

# Endothelial-Leukocyte Adhesion Molecule 1 Stimulates the Adhesive Activity of Leukocyte Integrin CR3 (CD11b/CD18, Mac-1, $\alpha_m\beta_2$ ) on Human Neutrophils

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## Summary

Two classes of adhesion molecules have well-defined roles in the attachment of unstimulated polymorphonuclear leukocytes (PMN) to cytokine-treated endothelial cells. Endothelial-leukocyte adhesion molecule 1 (ELAM-1) on endothelial cells interacts with specific carbohydrate residues on the PMN, and the leukocyte integrins (CD18 antigens) on PMN interact with intracellular adhesion molecule 1 and other structures on endothelium. Here we show that these two classes of molecules can act sequentially in an "adhesion cascade". Interaction of PMN with ELAM-1-bearing endothelial cells causes PMN to express enhanced adhesive activity of the integrin CR3 (CD11b/CD18). Expression of ELAM-1 on the cytokine-treated endothelium appears both necessary and sufficient for the stimulation of CR3 activity since blockade of ELAM-1 with mAbs prevents the activation of CR3 by cytokine-treated endothelium, and immobilized recombinant ELAM-1 activates CR3. The ability to activate CR3 is shared by chemattractants, suggesting that ELAM-1 may serve as a "tethered chemattractant." This hypothesis is strengthened by the observation that recombinant soluble ELAM-1 directs movement of PMN in chemotaxis chambers. These results suggest a mechanism by which multiple adhesive molecules may function together in diapedesis. ELAM-1 serves both as an adhesion and as a trigger that recruits the participation of additional adhesion molecules. Our results also suggest that ligands for adhesion molecules may also be "receptors" capable of generating intracellular signals.

Several classes of molecules have been described that may serve to direct PMN from the blood to sites of inflammation in tissues. Brief (5 min) stimulation of endothelial cells (EC)<sup>1</sup> with inflammatory molecules such as PMA or thrombin causes the expression of platelet activating factor (PAF) (1) and GMP140 (also known as PADGEM; reference 2) on the endothelial surface. Both of these molecules can serve as adhesins for PMN (3, 4). Longer incubation (3 h) of EC with inflammatory cytokines such as IL-1 or TNF causes expression of endothelial-leukocyte adhesion molecule 1 (ELAM-1) (5), a lectin-like molecule (6) that mediates attachment of PMN by binding to SLe<sup>x</sup> carbohydrate-related structures present on the surface of PMN (7-9). Treatment of EC with cytokines in this way causes dramatically enhanced binding of PMN in vitro (5, 10). ELAM-1, GMP-140, and PAF may serve in the crucial first step of diapedesis, attachment to endothelium.

<sup>1</sup> Abbreviations used in this paper: EC, endothelial cells; ELAM-1, endothelial-leukocyte adhesion molecule 1; fNLLP, formyl-norleucyl-leucyl-phenylalanine; ICAM-1, intracellular adhesion molecule 1; LAD, leukocyte adhesion deficiency; PAF, platelet activating factor.

PMN also express molecules actively involved in binding to endothelium, but since cells in the flowing blood are only briefly in the vicinity of an inflammatory focus, specialized means are necessary for regulating their adhesive activity. The leukocyte integrins ( $\beta_2$  integrins, CD18 antigens) are expressed on the surface of PMN in an inactive form (11). Upon stimulation of the cells with chemattractants, these receptors transiently acquire adhesive activity, then revert to an inactive state (11, 12). Leukocyte integrins may function in multiple, successive cycles of adhesion, and we have proposed that the cycles of adhesion and release enable locomotion of cells with adhesion occurring at the leading front of the cell and release occurring at the uropod (13). The leukocyte integrin LFA-1 ( $\alpha_1\beta_2$ , CD11a/CD18) binds to intracellular adhesion molecule (ICAM) on endothelial cells (14, 15), and CR3 ( $\alpha_m\beta_2$ , CD11b/CD18, Mac-1) binds to uncharacterized structures on endothelium (15).

The leukocyte integrins play a vital role in movement of cells to inflammatory sites. Binding of chemattractant-stimulated PMN to endothelium in vitro is completely blocked by anti-CD18 mAbs (12, 14, 16) and is nearly absent in PMN

from patients with a genetic defect in CD18 (leukocyte adhesion deficiency [LAD]; reference 17). Additional studies indicate that CD18 molecules are crucial for movement of PMN through an endothelial monolayer. Transmigration of PMN in vitro is completely blocked by anti-CD18 mAbs (14). Anti-CD18 mAbs also completely block the movement of PMN to inflammatory sites in the skin (18, 19), brain (20), and peritoneal cavity (21) of animals, and movement of PMN to sites of inflammation is virtually absent in patients with LAD.

The above observations raise question regarding how several apparently redundant receptor systems work together to mediate movement of cells. In principle, the receptors could act in parallel, with each type of adhesion molecule adding a partial contribution to the adhesive strength needed for binding. Alternatively, they could act in series, with one adhesion molecule acting first and enabling or inducing the function of the second. Here we provide evidence supporting a sequential model. Interaction of unstimulated PMN with ELAM-1 on endothelial cells or with purified recombinant ELAM-1 on a culture surface activates the adhesive activity of CR3, which may then participate in subsequent adhesion events. We further show that soluble ELAM-1 serves as a chemattractant.

## Materials and Methods

**Reagents.** Glycophorin A, formyl-norleucyl-leucyl-phenylalanine (fNLLP), PMA, and aprotinin were obtained from Sigma Chemical Co. (St. Louis, MO), fibronectin was from the New York Blood Center, and human serum albumin was from Armour Pharmaceuticals (Kankakee, IL). EC were stimulated with Re 595 LPS from List Biologicals (Campbell, CA). Experiments with LPS-coated erythrocytes used the LPS precursor, lipid IVA, a generous gift of Dr. C. R. H. Raetz (Rahway, NJ). Dulbecco's PBS and PBS deficient in divalent cations (PD) was from Whitaker MA Bioproducts (Walkersville, MD). Recombinant TNF- $\alpha$  was a gift of Genentech, and recombinant IL-1 $\beta$  was from R&D Systems (Minneapolis, MN).

Production and characterization of recombinant soluble (rs) ELAM-1 is described elsewhere (21a). Briefly, insertion of a stop codon at residue THR531 (6) generated a construct, designated rsELAM1, lacking putative transmembrane and cytoplasmic domains (6, 22). After stable expression in CHO cells according to standard techniques (23), rsELAM1 was purified to homogeneity from CHO cell-conditioned medium by immunoaffinity chromatography using mAb BB11 (24). rsELAM, when immobilized on plastic, is functional as an adhesive protein, and selectively binds only cell types known to bind to ELAM-1 on the surface of EC. These cells include PMN, HL60, and HT29 (21a).

**Monoclonal Antibodies.** mAbs OKM9 and OKM10 directed against CD11b (25) were a gift of Ortho Pharmaceuticals (Raritan, NJ), mAb LB-2 directed against ICAM-1 (26) was a gift of Dr. E. Clark (Seattle, WA), mAb H18/7 directed against ELAM-1 (5) was a gift of Dr. M. Bevilacqua (Boston, MA), mAb 3G8 directed against FcRIII (CD16) was as described (27), and mAb W6/32 against class I histocompatibility antigens was from the American Type Culture Collection (Rockville, MD). mAb BB11 directed against ELAM-1 was as described (24). All antibodies were purified IgGs.

**Cells.** Human PMN were isolated from adult blood on Ficoll-Hypaque gradients (28). Cells were suspended at  $2 \times 10^6$ /ml in

the buffer HAP (PBS containing 0.5 mg/ml HSA and 0.3 U/ml aprotinin). To allow ready identification of the PMN on endothelial monolayers, they were fluorescently labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate as described (12).

EC were isolated from human umbilical cords and seeded on fibronectin-coated Terasaki wells at either first or second passage, as described (12). Fixed monolayers of EC were obtained by incubating washed, live monolayers in Terasaki plates with 0.25% glutaraldehyde for 5 min at 0°C. The monolayers were extensively washed and incubated for 1 h with 50 mM glycine to quench unreacted glutaraldehyde.

Sheep erythrocytes were coated with IgG (29), C3bi (30), or the LPS precursor, lipid IVA (31), as previously described. The amount of IgG used to coat erythrocytes was adjusted to give submaximal binding (maximal binding yields an attachment index >1,200). Thus, either increases or decreases in binding could be observed.

**Assays.** The binding of fluorescently labeled PMN to EC was as described (12). Briefly, PMN were incubated with EC for 15 min at 37°C, then unbound cells were removed by vigorous agitation. Adherent PMN were enumerated by fluorescence microscopy.

Binding of ligand-coated erythrocytes to PMN was measured as previously described (11). Monolayers of PMN were established on protein-coated surfaces by adding  $10^4$  PMN in HAP and incubating for 30 min. Erythrocytes were added to the washed monolayers and, after an additional 30 min incubation, plates were washed and the attachment of erythrocytes to PMN was enumerated by microscopy. Briefly, in randomly chosen  $40\times$  microscope fields, all PMN and all erythrocytes bound to PMN were counted. At least 50 PMN were counted in each of duplicate wells. Results are expressed as attachment index, the number of erythrocytes bound per 100 PMN. While the pattern of responses to experimental conditions is extremely consistent, the precise level of binding observed varies considerably from experiment to experiment because of donor-to-donor variability in the PMN and variations in the amount of ligand per erythrocyte. For this reason, it is seldom possible to average separate experiments. Results of a single experiment, representative of several experiments on different donors, are usually presented.

To measure the binding of ligand-coated erythrocytes to PMN adherent to endothelial monolayers,  $10^4$  labeled PMN and  $5 \times 10^5$  erythrocytes were added to each well of a Terasaki plate. Cells were allowed to settle for 10 min at 0°C, then were warmed to 37°C for 20 min. The plates were then turned upside down to allow gravity to remove unbound erythrocytes from the monolayer, and the plate was gently dipped. This procedure allows retention of PMN weakly adherent to the endothelium. Attachment of erythrocytes to PMN was enumerated as described above. In experiments using antibodies against endothelial antigens, the antibodies (10  $\mu$ g/ml) were incubated for 30 min with the endothelium then washed away before the addition of PMN.

**Chemotaxis.** Migration of PMN across 2- $\mu$ m polycarbonate filters (Nucleopore Corp., Pleasanton, CA) was measured in a 48-well Boyden chamber (Neuroprobe Inc., Cabin John, MD) by the method of Deuel et al. (32). The upper wells contained  $2.5 \times 10^4$  PMN in HAP buffer and the lower chambers contained chemattractant diluted in HAP buffer. After a 2-h incubation at 37°C, the cells that had migrated through the 2- $\mu$ m pores into an underlying 0.45- $\mu$  nitrocellulose filter were enumerated by microscopy. Results are expressed as the number of migrated PMN observed per  $20\times$  field. All conditions were assayed in six replicate wells.

For observation of the behavior of single cells, glass coverslips

were coated with 100  $\mu\text{g/ml}$  fibrinogen (KabiVitrum) for 60 min, washed, and a monolayer of PMN was established on the coated coverslip by adding 0.25 ml of PMN diluted to  $2 \times 10^6/\text{ml}$  in HAP and incubating for 60 min at  $37^\circ\text{C}$ . The coverslip was fitted into a pre-warmed Zigmund apparatus (Neuroprobe Inc.) containing HAP buffer on both sides of the chamber. A time lapse video recording of a  $20\times$  field was then made to verify that the cells were immobile. Recordings were made at  $37^\circ\text{C}$  using a Nikon Diaphot equipped with temperature-controlled stage. After the initial recording, chemattractant was added to one side of the chamber and an additional recording was made for 20 min. Manual tracings of the paths of individual cells were made from the recorded video images.

## Results

*Attachment of PMN to Cytokine-treated Endothelium Activates CR3.* CR3 recognizes complement protein C3bi (25), fibrinogen (33, 34), and LPS (31), as well as molecules on endothelium. The binding activity of CR3 may thus be measured by observing the binding of erythrocytes coated with C3bi (EC3bi) or LPS (ELPS) to the PMN or by measuring attachment of PMN to endothelium. Suspensions of resting PMN express CR3 that is incapable of binding any of its known ligands. Thus, the cells fail to bind EC3bi (11) or ELPS (35) and fail to adhere firmly to endothelial cells (12). CR3 remains inactive when PMN attach to protein-coated plastic surfaces since monolayers of PMN fail to recognize EC3bi (Table 1) (11). We found that incubation of PMN with resting EC also does not affect CR3 activity since the weakly adherent PMN show low binding of EC3bi similar to that seen with parallel populations of PMN plated on serum albumin-coated plastic (Table 1). In contrast, attachment of PMN to endothelium stimulated with TNF- $\alpha$ , IL-1 $\beta$ , or LPS caused a 4–12-fold enhancement of CR3 activity measured by binding of EC3bi. The extent of activation was similar to that observed with fNLLP, a chemattractant known to strongly activate CR3. These data suggest that interaction of PMN with cytokine-treated endothelium results in the activation of the binding activity of CR3.

Several experiments indicate that the enhanced binding of EC3bi caused by adhesion of PMN to cytokine-treated endothelium is the specific result of increased activity of CR3. Alterations in nonspecific adhesion of erythrocytes is unlikely to underlie our results since binding of control IgG-coated erythrocytes to Fc receptors on PMN was unaffected by the activation state of the endothelium (Table 1). Previous studies showed that the binding activity of CR3 is completely blocked by removal of divalent cations (29) or by inclusion of the anti-CR3 mAb OKM10 (25). We confirmed that both of these treatments also completely blocked binding of EC3bi to PMN adherent to TNF-treated EC (data not shown). Finally, enhanced binding of EC3bi requires active metabolism of PMN since no binding could be observed in preparations held at  $0^\circ\text{C}$  (data not shown).

CR3 expresses two distinct binding sites, one for proteinaceous ligands such as C3bi and an additional binding site

**Table 1.** Adhesion of PMN to Cytokine-treated Endothelium Activates CR3

Substrate	Attachment of erythrocytes to PMN*		
	EC3bi	ELPS	ElgG
Endothelium	18	23	316
Endothelium, fixed	31	ND	ND
Endothelium + TNF $\alpha$	313	169	339
Endothelium + TNF $\alpha$ , fixed	402	ND	ND
Endothelium + LPS	243	78	281
Endothelium + IL-1 $\beta$	320	80	339
Albumin-coated plastic	19	ND	296
Albumin-coated plastic + fNLLP $^\dagger$	383	ND	ND

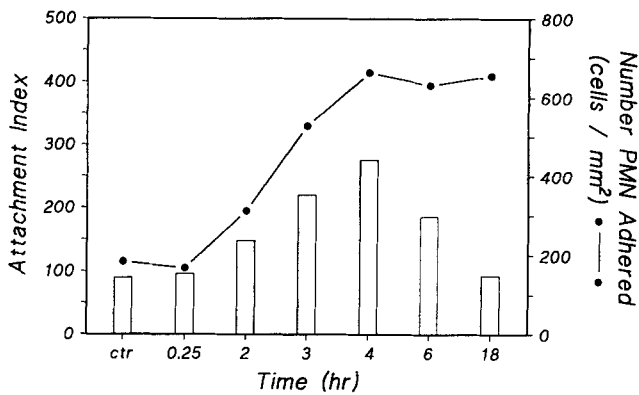
\* Monolayers of endothelial cells were incubated for 3 h with 10 ng/ml TNF- $\alpha$ , 50 ng/ml LPS, 5 U/ml IL-1 $\beta$ , or with medium alone. Control albumin-coated plastic wells contained no endothelial cells. Wells were washed and mixtures of erythrocytes, and PMN were added and incubated as described in Materials and Methods. Attachment of erythrocytes to adherent PMN was enumerated and reported here as attachment index, the number of erythrocytes per 100 PMN. Results of a single experiment, representative of seven separate experiments, are reported.

$^\dagger$  fNLLP ( $10^{-7}$  M) was added with the PMN.

that recognizes LPS (36). The binding activity of both sites can be strongly enhanced by chemattractants such as NAP-1/IL-8 (35). We found that attachment of PMN to TNF-treated EC, but not unstimulated EC, also enhanced the binding of ELPS (Table 1). Thus, attachment of PMN to cytokine-treated EC activates both of the binding sites of CR3.

Previous studies indicate that chemattractants enhance adhesive activity of CR3 by two mechanisms. Fusion of intracellular membranes with the plasma membrane results in a two- to threefold rise in expression of CR3, and simultaneous qualitative changes cause a 5–10-fold increase in the adhesive activity of existing CR3 (11, 12, 35, 37, 38). Preliminary studies suggest that interaction of PMN with cytokine-treated endothelium for 20 min causes an  $\sim 10\%$  rise in cell surface CR3 (S.K. Lo and S.D. Wright, unpublished observations) and thus qualitative changes in existing CR3 may be principally responsible for the increased adhesive activity. However, the precise contribution of newly recruited CR3 is not established by the current studies.

The time course for the acquisition of CR3 activating capacity was explored by incubating monolayers of EC for different intervals with TNF before fixation and incubation with PMN (Fig. 1). Incubation of EC with TNF- $\alpha$  for 15 min caused no increase in the activity of CR3 on adherent PMN. This observation suggests that TNF is not having its effect by being “carried over” in the cultures and directly stimulating the PMN. Rather, TNF must act by inducing a response in the EC. The EC became capable of activating PMN after 2 h of exposure to TNF, but activity declined to control levels by 17 h. Thus, the capacity to activate PMN is transiently induced by TNF.

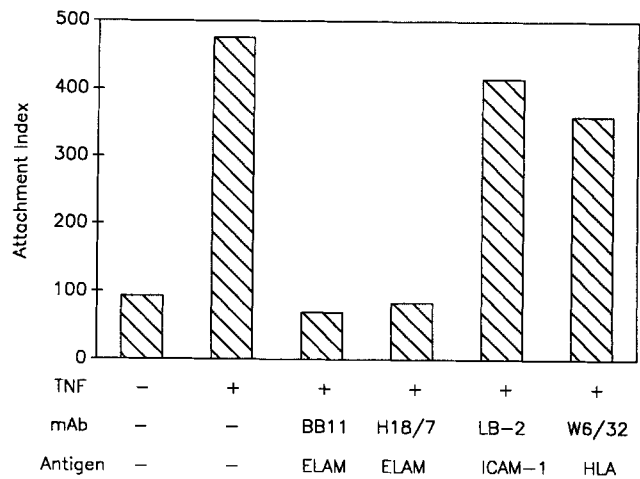


**Figure 1.** TNF- $\alpha$  transiently enables endothelial cells to activate CR3 on adherent PMN. EC were incubated for various times with 10 ng/ml TNF- $\alpha$ , then washed and fixed. Cells were incubated with the monolayers for 15 min and binding of PMN to the EC, expressed as cells/mm<sup>2</sup>, was determined after a vigorous wash. In parallel wells, a mixture of PMN and EC3bi was incubated with the EC, and the attachment of erythrocytes to PMN was measured as described in Materials and Methods. Results are representative of two experiments.

In agreement with other studies (39, 40) incubation with TNF caused enhanced attachment of PMN, and enhanced attachment was approximately equal from 3 to 17 h of incubation. Since binding of PMN to EC at 17 h is avid but binding of EC3bi to the PMN is low, it appears that activation of CR3 is not simply influenced by the extent of adhesion of PMN to EC.

**Anti-ELAM Blocks the Activation of CR3 by Cytokine-activated EC.** The time course for the acquisition of CR3-activating ability differs from the time course of expression of the adhesion molecule ICAM-1, which remains elevated for >17 h, but corresponds precisely with that for the expression of ELAM-1 on EC (41). To determine if ELAM-1 on the surface of EC plays a role in the activation of CR3, monolayers of TNF-treated EC were incubated with mAbs against a variety of endothelial surface structures (Fig. 2). mAbs against HLA or ICAM-1 had no effect on the activation of CR3 by EC. However, two anti-ELAM mAbs, BB11 and H18/7, each completely blocked the activation of CR3. This observation suggests that expression of ELAM-1 is necessary for EC to activate CR3 on PMN.

EC are known to synthesize IL-8 (42) and PAF (1), both potent activators of CR3 (35; and S.D. Wright, unpublished observations). We thus considered the possibility that these secreted molecules may contribute to the effects of cytokine-treated EC on CR3 activity. IL-8 is unlikely to play a role since fixed EC, which cannot secrete, are as active as unfixed cells in stimulating PMN (Table 1). Moreover, IL-8 production by EC is strongly induced after 17 h of incubation with TNF (43), but EC stimulated in this way do not activate CR3 (Fig. 1). PAF binds to the plasma membrane of the EC and may be retained after fixation (3). To explore a role for PAF, the strong PAF antagonist, WEB 2086, was included in cultures of PMN and TNF-treated EC. We used concentrations of WEB 2086 (10  $\mu$ M) that completely blocked the ability of purified PAF to activate CR3 (Hermanowski-Vosatka,



**Figure 2.** Anti-ELAM-1 blocks the activation of CR3 by cytokine-treated endothelium. Endothelial monolayers were treated with medium alone or 10 ng/ml TNF- $\alpha$  for 3 h at 37°C. 10  $\mu$ g/ml of the indicated mAbs were added followed by PMN and erythrocytes, and attachment of EC3bi to PMN was determined as described in Materials and Methods. The control antibodies, anti-ICAM-1 and anti-HLA, each react strongly with cytokine-treated EC (43), and our previous results showed that the anti-ICAM-1 effectively blocks ICAM-1-dependent binding of PMN events under similar conditions (15). Results are representative of four separate experiments.

A., and S.D. Wright, manuscript in preparation). This antagonist had no effect on the stimulation of CR3 activity by cytokine-treated endothelium, suggesting that CR3 is not activated by PAF on the surface of cytokine-treated endothelium.

**Purified ELAM Activates CR3.** We have recently generated a recombinant soluble form of ELAM-1 lacking the transmembrane and cytoplasmic domains (21a). The protein is a functional adhesion molecule since surfaces coated with rsELAM-1 avidly bind neutrophils and HL-60 cells (21a). To determine if ELAM-1 is sufficient to activate CR3, we observed PMN adherent to the immobilized rsELAM-1. Binding of PMN to ELAM-1-coated surfaces caused strong activation of CR3 (Table 2). In contrast, surfaces coated with albumin or glycophorin had no effect on CR3 activity. Activation of CR3 is unlikely to be caused by nonspecific interaction with an adhesive surface since attachment of PMN to a substrate coated with mAb 3G8 against the abundant surface glycoprotein, Fc $\gamma$ RIII, caused no alteration in CR3 activity (Table 2). Activation of CR3 is also unlikely to be caused by contaminants of ELAM-1 such as LPS, since boiled rsELAM-1 was ineffective in stimulating CR3 activity, and polymyxin B sulfate, a drug that neutralizes LPS, had no effect on activation of CR3 by rsELAM-1 (data not shown). Moreover, addition of anti-ELAM mAbs to the coated surface completely blocked the activation by purified ELAM-1 (Table 2). These experiments indicate that interaction of ELAM-1 with its counter structures on PMN is sufficient to cause activation of CR3.

**Soluble ELAM-1 Is a Chemattractant for PMN.** All known chemattractants of PMN activate CR3 perhaps because adhe-

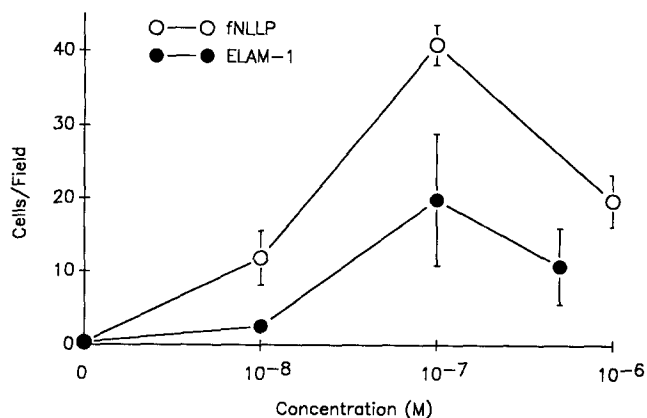
**Table 2.** Attachment of PMN to ELAM-1-coated Surfaces Activates CR3

Substrate	mAb	Attachment of EC3bi to PMN*
Glycophorin	-	66 ± 42
Glycophorin + fNLLP <sup>2</sup>	-	504 ± 103
ELAM-1	-	245 ± 52
ELAM-1	Anti-ICAM-1	375 ± 179
ELAM-1	Anti-HLA	396 ± 134
ELAM-1	Anti-ELAM (BB11)	85 ± 24
ELAM-1	Anti-ELAM (H18/7)	63 ± 25
HSA	-	88 ± 28
HSA + fNLLP <sup>2</sup>	-	554 ± 244
mAb 3G8	-	65 ± 55
ELAM-1	-	425 ± 244

\* Terasaki wells were coated with 10 µg/ml of glycophorin, HSA, 3G8 (anti-FcγRIII), or ELAM-1 for 2 h at 20° C. 5 mg/ml HSA was added for an additional 1 h, and plates were washed. 10 µg/ml of the indicated mAbs was added to the substrates for 40 min at 20° C. After a wash, a monolayer of PMN was established on the surfaces, EC3bi were added, and the attachment index was measured as described in Materials and Methods. The series of experiments in the upper panel shows that anti-ELAM-1 mAbs block the activation of PMN caused by surface-bound ELAM-1. A separate series of experiments in the lower panel shows that attachment of PMN to a nonbinding surface (HSA) or a binding surface (3G8) does not affect CR3 activity on adherent PMN. The day-to-day variations in levels of binding were relatively small in these experiments, thus permitting averaging of independent experiments. Results are averaged from three to five separate studies and are presented with SD. † fNLLP (10<sup>-7</sup> M) was added with the PMN.

sive interactions of CR3 with the substrate at the leading edge of the cell are needed for locomotion (13). Our observations show that ELAM-1 exhibits this characteristic of chemotactants and suggests that ELAM-1 may serve as a "tethered chemotactant" to direct the movement of PMN. The availability of a soluble (untethered) form of ELAM-1 allowed us to test this hypothesis. We found that PMN migrated across microporous filters in response to rsELAM-1 (Fig. 3). The specificity of this response is indicated by the finding that treatment of the rsELAM-1 with mAb BB11 completely abolished its chemotactant activity. The dose of rsELAM-1 needed for maximal chemotactant activity (10<sup>-7</sup> M) was similar to the optimal dose of fNLLP assayed in parallel. Direct comparisons also showed that the magnitude of the chemotactic response to rsELAM-1 varied from equal to one-third the magnitude of response to fNLLP.

The response of PMN to rsELAM-1 was found to be chemotactant, not merely chemokinetic, by direct observation in a Zigmond chamber. In the absence of chemotactant, PMN in the chamber remained round and immobile. Within 2 min of the introduction of 10<sup>-7</sup> rsELAM-1 into

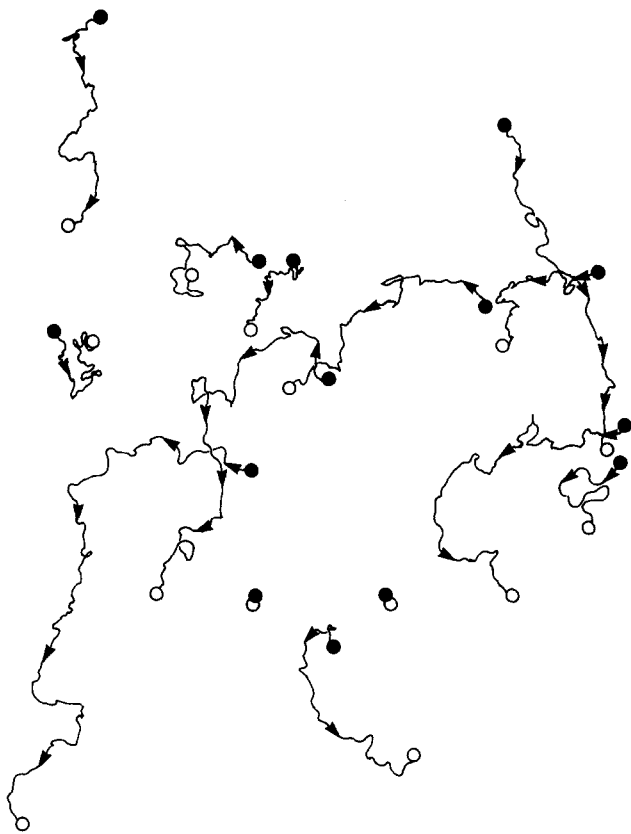


**Figure 3.** rsELAM-1 stimulates movement of PMN across filters. A Boyden chamber was assembled as described in Materials and Methods with the indicated doses of fNLLP or rsELAM-1 in the lower chamber. Migration in response to 10<sup>-7</sup> M rsELAM-1 neutralized with a 10-fold weight excess of BB11 was 1.0 ± 0.6 cells/field. Endotoxin contamination of the ELAM-1 is unlikely to be responsible for the results since boiling the ELAM-1 completely eliminated its chemotactic activity, and purified endotoxin (1 ng/ml) had no chemotactic activity in the assay. Similarly, it is unlikely that adsorption of the ELAM-1 to the filters caused haptotaxis since the filters are soaked in protein-containing buffer (HAP) before assembly into the apparatus. Finally, inclusion of 10<sup>-7</sup> M ELAM-1 in the upper chamber eliminated chemotaxis to 10<sup>-7</sup> M ELAM-1 in the lower chamber. Results are presented as the number of migrated cells per 20× field ± SD. The experiment shown is representative of nine separate studies. When no chemoattractant was added, the error bars were smaller than the symbol (0.33 ± 0.51 cells/field). *t* Test statistics comparing rsELAM-1 with no chemoattractant showed that all concentrations of rsELAM-1 shown here caused a highly significant increase in cell migration (*p* < 0.0005). The decrease in movement observed from 10<sup>-7</sup> to 5 × 10<sup>-7</sup> M rsELAM-1 was also significant (*p* = 0.068).

one side of the chamber, PMN began to orient and move. The movement induced by rsELAM-1 was predominantly in the direction of the source of chemotactant (Fig. 4). Directed movement of PMN toward ELAM-1 was detected using source concentrations as low as 10<sup>-9</sup> M, but rsELAM-1 neutralized with a 10-fold excess of the anti-ELAM antibody BB11 showed no chemotactant activity (data not shown).

## Discussion

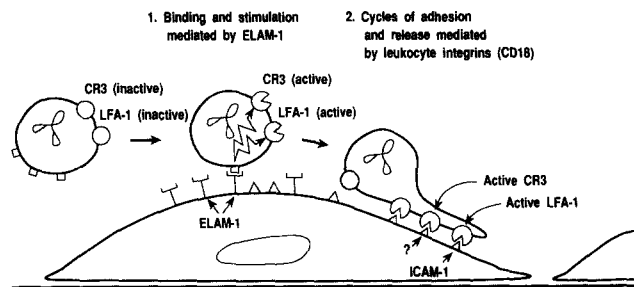
ELAM-1 has been described as an adhesion molecule, binding PMN to cytokine-treated endothelial cells (5). Here we show that ELAM-1 serves a second function. It recruits the participation of an additional class of adhesion molecules, the leukocyte integrins, in the adhesion event. These and other results suggest that ELAM-1 and the leukocyte integrins may normally work in a sequence or cascade (Fig. 5). Under conditions of flow, ELAM-1 is far more effective than CD18 molecules in initiating adhesion between PMN and EC (44). Thus, at an established site of inflammation, the first binding of PMN to EC is likely to be mediated by ELAM-1. The function of leukocyte integrins induced by binding to ELAM-1 is essential for the next events, the movement of PMN to a junction between endothelial cells and diapedesis (14, 18). The sequential nature of the functioning of these molecules



**Figure 4.** rsELAM-1 stimulates directed movement of PMN. A Zigmund chamber was assembled as described in Materials and Methods with 10  $\mu\text{g/ml}$  rsELAM-1 ( $10^{-7}$  M) in one chamber. The positions of individual PMN at the beginning of the recording are depicted as filled circles, and their positions after 20 min are depicted as open circles. The lines trace the path of migration with arrows showing direction of movement added for clarity. The drawing is oriented such that the chamber containing rsELAM-1 is at the bottom of the page. Cells exhibited no movement before the addition of rsELAM-1, and parallel studies showed no movement in response to 10  $\mu\text{g/ml}$  ELAM-1 neutralized with 100  $\mu\text{g/ml}$  BB11. Results depict the image from a single 20 $\times$  field and are representative of three separate studies.

may explain why anti-CD18 antibodies completely block inflammatory movement of PMN in animals despite the presence of ELAM-1, and suggest that anti-ELAM-1 mAbs may have similar *in vivo* potency in inflammatory conditions in which ELAM-1 is present. The adhesion cascade proposed here resembles in some ways the interaction of cytotoxic T cells with their targets. The TCR is known to mediate weak binding of cytotoxic T cells to targets, and crosslinking of the TCR may recruit the participation of LFA-1 to strengthen adhesion (45).

The experiments described here may provide a model for understanding the individual contribution of multiple adhe-



**Figure 5.** Model of the sequential function of ELAM-1 and leukocyte integrins in the movement of PMN across endothelial monolayers at a site of inflammation. See text for details.

sion molecules in complex events such as lymphocyte homing and in the movements of embryogenesis. The ability of an adhesion molecule such as ELAM-1 to induce a response in a neighboring cell also suggests that the ligands for adhesion molecules may themselves be “receptors” capable of generating responses in the cells that bear them. Since ELAM-1 initiates strong responses in PMN, we predict that at least a subset of the glycoproteins recognized by ELAM-1 are such signaling receptors.

Gradients of soluble rsELAM-1 caused directed movement of PMN (Figs. 3 and 4). Chemotaxis is a complex event requiring orientation of the cell, increased adhesion at the leading edge, and vigorous cytoskeletal activity. Thus, the enhanced activity of CR3 we observed is likely to represent one of several responses induced by ELAM-1. The ability of ELAM-1 to serve as a chemattractant suggests that this molecule may carry directional information and may explain the observation that migration of PMN through unstimulated endothelium is dependent on an applied gradient of soluble chemattractant but that rapid movement of PMN through monolayers of cytokine-treated endothelium occurs without a requirement for such a gradient (46, 47). Since ELAM-1 is normally observed as a cell-bound molecule, it may serve as a “tethered chemattractant” to precisely direct the movement of leukocytes. Our observations also suggest that soluble ELAM-1 may have an effect on leukocytes. Indeed, addition of soluble ELAM-1 to monolayers of PMN causes enhanced CR3 activity (S.D. Wright, unpublished observations), and addition of soluble ELAM-1 to suspensions of PMN causes enhanced adhesion to ICAM-1-bearing cells (S.K. Lo and M. Bevilacqua, manuscript in preparation). The observation of a soluble form of ELAM-1 in serum (W. Newman, personal communication) raises the possibility that ELAM-1 could also serve as a conventional chemattractant upon release from the cells.

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