., with a peak at 4–6 h after cytokine addition (Kuijpers et al., 1991a; data not shown).

The percentage of neutrophils migrating into the lower, FMLP-containing chamber was high. PAF receptor antagonists (WEB 2086 or L-652,731) did not affect the FMLP-dependent migration (Fig. 2). When EC were prestimulated for about 4 h with rIL-1 $\beta$  or rTNF $\alpha$  and subsequently washed several times, neutrophils were found to adhere avidly (Fig. 2). Migration across the monolayer into the lower chamber was also considerable (Fig. 2). The PAF receptor antagonists significantly inhibited the neutrophil migration under these conditions ( $P < 0.005$ ), without affecting adherence. However, titration curves reproducibly showed that the PAF

**Table I. Endothelial De Novo Synthesis of PAF upon Stimulation**

Addition	PAF (mol/10 <sup>6</sup> cells)
No stimulus	0
rIL-1 $\beta$ (10 U/ml)	0.12 $\times$ 10 <sup>-12</sup>
thrombin (2 U/ml)	0.30 $\times$ 10 <sup>-12</sup>

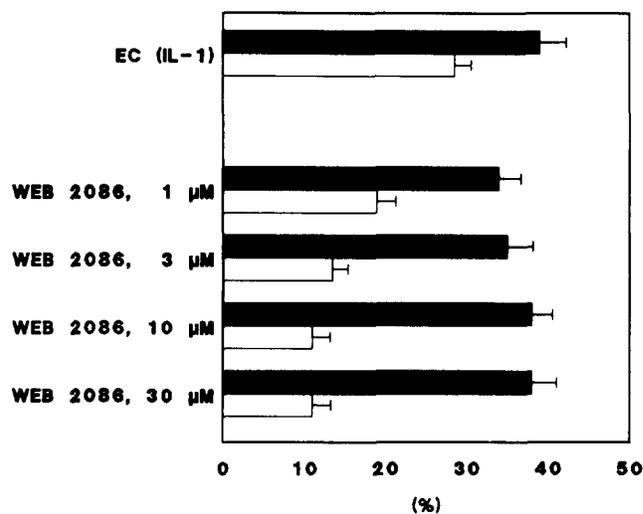
EC were stimulated with rIL-1 $\beta$  (10 U/ml) for 4 h, or with thrombin (2 U/ml) for 7 min, i.e., the time point at which the endothelial PAF synthesis was found to be maximal. Activation of the EC was stopped with methanol (see Materials and Methods). Results are expressed as the mean for three different experiments in duplicate.



**Figure 2.** Neutrophil adherence to and migration across confluent monolayers of EC either resting or prestimulated by rIL-1 $\beta$  or rTNF $\alpha$  for 4 h. In case of resting monolayers of EC, neutrophils were attracted to the lower chamber by a chemotactic amount of FMLP (10<sup>-8</sup> M). The PAF receptor antagonists WEB 2086 (10  $\mu$ M) or L-652,731 (100  $\mu$ M) significantly inhibited neutrophil migration across EC pretreated with rIL-1 $\beta$  or rTNF $\alpha$  ( $P < 0.005$ ). Results expressed as the mean  $\pm$  SEM for six to ten experiments on different occasions. (■) Adherence at 30 min; (□) migration at 30 min.

receptor antagonists did not completely block neutrophil migration, as shown here for WEB 2086 (Fig. 3).

The potential role of IL-8 (Baggiolini et al., 1989), a neutrophil agonistic stimulus generated by (e.g.) cytokine-treated EC (Wheeler et al., 1988; Schröder and Christophers, 1989), was also investigated. We used a 1:1,000 dilution of a murine anti-IL-8 antiserum; this completely prevented neutrophil migration along a gradient of rIL-8 (10<sup>-8</sup> M) (not



**Figure 3.** Neutrophil adherence to and migration across rIL-1 $\beta$ -pretreated EC in the presence of different concentrations of the PAF receptor antagonist WEB 2086. Results are expressed as the mean  $\pm$  SEM for three experiments on different occasions. (■) Adherence at 30 min; (□) migration at 30 min.

**Table II. Role of Endothelial PAF and IL-8 in the Neutrophil Adherence to, and Migration across, Monolayers of EC**

Addition	Adherence	Transmigration
	(%)	(%)
-	2 ± 1.1	2 ± 0.8
FMLP (10 nM)	12 ± 2.7	35 ± 4.5
αIL-8	12 ± 3.2	36 ± 5.6
WEB 2086	13 ± 4.2	37 ± 6.2
αIL-8 + WEB 2086	12 ± 3.8	35 ± 4.9
EC (IL-1)	36 ± 3.4	29 ± 2.7
αIL-8	38 ± 5.1	12 ± 6.2 ( <i>P</i> < 0.005)
WEB 2086	35 ± 4.8	13 ± 4.6 ( <i>P</i> < 0.005)
αIL-8 + WEB 2086	37 ± 5.4	3 ± 2.9 ( <i>P</i> < 0.001)
EC (TNF)	50 ± 7.4	25 ± 4.5
αIL-8	49 ± 5.1	11 ± 3.9 ( <i>P</i> < 0.005)
WEB 2086	49 ± 8.8	10 ± 6.1 ( <i>P</i> < 0.005)
αIL-8 + WEB 2086	47 ± 9.3	4 ± 2.6 ( <i>P</i> < 0.001)

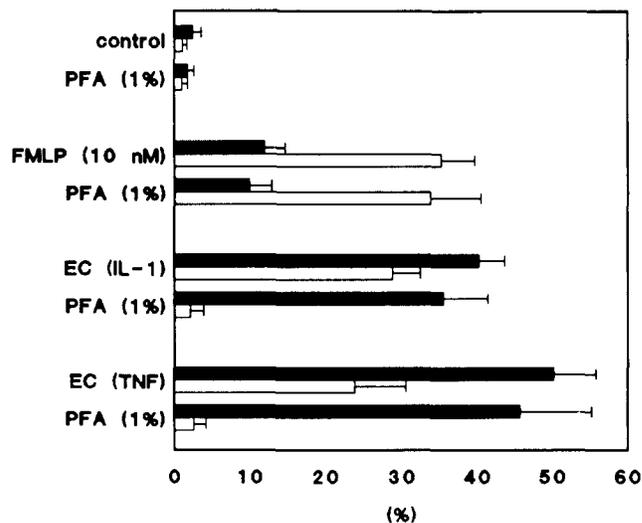
The murine anti-IL-8 antiserum (αIL-8) was used at a fully inhibitory dilution of 1:1,000. The PAF receptor antagonist WEB 2086 was used at a concentration of 10 μM. The reagents remained present during the transmigration. Normal mouse serum did not have any effect. Results are expressed as mean ± SEM of three to six separate experiments.

shown). Whereas the FMLP-dependent transmigration was unaffected in the presence of the anti-IL-8 antiserum, the neutrophil migration across cytokine-activated EC was inhibited by ~60% (Table II). In combination with PAF receptor antagonists, transmigration was totally blocked. However, in the presence of WEB 2086 and the anti-IL-8 antiserum, neutrophils still migrated across the activated EC when FMLP was included in the lower chamber (43% ± 8.4%, mean ± SEM, *n* = 3 at 30 min).

Fixation of monolayers of cytokine-activated EC with PFA (1%, wt/vol) made the EC impermeable to the neutrophils (Fig. 4), unless the exogenous chemoattractant FMLP was added to the lower compartment. The number of neutrophils adhering the cytokine-activated EC was almost unchanged, indicating that PFA-sensitive products of EC (primarily) determine the neutrophil migration across cytokine-activated EC.

### Changes in Neutrophil Surface Antigen Expression

We further investigated whether the change in surface expression of the adhesion molecules CD11b/CD18 and LAM-1 on transmigrating neutrophils (Kuijpers et al., 1992), was a result of the action of PAF or IL-8. Hence, migrated neutrophils in the lower compartment were compared with the neutrophils in the upper compartment. The effects of FMLP (10<sup>-8</sup> M) present in the lower compartment of the two chamber system were related to the changes induced by an identical concentration of FMLP on neutrophils held in suspension during the same period. In suspension, the expression of CD11b (the α-chain of CR3), and CD18 (the common β-chain of LFA-1, CR3 and p150,95) was significantly increased by FMLP, whereas the expression of LAM-1 and CD44 significantly decreased (Table III). The expression of CD32 (FcγRII) (Table III) or HLA class I (not shown) did not change under these conditions.



**Figure 4.** Neutrophil adherence to and migration across mildly fixed confluent monolayers of either resting EC or EC prestimulated for 4 h with rIL-1β or rTNFα before fixation. Monolayers were fixed with prewarmed PFA 1% (wt/vol) in basal incubation medium for 10 min in the incubator. Thereafter, the monolayers were thoroughly washed four times by excess of prewarmed incubation medium before further use. Results are expressed as the mean ± SEM of four different experiments. (■) Adherence at 30 min; (□) migration at 30 min.

The neutrophils that had migrated showed an identical modulation of these surface antigens (Table III). The neutrophils in the upper compartment appeared to be affected as well, as indicated by the significantly changed expression (*p* < 0.05) of CD11b, CD18, CD44, and LAM-1. These last effects can be ascribed to FMLP that diffuses from the lower compartment into the upper chamber. Neutrophils added to the upper chamber in the absence of FMLP did not show the changes in surface antigen expression.

The neutrophils that had migrated across rIL-1β-prestimulated EC showed similar changes in surface antigen expression (Table IV). The relatively larger increases in CD18 expression as compared to CD11b on transmigrated neutro-

**Table III. Changes in Surface Antigen Expression on Neutrophils in Suspension or Migrated across EC Monolayers along a Gradient of FMLP**

	Control (susp)	FMLP (susp)	Luminal	Abluminal
Control	10 ± 0.3	10 ± 0.5	10 ± 0.4	10 ± 0.3
CD11b	289 ± 24.8	455 ± 32.6*	389 ± 47.6	528 ± 57.7*
CD18	61 ± 18.4	193 ± 24.1*	89 ± 9.7	208 ± 40.1*
CD32	108 ± 12.1	101 ± 6.8	115 ± 11.2	112 ± 5.7
CD44	368 ± 13.7	275 ± 15.9*	348 ± 16.4	299 ± 15.4*
LAM-1	121 ± 10.9	17 ± 2.0*	41 ± 6.5	15 ± 1.8*

Neutrophils in suspension were activated with FMLP (10<sup>-8</sup> M) for the same period (30 min) during which migration took place. Results are expressed as MFI ± SEM for experiments on six to ten different occasions. The surface antigen expression of untreated neutrophils in suspension was compared with the expression of the same antigen on FMLP-treated neutrophils in suspension. Similarly, luminal neutrophils were compared with abluminal (transmigrated) neutrophils. Anti-TNP was used as an isotype-matched "control" antibody. Data marked by an asterisk (\*) were significantly different (*P* < 0.05).

**Table IV. Changes in Surface Antigen Expression on Neutrophils Migrated across EC Monolayers Prestimulated for 4 h with rIL-1 $\beta$**

	Luminal –	Abluminal –	Luminal + WEB	Abluminal + WEB	Luminal + $\alpha$ IL-8	Abluminal + $\alpha$ IL-8
Control	10 $\pm$ 0.3	10 $\pm$ 0.5	10 $\pm$ 0.4	10 $\pm$ 0.3	10 $\pm$ 0.5	10 $\pm$ 0.4
CD11b	321 $\pm$ 75.2	465 $\pm$ 34.3*	304 $\pm$ 68.3	342 $\pm$ 87.4	298 $\pm$ 66.6	339 $\pm$ 75.9
CD18	97 $\pm$ 24.1	201 $\pm$ 31.2*	77 $\pm$ 14.5	138 $\pm$ 17.9*	82 $\pm$ 20.7	119 $\pm$ 33.1
CD32	119 $\pm$ 13.3	111 $\pm$ 12.8	107 $\pm$ 7.9	110 $\pm$ 15.1	111 $\pm$ 14.5	115 $\pm$ 16.4
CD44	343 $\pm$ 18.4	275 $\pm$ 7.6*	350 $\pm$ 16.4	317 $\pm$ 46.2	377 $\pm$ 33.3	311 $\pm$ 50.0
LAM-1	83 $\pm$ 11.6	18 $\pm$ 2.2*	85 $\pm$ 11.3	73 $\pm$ 11.8	90 $\pm$ 22.0	69 $\pm$ 16.9

Results are expressed as MFI  $\pm$  SEM for experiments on four to ten different occasions. The surface antigen expression on luminal neutrophils was compared with the expression of the same antigen on abluminal (Transmigrated) neutrophils. The PAF receptor antagonist WEB 2086 was present at 10  $\mu$ M. L-652, 731 was not tested in this set of experiments. The murine anti-IL-8 antiserum ( $\alpha$ IL-8) was used at a fully inhibitory dilution of 1:1,000. Data marked by an asterisk (\*) were significantly different ( $P < 0.05$ ).

phils (Tables III and IV) can be explained by the simultaneous upregulation of the  $\alpha$ -subunit CD11c of p150,95 (CD11c/CD18), whereas LFA-1 (CD11a/CD18) remained unchanged (not shown).

In the presence of the PAF receptor antagonist WEB 2086, the abluminal transmigrated neutrophils showed a strongly reduced up-regulation of the surface antigens CD11b and CD18 compared to neutrophils at the luminal side. WEB 2086 apparently interfered with the (extent of) modulation of surface antigen expression, as was also demonstrated by the significantly reduced down-modulation of LAM-1 and CD44 during diapedesis ( $P < 0.05$ ). However, the neutrophils still displayed a diminished expression of LAM-1 under these conditions of cytokine-activated EC (Table IV) as compared to control neutrophils in suspension (Table III). Results obtained with neutrophils migrated across rTNF $\alpha$ -pretreated EC monolayers were identical (not shown). Moreover, the effects on surface antigen expression observed in the presence of the anti-IL-8 antiserum, gave similar results as noted with WEB 2086 (Table IV). No effect on the surface antigen expression was observed with WEB 2086 or anti-IL-8 antiserum when the neutrophils had migrated to FMLP (10 nM), or when suspended neutrophils were activated with FMLP (1 nM) (not shown).

## Discussion

The entry of neutrophils into inflamed tissues is a prerequisite for an early and adequate elimination of invading microorganisms. Neutrophil adherence to the vascular cell lining is followed by diapedesis, a process of complex movements of the neutrophils between the EC into the subendothelial area. The cellular movements are fueled by the presence of a chemotactic gradient of mediators generated at the site of inflammation, e.g., bacterial FMLP-like peptides, lipid-derived mediators such as PAF and leukotrienes, or chemotactic cytokines (Snyder, 1987; Williams and Higgs, 1988; Baggiolini et al., 1989; Prescott et al., 1990). However, without proof of such a gradient, in case of IL-1-pretreated endothelium, neutrophil migration across these EC monolayers has also been established (Furie and McHugh, 1989; Moser et al., 1989; Hakkert et al., 1990, 1991).

Pretreatment of EC with IL-1 induced the synthesis of PAF (Table I), as was reported before (Bussolino et al., 1986; Breviario et al., 1988). For unknown reasons, Zavoico et al. (1989) did not find this cytokine-induced PAF generation in

EC. The form of PAF we measure is strictly membrane-associated and induces rapid rises of  $[Ca^{2+}]_i$  in neutrophils adhering to rIL-1 $\beta$ -pretreated EC, yet without exerting much influence on adherence as such (Kuijpers et al., 1991a). The present study shows that an EC-associated, PAF-dependent mechanism is operative in neutrophil passage across the rIL-1 $\beta$ -pretreated EC monolayers. The same appeared to be true in case of rTNF $\alpha$ -pretreated EC monolayers. The specific PAF receptor antagonists WEB 2086 and L-652,731 did not affect adherence significantly, whereas neutrophil migration across the cytokine-activated EC monolayers was inhibited by  $\sim$ 60% (Fig. 2 and Table II). In contrast, blockade of the PAF receptor does inhibit the adherence of neutrophils to thrombin or histamine-treated EC (Lorant et al., 1991), probably because these EC do not express ELAM-1. The FMLP-driven migration of neutrophils across resting untreated EC was not influenced by the PAF receptor antagonists.

PAF was not the only mediator involved in the neutrophil migration, as indicated by the incomplete reduction of diapedesis at high concentrations of WEB 2086 (Fig. 3). The second mechanism involved in neutrophil migration across cytokine-activated EC proved to be the neutrophil agonistic stimulus IL-8, as can be concluded from the preferential interference in this migration by a specific and highly potent anti-IL-8 antiserum (Table II). Huber et al. (1991) have recently reported that neutrophil migration was blocked by 75–80% in the presence of anti-IL-8. Neutrophil adherence to IL-1 $\beta$ -pretreated EC was almost completely prevented by anti-IL-8 as well, suggesting a very limited contribution of ELAM-1. In contrast, our inhibitory anti-IL-8 antiserum resulted in  $\sim$ 60% inhibition of the migration without any effect on adherence (Table II). As expected under these conditions, neutrophil adherence was strongly reduced by anti-ELAM-1 mAb (not shown). Binding of neutrophils to ELAM-1 occurs in a very avid way, as also indicated by the CD18 independency in adherence under flow conditions (Lawrence et al., 1990). Whereas we cultured EC on fibronectin for  $\sim$ 5 d before use, Huber et al. (1991) have used EC cultured for 21 d on collagen matrices. It is not known to which extent PAF or ELAM-1 can be induced in these long-term cultures upon cytokine or LPS activation. The difference in EC cultures may thus explain the prominent role for IL-8 in their migration system. In our system the contribution of endothelial IL-8 and PAF is more balanced.

Whereas soluble IL-8 has been reported to down-regulate

neutrophil adherence to cytokine-activated EC (Wheeler et al., 1988; Gimbrone et al., 1989), the pro-inflammatory response predominates in our system. This might be explained by the fact that all soluble IL-8 present was rinsed away before the addition of neutrophils to the upper compartment (Wheeler et al., 1988). It is as yet unclear whether the IL-8 is localized underneath the EC from where it slowly diffuses, thereby creating a kind of chemotactic gradient. These issues of localization and the precise time course will be further investigated.

We observed a significant down-modulation of CD44 and LAM-1, in the FMLP-dependent transmigration as well as in the migration across cytokine-treated EC (Tables III and IV). The down-modulation of these antigens in the FMLP-driven migration is a result of the neutrophil activation by the chemotactic mediator itself (Table II). Both molecules are down-modulated by shedding. Shedding of CD44 is the result of a DFP-sensitive proteolytic activity. CD44 can be immunoprecipitated from the supernatant of FMLP- or PMA-activated neutrophils, with a slightly reduced molecular weight compared to intact CD44 from neutrophil lysates (Kuijpers, T. W., and M. Hoogerwerf, unpublished observations). The extreme sensitivity of LAM-1 for shedding from neutrophils upon their activation was previously demonstrated by Jutila et al. (1990). The down-modulation of LAM-1 on neutrophils observed after migration across cytokine-treated EC was partly prevented by WEB 2086 or the anti-IL-8 antiserum (Table IV). Probably, shedding of these proteins is the result of a combined PAF/IL-8 activation of the neutrophils.

In another study (Kuijpers et al., 1992), we have found that neutrophils upregulate not only CD11b and CD18 during transmigration but also CD45 and CD67, indicating that fusion of the specific (and tertiary) granules with the plasma membrane takes place during this process. This was found both with FMLP-induced diapedesis and with diapedesis across IL-1 $\beta$ -treated EC. In contrast, CD63 was not upregulated, indicating that fusion of azurophil granules does not occur under these conditions (Kuijpers et al., 1991b). However, pretreatment of the neutrophils with BAPTA/AM, an intracellular Ca<sup>2+</sup> chelator, blocked degranulation but not transmigration. Also the shedding of LAM-1 was not blocked (Kuijpers et al., 1992). Thus, degranulation is not necessarily involved in diapedesis.

In conclusion, the migration of neutrophils across monolayers of activated EC (shortly prestimulated with rIL-1 $\beta$  or rTNF $\alpha$ ) is mediated by an EC-associated form of PAF in combination with IL-8 generated by EC. The down-modulation of LAM-1 and CD44 during diapedesis may be the result of this process.

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