Neutrophil-Endothelial Cell Interactions

Modulation of Neutrophil Adhesiveness Induced by Complement Fragments C5a and C5a des arg and Formyl-Methionyl-Leucyl-Phenylalanine In Vitro

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Abstract. Neutrophil adherence to vascular endothelial cells is the initial event in the emigration of neutrophils through blood vessel walls to tissue sites of inflammation; this process is attributed to the generation of extravascular chemotactic factors. To investigate the effect of chemotactic factors on neutrophil adherence to endothelium, we developed a sensitive, reproducible in vitro microtiter adherence assay. Base-line nonstimulated adhesion of human neutrophils to cultured human umbilical vein endothelial cell monolayers was 35.2 ±0.9%, which is equivalent to three to four neutrophils per endothelial cell. Addition of either purified complement fragment C5a des arg, or formyl-methionyl-leucyl-phenylalanine (FMLP), in concentrations ranging from 10^{-10} to 10^{-6} M, increased neutrophil adherence to endothelium in a dose-dependent manner. Purified C5a and C5a des arg were essentially equal in their ability to enhance neutrophil adherence, in contrast to the previously described greater in vitro potency of C5a compared with C5a des arg in stimulating neutrophil chemotaxis and enzyme release. Nonstimulated neutrophils adhered preferentially to human endothelial cells compared with fibroblasts or smooth muscle cells, suggesting that endothelial cells may make a unique contribution to the base-line adhesive interaction. However, chemotactic factors appear to enhance neutrophil adherence to endothelium by exerting an effect primarily on the neutrophil. In the presence of chemotactic factor, neutrophils adhered equally well to different cell types or to protein-coated plastic. Pretreatment of endothelial cells with chemotactic factor for as long as 4 h failed to increase subsequent neutrophil adherence. In contrast, pretreatment of neutrophils with chemotactic factor increased adherence to endothelium. Chemotactic factor-stimulated neutrophil adherence to endothelium occurred rapidly (within 2 min), diminished upon removal of stimulus, but could be rapidly and maximally restimulated upon readdition of the original dose of chemotactic factor. Thus, adherence to endothelium stimulated by chemotactic factor would appear to be a dynamic neutrophil response capable of rapid modulation, possibly important to the ability of neutrophils to adhere to and then migrate through vessel walls to localize at sites of inflammation.

Introduction

Interaction of circulating polymorphonuclear leukocytes (neutrophils) with the vascular endothelium is an essential initial event in the induction of the acute inflammatory response. Although neutrophil adhesion and emigration have been studied extensively (1–4), the mechanisms by which neutrophils recognize and adhere to endothelial cells and subsequently migrate through interendothelial cell junctions are still not understood.

Early in vivo studies that used the rabbit ear chamber (5–7), mouse or rat mesentery (8, 9), and/or hamster cheek pouch (9) to enable direct visualization of the microvasculature provided detailed observations and elegant electron micrographs of the morphologic events involved in the process of leukocyte emigration. Since the first observable event was localized

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margination and sticking of leukocytes to endothelial cells in small blood vessels (5–7, 9), it was assumed that a localized alteration in the endothelium itself was an essential prerequisite for subsequent leukocyte adherence. However, no specific morphologic endothelial alterations could be detected, even with electron microscopy (7, 8), to explain the initial adhesive interaction.

Recently, numerous in vitro assays (cell-cell aggregation [10–13], cell-substratum adhesion [14–18], and cell-monolayer adhesion [14, 15, 17–20]) using a variety of species (human, bovine, porcine, and rabbit) have been designed to investigate various aspects of leukocyte-endothelial interactions. Adhesion of neutrophils has been shown to be dependent on divalent cations (18, 21–23), proportional to the initial neutrophil concentration (18), enhanced by the presence of erythrocytes and agents that increase cyclic AMP levels (19), and reduced by the presence of platelets (19) or by pretreatment with agents that interfere with the metabolism of arachidonic acid via noncyclooxygenase-dependent mechanisms (20). Neutrophils adhere preferentially to cultured endothelial cells compared with other cell types, including fibroblasts and smooth muscle cells (14, 15, 17, 18). Neutrophils have been demonstrated to move freely over the surfaces of endothelial cells (14, 24) and eventually to migrate beneath cultured endothelial cell monolayers (18, 24, 25).

Early observations by Allison et al. (5) of the inflammatory reaction induced by thermal injury in the rabbit ear chamber documented that adherence of leukocytes to endothelium was usually first noted on the side of the vessel closest to the site of injury and suggested that the vascular adhesive interaction might be caused by products of cell damage that had diffused to the vessel from the site of tissue injury. Since then, substantial in vivo and in vitro evidence has accumulated to implicate chemotactic factors, such as complement factor 5 (C5)[1] fragments, as key participants in the process of leukocyte adherence and emigration. In vivo, chemotactic factors infused intravenously, generated intravascularly, or activated extracorporeally and then reinfused intravenously (e.g., hemodialysis and leukapheresis) induce a profound but transient neutropenia by increasing granulocyte adhesiveness and causing sequestration in the microvasculature (26–32). Chemotactic factor deposition in a defined extravascular location results in neutrophil emigration from vessels and influx into tissues, as demonstrated in the hamster cheek pouch (9) and rabbit alveolus (33). Application of partially purified C5 fragment preparations to the exposed adventitial surface of the carotid artery in the anesthetized rabbit induces adherence of neutrophils to the endothelium and migration into the arterial wall (34 and Goins, A., and P. M. Henson, unpublished observations). Although most physiologic neutrophil emigration appears to occur through the endothelium of postcapillary venules, evidence that neutrophils, given an appropriate stimulus, are capable of adhering to and migrating through large vessel endothelium in vivo justifies to some extent the use of readily available human umbilical vein and bovine aorta and pulmonary artery endothelial cells in initial in vitro studies of neutrophil-endothelial cell interactions. In vitro chemotactic factors have been shown to enhance neutrophil adherence to substrata (27, 35, 36) and to cultured endothelial monolayers (17, 21, 36).

To define further the enhanced adhesive interaction between neutrophils and endothelial cells induced by chemotactic factors, we used a quantitative monolayer adhesion assay to investigate the effects of purified C5 fragments and formyl-methionyl-leucyl-phenylalanine (FMLP) on human neutrophil adherence to cultured human endothelial cells. Our results suggest that chemotactic factors act primarily on the neutrophil to stimulate adherence to the endothelium and that this enhanced adhesive response is capable of rapid modulation.

Methods

Isolation of neutrophils. Human peripheral blood neutrophils were isolated as previously described (37). Bovine peripheral blood neutrophils were isolated by initial lysis of erythrocytes with hypotonic saline followed by resuspension in 25% bovine plasma in saline and subsequent Ficoll-Hypaque density centrifugation.

^{51}Cr-labeling of neutrophils. Human or bovine neutrophils (5×10^7 cells/ml) were incubated with 100 μCi of ^{51}Cr (sodium chromate solution, 1 mCi/ml; New England Nuclear, Boston, MA) per milliliter of cells for 1 h at 37°C in a shaking water bath, washed three times, and finally resuspended in medium 199 (M199; Gibco Laboratories, Grand Island, NY) with 20 mM Hepes for addition to adherence assays.

Isolation and culture of endothelial cells, smooth muscle cells, and fibroblasts. Human endothelial cells were harvested from umbilical veins (38, 39), plated in M199 with 20% fetal calf serum (FCS; Sterile Systems Inc., Logan, UT) and maintained in a 37°C, 5% CO_2 humidified atmosphere. Within 3–4 d, the monolayered cells were transferred nonenzymatically (by scraping with a rubber policeman) directly into fibronectin-coated (Collaborative Research, Waltham, MA) microtiter tissue culture (Costar, Cambridge, MA) assay wells. These first passage cells were used for experimentation when they had formed a confluent monolayer. The presence of a characteristic contact-inhibited cobblestone monolayer and positive immunofluorescent staining for Factor VIII-related antigen (40) with fluorescein-conjugated goal anti-human Factor VIII antibody, IgG fraction (Atlantic Antibodies, Scarborough, ME) were used to identify cells in culture as endothelium.

Bovine endothelial cells were harvested in the absence of protease treatment according to the method of Ryan et al. (41), and plated in M199 with 10% FCS. Nonenzymatic cell passage was accomplished by scraping with a rubber policeman. First to ninth passage cells were used for experimentation when they had formed confluent monolayers. Within this range the degree of neutrophil adherence did not appear to alter with passage number.

1. *Abbreviations used in this paper:* C5, complement factor 5; C5fr, C5 fragments; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; FMLP, formyl-methionyl-leucyl-phenylalanine; M199, medium 199.

Human vascular smooth muscle cells were harvested by controlled trypsin/EDTA (Gibco Laboratories) digestion from umbilical veins previously used as a source of endothelium, a method modified from Gimbrone and Cotran (42), and plated in Dulbecco's minimal essential medium (DMEM, Gibco Laboratories) with 10% FCS. First to seventh passage cells were used for experimentation.

Human dermal fibroblasts were harvested by controlled trypsin/EDTA digestion from fresh minced newborn foreskins (43) and plated in DMEM with 10% FCS. First to ninth passage cells were used for experimentation.

Preparation of chemotactic factors. Biologically active C5a and C5a des arg were purified from human serum by a procedure modified from that of Fernandez and Hugli (44) as reported previously (45). Partially purified C5 fragments (C5fr) were prepared from yeast activated serum by CM Sepharose chromatography followed by Sephadex G100 chromatography. Further purification of C5a was achieved by subsequent CM Sepharose chromatography and then CM Sephadex chromatography. Purified C5a des arg was generated by exhaustive reaction of purified C5a with carboxypeptidase B covalently linked to Sepharose-6B beads (45). Macro (46) and micro (47) assays of neutrophil release of myeloperoxidase were used to determine the biological activity of each preparation. Concentration of partially purified C5fr preparations was expressed as micrograms per milliliter based on protein determination according to the method of Lowry et al. (48) using bovine serum albumin as standard. Molar concentration of purified C5a and C5a des arg was calculated by assuming a molecular weight of 10,000.

FMLP was obtained from Vega Biochemicals, Tucson, AZ.

Assay of neutrophil chemotaxis. In vitro assay of neutrophil chemotaxis was performed by the two-filter technique of Keller et al. (49). Cell migration was quantitated as the mean number of cells per high power field. Five measurements were made on each of triplicate filters for every concentration of stimulus tested.

Assay of neutrophil adherence to endothelial cell monolayers. The adherence assay developed for these studies was a modification of reported monolayer adhesion assays using radiolabeled cells in suspension incubated over cultured cell monolayers (17, 19, 50–52). In preliminary studies using endothelial cells monolayered in 16-mm tissue culture wells and a hand pipette wash technique, base-line (nonstimulated) neutrophil adherence was found to vary with degree of plate agitation, force of pipetting, thoroughness of each wash, and number of sequential washes. In addition, 16-mm well-size precluded performance of multiwell dose response or time course studies due to time and reagent limitations. Therefore, a microtiter adherence assay was developed that used glutaraldehyde fixation to stabilize loosely adherent cells and used an adaptation of a low flow gravity delivery, gentle suction aspiration, multiwell wash apparatus (53) to completely remove nonadherent neutrophils without disruption of the underlying cell monolayer.

First-passage human endothelial cells (or other cell types as described) were grown to confluence within 2–4 d in microtiter tissue culture wells (Costar, Cambridge, MA) in M199 with 20% FCS. 1–2 h before assay, the monolayers were washed twice and maintained in serum-free M199 with 20 mM Hepes. Quadruplicate wells were prepared for each experimental variable to be tested. In each assay, blank plastic wells, which had been coated with fibronectin and preincubated in serum-containing medium to simulate endothelial cell culture conditions, were processed in parallel with wells containing cell monolayers. Assessment of adherence to these protein-coated plastic wells provided a means to monitor both intra- and interexperimental variations in neutrophil reactivity.

After gentle suction aspiration of the serum-free medium from the assay wells, a 50-μl aliquot of ^{51}Cr-labeled neutrophils (6×10^6/ml in M199 with 20 mM Hepes [assay buffer]) was added to each well (a ratio of 10 neutrophils to one endothelial cell), followed by a 50-μl aliquot of an appropriate concentration of chemotactic stimulus dissolved in assay buffer, or assay buffer alone. The microtiter plates were incubated without agitation for 15 min (or for time intervals varying from 15 s to 45 min) at 37°C in a 5% CO_2 humidified tissue culture incubator. At the end of the incubation period, 100 μl of 2% glutaraldehyde in phosphate-buffered saline was carefully added to each well. After 10 min, the wells were washed twice with assay buffer to remove nonadherent neutrophils. Washing buffer was delivered gently by gravity flow through 25-gauge needles arrayed in parallel and aspirated by gentle suction to avoid disruption of the monolayer. A 75-μl aliquot of assay buffer was then added to each well. Before harvesting, representative wells were visually assessed by phase microscopy to confirm the integrity of the underlying monolayers and the distribution of the adherent neutrophils. The ^{51}Cr-labeled neutrophils adherent to the cell monolayers were then harvested by a thorough swabbing of each well with a cotton-tipped applicator. The applicators were placed in small plastic tubes and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, CA). Results were expressed as percent adherence = (cpm harvested)/(cpm added) $\times$ 100.

In general, average variation from the mean for quadruplicate determinations was <10%. However, due to donor-to-donor variability in neutrophil physiology (see below), experiments performed on different days with different donors were often difficult to compare directly. When such a comparison was considered appropriate, percent adherence determined in the presence of a chemotactic factor was normalized to control by dividing the cpm from stimulated samples by the cpm from base-line (control wells with buffer alone) samples for the same set of cells. Results were then expressed as: percent base-line adherence.

Control experiments were performed to ascertain whether coating the wells with fibronectin, radiolabeling the neutrophils with ^{51}Cr, or fixing the adherent cells with glutaraldehyde altered or adversely affected the adherence assay. A parallel experiment comparing base-line adherence and adherence stimulated by FMLP or C5fr to endothelial monolayers and serum-coated plastic yielded identical results whether or not the wells had been initially coated with fibronectin. Fibronectin coating was therefore retained in the assay procedure to facilitate endothelial cell plating efficiency and growth and to approximate more closely physiologic conditions since endothelial cells have been shown to synthesize and deposit fibronectin in blood vessel walls in vivo (54). A parallel experiment comparing percent adherence of ^{51}Cr-labeled neutrophils, determined by gamma counts of harvested adherent cells, to that of unlabeled neutrophils, determined by visual hemocytometer counts of aspirated nonadherent cells, yielded stimulated responses over base line that were not significantly different. ^{51}Cr-labeling of neutrophils was therefore retained in the assay procedure to enable large experiments with multiple variables to be assessed rapidly and efficiently.

Adherence after immediate glutaraldehyde fixation followed by two washes was compared with adherence after two immediate washes before the addition of glutaraldehyde, in both time course and dose response experiments carefully designed to permit rapid handling of individual sets of wells. Washing before fixation resulted in a decrement of ~25% in both base line and chemotactic factor-stimulated absolute percent adherence but did not alter either the pattern of response or the percent increase in adherence with stimulation; see Table I.

Table I.

% Adherence (±SD)	Immediate fixation	Fixation after two washes	% Decrease
Base line	29.0±6.2	21.4±5.5	26%
Stimulated (FMLP 10^{-6} M)	68.1±4.3	50.7±5.5	26%
% Increase	234%	237%	

Visual assessment of neutrophil adherence (see Fig. 1) consistently revealed a uniform even distribution of adherent neutrophils that had been immediately fixed with glutaraldehyde. Wells washed twice before fixation exhibited a central clear zone and a peripheral ring of variably decreased neutrophil density, presumably the result of a vortex effect of the wash fluid. Immediate glutaraldehyde fixation was therefore retained in the assay procedure to ensure a uniform and reproducible assessment of adherence.

The addition of bovine serum albumin in concentrations varying from 0.25 to 5 mg/ml did not alter either base-line adherence or adherence stimulated by FMLP (10^{-8}–10^{-6} M). FMLP also enhanced neutrophil adherence to endothelial cell monolayers in the presence of 50–100% heat-inactivated plasma or serum in experiments performed without glutaraldehyde fixation of adherent cells.

In time course experiments with or without glutaraldehyde, maximum adherence was achieved within 15 min and was sustained for 45 min (data not shown). Therefore, a 15-min incubation was used routinely to minimize neutrophil migration under the monolayer during the assay period.

Electron microscopy. Cell monolayers were grown on 13-mm plastic coverslips in tissue culture wells. At confluence, neutrophils (2×10^6 per coverslip) were added with or without a chemotactic factor stimulus. After a 15-min incubation at 37°C, the monolayers were fixed, washed, and processed for electron microscopy as previously described (55).

Results

Neutrophil adherence to endothelial cell monolayers. Freshly isolated nonstimulated human neutrophils adherent to a monolayer of human umbilical vein endothelial cells are illustrated in Fig. 1. The mean percent base-line (control with buffer alone) nonstimulated adherence of neutrophils to endothelial monolayers was 35.2±0.9% (±SEM) (with a range from 19.7 to 53.1%) in 69 assays. This was equivalent to three to four neutrophils per endothelial cell. Mean adherence to tissue-culture plastic-wells coated with fibronectin and serum was 30.7±0.8% (with a range from 16.8 to 45.9%, $n = 69$).

Addition of a chemotactic factor stimulus caused greater numbers of neutrophils to adhere, as illustrated in Fig. 1. Both FMLP and purified C5 fragments (either C5a or C5a des arg) in concentrations ranging from 10^{-10} to 10^{-6} M increased neutrophil adherence to endothelium in a dose-dependent manner (Fig. 2). In a homologous bovine system, bovine neutrophils demonstrated a similar dose-dependent increase in adherence to bovine pulmonary artery endothelial cell monolayers when stimulated with increasing doses of zymosan-activated bovine plasma ranging from 1 to 10%. In two representative experiments, mean adherence (±SD) increased

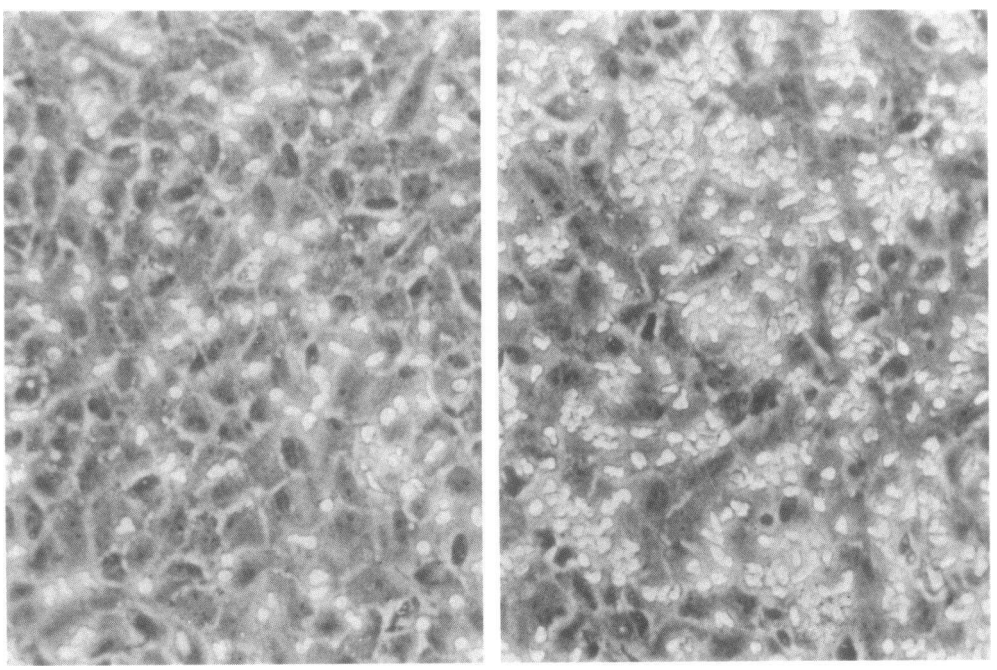

Figure 1. Human neutrophils adherent to monolayers of human umbilical vein endothelial cells. (*Left*) Base-line adherence after incubation in assay buffer alone. (*Right*) Stimulated adherence after incubation in the presence of FMLP, 10^{-6} M. × 1,200.

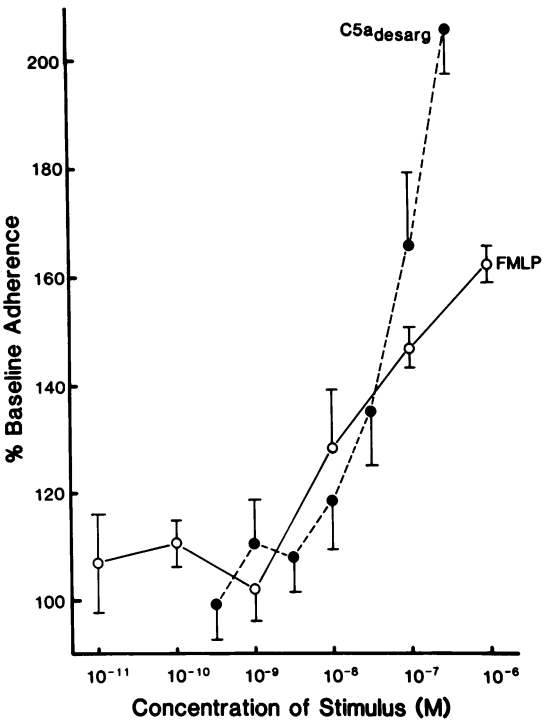

Figure 2. Effect of chemotactic factors on adherence of neutrophils to endothelium. Adherence of neutrophils (3×10^6/ml) to endothelial cell monolayers for 15 min at 37°C in the presence of increasing doses of either purified C5a des arg (●) or of FMLP (○) was compared with adherence in assay buffer alone (base line). Each line represents the mean±SEM of eight determinations.

from 59.7±8.4 to 83.6±14.8% in the presence of 10% zymosan-activated bovine plasma.

Visual assessment of both nonstimulated and stimulated wells, after washing, revealed confluent monolayers of endothelial cells with a uniform distribution of adherent neutrophils. Stimulated adherence could not be attributed to neutrophil-neutrophil aggregates but was clearly the result of increased coverage of the monolayer surface by adherent cells (Fig. 1). Direct observation and sequential photographs during a 45-min incubation period revealed that adherent neutrophils, in the presence or absence of stimulus, were not immobilized but were capable of moving about over the surface of the monolayer. In electron micrographs, neutrophils were observed both adherent to and beneath endothelial monolayers as well as in the process of migration between endothelial cells. (This latter process is under investigation [56].)

Comparison of FMLP, C5fr, and C5a and C5a des arg as stimuli of neutrophil adherence. Neutrophil adherence to endothelium induced by FMLP, C5fr or purified C5a or C5a des arg is shown in Fig. 3. The mean percent stimulated adherence and the range of response were similar for all the stimuli tested. Plots of maximal and minimal response to each stimulus depict the observed donor-to-donor variation and illustrate the fact that in some experiments up to 70–80% of the neutrophils were capable of being stimulated to adhere to endothelium (equivalent to seven to eight neutrophils per endothelial cell).

For each preparation, the ability of purified C5a to stimulate neutrophil adherence was compared with that of purified C5a des arg. In each instance, essentially identical dose-response curves were obtained. By contrast, C5a was consistently more potent in stimulating neutrophil enzyme release and chemotaxis than C5a des arg, as previously reported (45). The results for a typical preparation are shown in Table II.

Day-to-day variation in neutrophil adherence. Although the degree of base-line and stimulated adherence varied from donor to donor, values obtained for individual donors studied repeatedly were strikingly consistent. When neutrophils from five normal individuals were assayed on three successive days at 0, 24, and 48 h (data not shown), each individual's neutrophils demonstrated a characteristic base-line adherence that varied little from day to day. Therefore, the mean base-line adherence for the group was essentially constant over the 3-d period. Stimulation by four doses of chemotactic factor (C5fr, 50 and 25 μg/ml: FMLP, 10^{-6} and 10^{-7} M) generated reproducible increments in adherence. Each individual's neutrophils displayed the same relative response to the various doses of stimuli. Thus, for each dose of chemotactic factor, mean stimulated adherence exhibited only slight day-to-day variation.

Neutrophil adherence to different cell types. In agreement with previous reports (15, 17, 18), human neutrophils demonstrated preferential adherence for human umbilical vein endothelial cells compared with adherence to two other human cell types, umbilical vein smooth muscle cells and dermal fibroblasts, or to protein-coated tissue culture plastic (Table III). Adherence to smooth muscle cells was comparable but slightly less than adherence to fibroblasts.

However, in the presence of chemotactic factors (either FMLP or C5fr), this preferential interaction was markedly diminished. Stimulated neutrophils adhered equally well to all three cell types and to protein-coated plastic (Table III). In addition, in dose-response experiments (Fig. 4), neutrophil adherence to plastic stimulated by FMLP was essentially identical to stimulated adherence to endothelial cell monolayers at each dose of FMLP tested from 10^{-11} to 10^{-5} M.

Pretreatment studies. To investigate whether the increase in adherence induced by chemotactic factors could be attributed to an effect primarily on the neutrophil or on the endothelium, either neutrophil suspensions or endothelial monolayers were pretreated with various doses of chemotactic factor before the adhesion assay.

Fig. 5 illustrates the effect of pretreating endothelium or protein-coated plastic wells for 15 min with doses of C5fr ranging from 0.5 to 100 μg/ml, followed by two rapid washes before the addition of neutrophils, compared with the effect of simultaneous coincubation of the neutrophils with the dose of chemotactic factor. No effect of pretreatment of endothelium compared with plastic could be detected despite a dose-

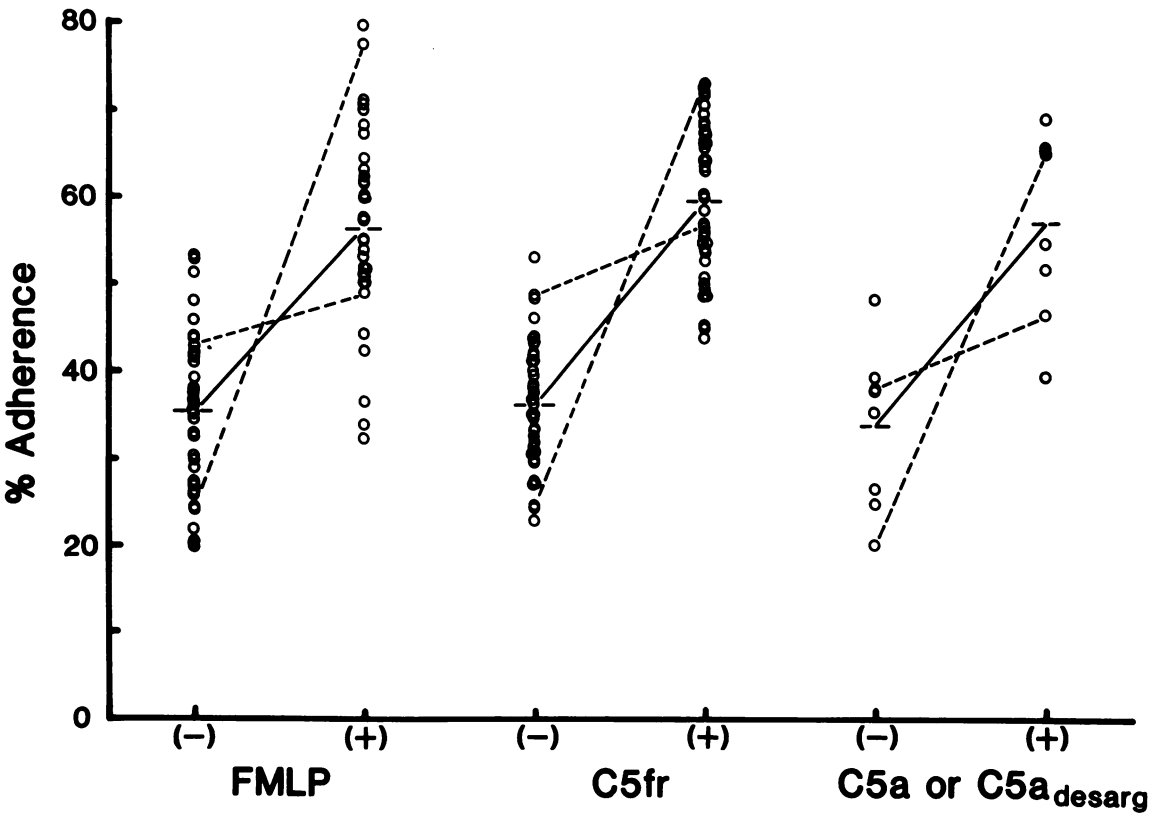

Figure 3. Comparison of the effect of different chemotactic factors on neutrophil adherence to endothelium. Percent base line and stimulated adherence were determined for FMLP (10^{-6} M, n = 43), C5 fragments (40–50 μg/ml, n = 43), and purified C5a and C5a des arg (2.8–10 μg/ml, n = 8). Mean adherence, (——); maximal and minimal responses, (- - -). Each experimental point represents the mean of quadruplicate determinations.

dependent increment in adherence with coincubation. Similarly, no increase in adherence was induced by pretreatment of the endothelium with FMLP in doses ranging from 10^{-10} to 10^{-6} M (data not shown). However, a slight increase above baseline adherence to both endothelial cells and plastic was apparent at the highest dose of stimulus used (Fig. 5). To determine whether this might have been due to the effect of residual stimulus remaining in the wells after washing, endothelial monolayers and protein-coated plastic wells were pretreated for 15 min with comparable doses of FML^{3}HP (New England Nuclear), washed twice, and then harvested. In both monolayered and protein-coated wells, 2% of the initial dose of FMLP

Table II. Comparison of C5a and C5a des arg Stimulation of Neutrophil Adherence, Enzyme Secretion, and Chemotaxis

Concentration of stimulus (*μg/ml*)		0.1	1	5	10
Adherence to endothelium (% *adherence*)	C5a	33.2±7.4	46.1±4.0	47.1±1.1	ND
	C5a des arg	36.1±5.2	45.7±12.7	49.8±5.3	ND
Enzyme secretion (% *myeloperoxidase release*)	C5a	37.1±0.6	47.7±0.1	53.9±2.3	71.8±0.2
	C5a des arg	0.2±0.3	0.1±0.1	0.3±0.1	0.9±0.2
Chemotaxis (*mean number cells/high power field*)	C5a	21±3	118±10	ND	152±30
	C5a des arg	14±2	60±3	ND	77±14

Data represent mean±SEM. ND, not determined.

Table III. Comparison of Base-line and Stimulated Neutrophil Adherence to Different Cell Types

Cell type	% Base-line adherence	% Stimulated adherence	
		FMLP*	C5fr*
Human umbilical vein endothelial cells	40.7±1.7 (24)‡	55.9±2.1 (8)	63.6±1.4 (4)
Protein-coated plastic	33.3±1.9 (8)	52.7±3.9 (4)	ND
Human dermal fibroblasts	28.0±1.0 (23)	48.8±1.8 (8)	63.4±1.9 (4)
Human umbilical vein smooth muscle cells	25.8±1.5 (8)	49.7±3.4 (4)	59.1±3.5 (4)

ND, not determined.
* Doses used: FMLP, 10^{-6} M; C5fr, 50 μg/ml.
‡ Data represent mean±SEM (*n*).

remained after washing, enough to account for the slight increment in neutrophil adherence seen with the highest doses of stimulus.

To investigate the possibility that an effect of chemotactic factor on endothelium might be induced slowly and thus might not have been detected in the 15-min pretreatment assays described above, endothelial monolayers were pretreated with either FMLP (5×10^{-7} M) or purified C5a (2.8 μg/ml) for either 1 h or 4 h and then washed before assay (data not shown). No increase in neutrophil adherence to the pretreated endothelium was detected for either time point.

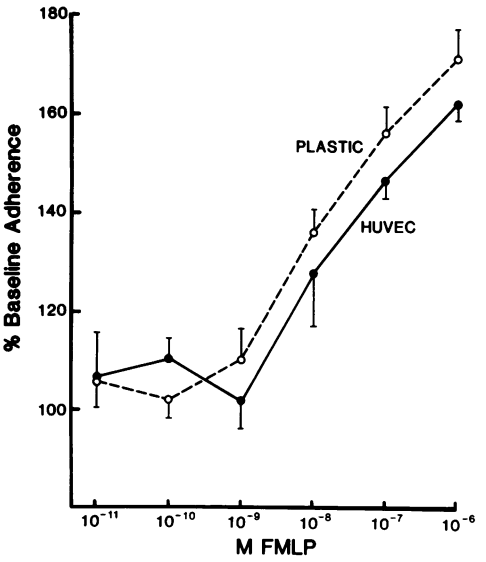

Figure 4. Comparison of neutrophil adherence to endothelium and to plastic stimulated by FMLP. Adherence of neutrophils to human umbilical vein endothelial cell (HUVEC) monolayers (●) and to protein-coated plastic (○) was determined in the presence of increasing doses of FMLP. Data represent the mean±SEM of eight determinations.

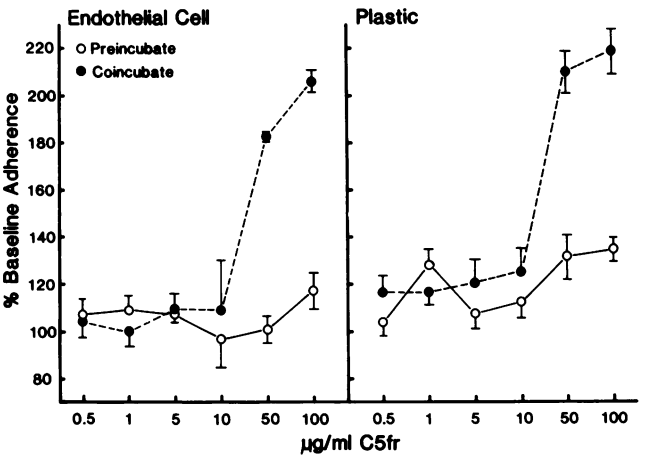

Figure 5. Effect of pretreatment of endothelium with chemotactic factor. Endothelial cell monolayers or protein-coated plastic wells were either pretreated with chemotactic factor for 15 min at 37°C and then washed twice before incubation with neutrophils (○) or pretreated with assay buffer, washed twice, and then incubated with chemotactic factor and neutrophils (●). Data are expressed as mean±SEM of eight determinations.

As shown in Fig. 6, increased adherence was usually but not always observed when neutrophils were pretreated with various doses of either FMLP, C5fr, or C5a and then washed before assay. This variable response also occurred with pretreatment times ranging from 5 to 30 min. In contrast, as

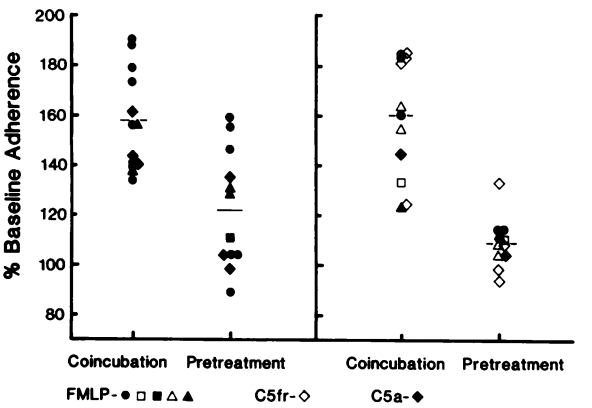

Figure 6. Comparison of effects of neutrophil (*left*) or endothelial cell (*right*) pretreatment with chemotactic factor on neutrophil adherence. Either neutrophils or endothelial monolayers were pretreated with indicated doses of chemotactic factor and then washed before the adhesion assay. Results were compared with neutrophils or endothelial monolayers pretreated with assay buffer, washed, and then coincubated with cells and chemotactic factor. Doses of chemotactic factor: ●, FMLP, 10^{-6} M; □, FMLP, 5×10^{-7} M; ■, FMLP, 10^{-7} M; △, FMLP, 5×10^{-8} M; ▲, FMLP, 10^{-8} M; ◇, C5fr, 40–50 μg/ml; ◆, C5a 2.8, μg/ml. Each symbol represents the mean of four replicate determinations.

described, pretreatment of endothelium consistently failed to increase adherence above base line. Pretreatment of both endothelial cells and neutrophils (data not shown) resulted in enhanced adherence equal to but no greater than that achieved by pretreatment of the neutrophils alone.

Of interest is the fact that when pretreatment experiments were conducted at 25° instead of at 37°C, a striking and more consistent neutrophil pretreatment effect was observed. The mean increase in adherence for pretreated neutrophils was 170.8±6.5% of base line compared with 102.4±5.6% for pretreated endothelial cells in five experiments using FMLP (10^{-7} to 10^{-6} M) as stimulus. These data suggested that chemotactic factors did act primarily on the neutrophil but that at 37°C the effect was transient and not always captured by the experimental system.

Rapid modulation of chemotactic factor-stimulated neutrophil adherence to endothelium. To determine how rapidly neutrophils could be stimulated by chemotactic factor to adhere to endothelium, time course experiments were performed in which neutrophils were allowed to settle in the assay wells for 10 min at 37°C before the addition of stimulus. As shown in Fig. 7, the onset of stimulated adherence occurred within 30 s after the addition of chemotactic factor. Maximal adherence was attained within 2 min and persisted for 15 min. Stimulated adherence could be demonstrated to persist for up to 45 min (data not shown).

To investigate whether chemotactic factor-induced neutrophil adherence to endothelium was capable of rapid modulation, neutrophils were pretreated with chemotactic factor, quickly washed twice (expired time: 10–15 min) or diluted 10-fold (expired time: 1–2 min), and then added to endothelial monolayers in the presence or absence of the same dose of stimulus (settling time of cells in assay wells: 5–10 min). Results obtained for FMLP and C5fr are illustrated in Fig. 8. Neutrophils pretreated in assay buffer alone, washed or diluted, and then incubated in the presence of stimulus (coincubation) demonstrated the expected stimulated increase in adherence over base line. Rapid loss of stimulated activity occurred upon removal of stimulus: neutrophils pretreated with chemotactic factor, washed or diluted, and then incubated in the absence of stimulus demonstrated diminished adherence. Rapid restimulation of activity occurred upon readdition of stimulus: neutrophils pretreated with chemotactic factor, washed or diluted, and then incubated in the presence of the same dose of stimulus demonstrated maximal increase in adherence.

Thus, neutrophil adherence to endothelium stimulated by chemotactic factor occurred rapidly upon addition of stimulus, decreased upon removal of stimulus, but could be maximally restimulated upon immediate readdition of the same stimulus.

Discussion

In this study, a quantitative monolayer adherence assay was used to investigate human neutrophil adherence to cultured human endothelial cell monolayers under the influence of chemotactic C5 fragments or FMLP as stimuli. The data presented here suggest that chemotactic factors act primarily on the neutrophil to enhance adherence to the endothelium and that the stimulated adhesion is capable of rapid modulation.

Under our experimental conditions, base-line adhesion of nonstimulated neutrophils to endothelial cell monolayers was

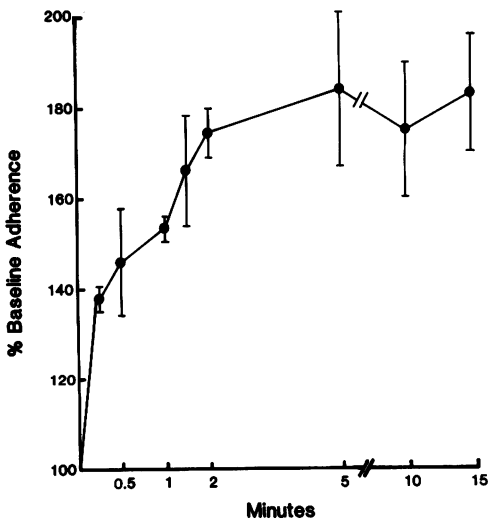

Figure 7. Time course of chemotactic factor-stimulated neutrophil adherence to endothelium. Neutrophils were allowed to settle in the assay wells for 10 min at 37°C before the addition of stimulus (FMLP, 10^{-6} M). The assay was halted at the indicated times of incubation, from 15 s to 15 min, by the addition of glutaraldehyde. Data are expressed as mean±SEM for eight determinations.

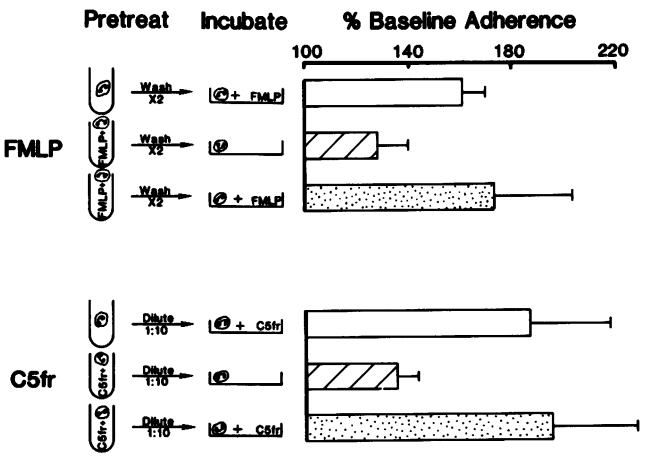

Figure 8. Rapid restimulation of neutrophil adherence to endothelium. Neutrophils were pretreated with either chemotactic factor (FMLP, 10^{-6} M or C5fr, 40 μg/ml) or assay buffer, and then either washed twice or diluted 1:10 before addition to assay wells and subsequent coincubation with either the same dose of chemotactic factor or assay buffer alone. Results for FMLP represent mean±SEM of four experiments. Results for C5fr represent mean±SEM of three experiments.

35.2±0.9% (±SEM). This was equivalent to three to four neutrophils per endothelial cell since the neutrophils were initially added in a ratio of 10 neutrophils per endothelial cell, and is in agreement with Buchanan and Gimbrone (20) who reported a basal adhesion of four to five human neutrophils per bovine aortic endothelial cell as determined by direct visual counts after a sequential immersion wash technique. Nonstimulated neutrophils demonstrated a relative specificity for adherence to human umbilical vein endothelial cells (three to four per cell) compared with human dermal fibroblasts or human umbilical vein smooth muscle cells (two to three per cell). Although not large, this difference was reproducible and is consistent with previous reports from other laboratories using comparable systems to study adherence of human, rabbit, bovine, or porcine neutrophils to a variety of cell types and lines from different species (14, 15, 17, 18). This report extends preferential neutrophil adhesion to endothelium to include human neutrophil interaction with freshly isolated low passage human fibroblasts and smooth muscle cells compared with human endothelial cells. This preferential interaction, in addition to the observed motility of adherent neutrophils over the surface of cultured endothelial monolayers, (also noted by other investigators [14, 18, 24]) is consistent with the concept of a marginated pool in which one-half or more of intravascular neutrophils are believed to be, at any one time, reversibly associated with (i.e., adherent to) the vascular endothelial surface (25, 57, 58). Our assay, which used static conditions and immediate glutaraldehyde fixation of adherent neutrophils, was designed to study the low affinity adhesion (which ranged from 20 to 53%) presumably occurring during periods of stasis in dilated microvessels near a site of inflammation and apparently necessary for subsequent cell migration; in contrast, other investigations have specifically used an intensive washing procedure to assay only very tightly attached cells, representing <5% of the population under base-line conditions (59). (The degree of base-line adhesion has been demonstrated to decrease in the presence of low degrees of shear stress [60]). That percent adherence decreased on sequential incubation of a nonstimulated neutrophil population with successive endothelial monolayers also suggests the probable existence of a subpopulation of more adherent neutrophils (34).

The addition of a chemotactic factor stimulus (purified C5fr or FMLP) caused a dose-dependent increase in neutrophil adherence, confirming earlier reports of increased adherence to endothelium in response to zymosan-activated serum, C5a, or FMLP (17, 21, 36). Under our experimental conditions, maximal stimulated adherence averaged 55–59%, equivalent to five to six neutrophils per endothelial cell. In individual experiments, stimulated adherence was as high as 70–80%, indicating that a major proportion of circulating neutrophils are capable of being stimulated by chemotactic factors to adhere to endothelium. In experiments in which endothelial cells were radiolabeled with ^{111}Indium, <1% of the label was released during 1-h incubations with neutrophils and FMLP, 10^{-6} M, in the absence of albumin, indicating that substantial neutrophil-dependent endothelial injury was not occurring under the assay conditions used. Experiments using a homologous bovine system demonstrated that neutrophils could be stimulated by chemotactic factors to adhere to adult arterial endothelium as well as to fetal venous endothelium. The fact that stimulated adherence also occurred in the presence of 50–100% heat-inactivated plasma or serum emphasizes the likelihood that chemotactic factor enhancement of neutrophil adhesion to endothelium might occur in vivo.

The increase in adherence induced by chemotactic factors was interpreted as representing a true enhancement of neutrophil adhesiveness to the surface of the monolayered endothelial cells, and not of neutrophil-neutrophil aggregation, for several reasons. Neutrophil-neutrophil aggregation has been shown to occur in suspension upon the addition of chemotactic factor, to peak within 2 min, to abate within 10–15 min, and to undergo the phenomenon of desensitization, a decrease in response upon rapid restimulation (61). In our experimental system, stimulated adherent neutrophils visually covered a greater percent of the monolayer surface than under nonstimulated conditions, and were not observed to form aggregates. The adherence did not diminish within 10–15 min but was sustained at a constant level for up to 45 min. Stimulated neutrophils did not become desensitized to adherence, but after initial pretreatment with chemotactic factor, could be rapidly restimulated to adhere maximally to endothelium.

The microtiter adherence assay used in these studies proved to be sensitive and reproducible and, because of the small volumes required, enabled multiple variables to be assessed concurrently, thus facilitating time course, dose response, and cell comparison experiments. The reproducibility of the assay was demonstrated when neutrophils from five normal individuals were assayed on each of three successive days. Base-line and stimulated adherence to four different doses of chemotactic factor were strikingly constant for each individual donor, despite donor-to-donor variability. Therefore, mean values for the group of five individuals exhibited only slight day-to-day variation.

Purified C5a and C5a des arg, on a molar basis, demonstrated an essentially equal ability to enhance neutrophil adherence to endothelium, in marked contrast to the relatively greater potency of C5a compared with C5a des arg in stimulating neutrophil chemotaxis, enzyme release, and superoxide anion production, as confirmed in this study and as previously reported (45). Although the means by which C5a des arg stimulates neutrophil function are unknown, the greater potency of C5a des arg in enhancing adherence suggests that adherence might be mediated by different intracellular mechanisms than those of chemotaxis or enzyme release. These data are also consistent with our previous observations of the effectiveness of C5a des arg in attracting neutrophils into pulmonary alveoli (62).

The preferential interaction of nonstimulated human neutrophils with human endothelium suggests that endothelial cells may make a unique contribution to the base-line adhesive

interaction. However, the data presented here support the concept that chemotactic factors enhance neutrophil adherence to endothelial cells by exerting an effect primarily on the neutrophil. In the presence of various doses of chemotactic factor, neutrophils no longer displayed a preferential interaction with human endothelial cells but adhered equally well to different cell types or to protein-coated plastic. In addition, pretreatment of endothelial cells with chemotactic factor for as long as 4 h before assay failed to increase subsequent neutrophil adherence, suggesting that chemotactic factors did not cause a persistent alteration in the endothelial cell. However, our data cannot exclude the possibility that chemotactic factors might induce a transient (or low affinity) alteration in the endothelial cell surface to enhance neutrophil adhesion. In contrast, pretreatment of neutrophils with chemotactic factor variably increased their subsequent adherence to endothelium at 37°C. This neutrophil pretreatment effect was consistently present at 25°C. These findings are in disagreement with reports by Hoover et al. (17, 21) that pretreatment of either endothelial cells or neutrophils increased subsequent adherence. The reasons for this discrepancy are not clear. However, it may be in part a result of differences in the washing procedures used in the two assay systems. In our system, two washes left behind in each well ~2% of the added dose of stimulus, as determined in tritiated FMLP experiments. Therefore, our pretreatment experiments were performed with various doses of the chemotactic factor stimuli and were always designed to permit direct comparison of pretreatment and coincubation conditions. Nevertheless, in our assay system, optimal adhesion was consistently attained when neutrophils, endothelial cells, and chemotactic factor were all present together in the assay well (coincubation). These observations suggested that the adhesive capacity of the neutrophil, once stimulated, might be transient.

The data presented above are consistent with the concept that chemotactic factors derived from tissue sites of inflammation might act directly on the neutrophil in the circulation to enhance adherence to the endothelium. For this to be feasible, the enhanced neutrophil-endothelial cell adhesive interaction would have to be capable of rapid modulation to ensure proper localization of neutrophil emigration. Time course experiments designed to test this hypothesis demonstrated that chemotactic factor-stimulated neutrophil adherence to endothelium occurred rapidly (within 2 min), diminished upon removal of stimulus (10-fold dilution), but could be rapidly and maximally restimulated upon immediate readdition of the original dose of chemotactic factor. It is of interest that chemotactic factor-induced neutrophil adherence did not undergo the process of desensitization previously demonstrated to occur for other chemotactic factor-induced neutrophil functions such as chemotaxis and enzyme release (63).

Neutrophil adherence to endothelium stimulated by chemotactic factor would appear to be a dynamic neutrophil response capable of rapid modulation, possibly important to the ability of neutrophils to adhere to and then migrate through vessel walls to localize at sites of tissue inflammation. Our data certainly do not eliminate the possibility that chemotactic factors or other agents might induce a proadhesive alteration in the endothelial cell, but do support the concept that chemotactic factors act primarily on the neutrophil. Thus, the specificity and time course of cell recruitment in an acute inflammatory reaction might be dependent upon interactions of circulating leukocytes with specific chemotactic factors.

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