

# Adherence of Neutrophils to Cultured Human Microvascular Endothelial Cells

## Stimulation by Chemotactic Peptides and Lipid Mediators and Dependence upon the Mac-1, LFA-1, p150,95 Glycoprotein Family

Marcia G. Tonnesen,<sup>\*\*§</sup> Donald C. Anderson,<sup>§</sup> Timothy A. Springer,<sup>\*\*</sup> Ann Knedler,<sup>\*</sup> Natalie Avdi,<sup>\*</sup> and Peter M. Henson<sup>\*\*||</sup>  
<sup>\*</sup>Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, <sup>‡</sup>Dermatology Service, Veterans Administration Medical Center, <sup>§</sup>Departments of Dermatology, <sup>||</sup>Pathology and Medicine, University of Colorado School of Medicine, Denver, Colorado 80206; <sup>§</sup>Departments of Pediatrics, Cell Biology and Microbiology and Immunology, Baylor College of Medicine and the Speros P. Martel Leukocyte Biology Laboratory, <sup>†</sup>Texas Children's Hospital, Houston, Texas 77054; <sup>‡</sup>Department of Pathology, Harvard Medical School and Laboratory of Membrane Immunochemistry, <sup>\*\*</sup>Dana-Farber Cancer Institute, Boston, Massachusetts 02115

### Abstract

The process of neutrophil adhesion to and migration through the microvascular endothelium, an early event in the induction of the acute inflammatory response, has been attributed to the generation of extravascular chemoattractants. Although both chemotactic peptides and lipid mediators enhance neutrophil adherence *in vitro* and *in vivo*, the mechanism(s) involved in the interaction between circulating neutrophils and microvascular endothelial cells is still not completely understood. In a microtiter well adherence assay, the chemotactic peptides, FMLP and C5a, and the lipid mediators, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and platelet activating factor (PAF), enhanced human neutrophil adherence to cultured human microvascular endothelial cells as well as to human umbilical vein endothelial cells in a dose-dependent manner with a rapid time course. This stimulated adhesive interaction between neutrophils and cultured human endothelial cells was dependent on the expression of the Mac-1, LFA-1, p150,95 glycoprotein family on the neutrophil surface since neutrophils from patients with leukocyte adhesion deficiency, lacking surface expression of the adhesive glycoproteins, exhibited markedly diminished adherence to human endothelial cells in response to stimulation with chemotactic factors compared to normal control neutrophils. All four mediators enhanced expression of the glycoprotein family on the surface of normal neutrophils as determined by flow cytometry using a monoclonal antibody (TS1/18) to the glycoprotein common beta subunit. In addition, TS1/18 inhibited up to 100% the adherence of normal neutrophils to endothelial cells stimulated by maximal concentrations of FMLP, C5a, LTB<sub>4</sub>, or PAF. Moreover, HL-60 cells, human promyelocytic leukemia cells, neither increased glycoprotein surface expression nor adherence in response to stimulation. Thus, peptide and lipid mediators of the acute inflammatory response

appear to enhance adherence of circulating neutrophils to the microvascular endothelium by a mechanism dependent on expression of the Mac-1, LFA-1, p150,95 glycoprotein family on the neutrophil surface.

### Introduction

Increased adherence of circulating neutrophils to the microvascular endothelium is an essential early event in the initiation of the acute inflammatory response, regularly preceding neutrophil migration through vessel walls and accumulation at sites of tissue injury (1, 2). For nearly a century, chemotactic factors have been implicated as key participants in the process of leukocyte diapedesis (2-4). *In vivo*, intravascular chemoattractants induce a profound neutropenia with sequestration of neutrophils in the microvasculature, presumably the result of enhanced interaction between neutrophils and the microvascular endothelium (5-8). *In vitro* studies have demonstrated that neutrophil adherence to cultured large vessel endothelial cells (human umbilical vein, bovine aorta, porcine aorta) is increased in the presence of a chemotactic stimulus (either peptide or lipid) (9-17). However, the mechanism(s) by which chemotactic factors enhance the adhesive interaction between circulating neutrophils and the microvascular endothelium has not yet been defined.

Nevertheless, significant progress has been made in our understanding of mechanisms of human neutrophil adherence as a result of the recent definition of the critical role of a family of leukocyte surface adhesive glycoproteins in neutrophil adherence-dependent functions (18, 19). Termed the Mac-1, LFA-1, p150,95 glycoprotein family by Springer and colleagues (20, 21) or CDw18 by the Second International Workshop on Leukocyte Differentiation Antigens (22), the family is composed of three structurally and functionally related glycoproteins: Mac-1 (the C3bi complement receptor), LFA-1 (lymphocyte function-associated antigen),<sup>1</sup> and p150,95. Each consists of an immunologically distinct alpha subunit that is noncovalently associated with a common beta subunit (20). The distribution of these glycoproteins is limited to white blood cells and hematopoietic precursor cells (19). Leukocyte adhesion deficiency, a recently recognized autosomal recessive

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Address reprint requests to Dr. Tonnesen, Department of Pediatrics, 9GB, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206.

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1. *Abbreviations used in this paper:* HMVEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; KRPD, Krebs-Ringer PBS with 0.2% dextrose; LFA-1, lymphocyte function-associated antigen; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; MPO, myeloperoxidase; PAF, platelet activating factor; RFI, relative fluorescence intensity; TNF, tumor necrosis factor.

disorder characterized by recurrent bacterial infections, impaired pus formation, and delayed wound healing, has been attributed to deficiency (or complete absence) of cell surface expression of the Mac-1 adhesive glycoprotein family (reviewed in 19). Neutrophils from such patients demonstrate abnormalities in adherence-dependent functions *in vitro* (18, 23) including one report of markedly reduced adherence of neutrophils from two patients to cultured large vessel endothelial cells (bovine aortic and human umbilical vein) compared to that of normal neutrophils, when stimulated by phorbol myristate acetate (PMA) or the calcium ionophore A23187 (24). In addition, a monoclonal antibody (60.3) to the common beta subunit of the glycoprotein family inhibits normal neutrophils stimulated by a variety of mediators including PMA, A23187, FMLP, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), platelet-activating factor (PAF) and tumor necrosis factor (TNF), from adhering to large vessel endothelial cells (24, 25).

Thus it has been proposed that the inability of patient neutrophils, when stimulated, to express surface adhesive glycoproteins and thereby increase adherence to microvascular endothelium may account for the failure of patient neutrophils to migrate to sites of inflammation *in vivo* (24, 26). Evidence to support this concept further derives from the demonstration that bone marrow transplantation completely restored leukocyte function and reversed symptoms in two patients with leukocyte adhesion deficiency (19, 27) and from animal models in which monoclonal antibody 60.3 inhibited neutrophil adherence to venules and accumulation in rabbit muscle after local application of LTB<sub>4</sub> or zymosan-activated serum (28) and blocked neutrophil accumulation in polyvinyl sponges placed subcutaneously in the rabbit to induce inflammation (29). Demonstration of an essential role for surface expression of the Mac-1 family of adhesive glycoproteins in neutrophil adherence to human microvascular endothelium induced by physiologically relevant chemotactic stimuli would serve to validate the critical role of the Mac-1 glycoprotein family in neutrophil diapedesis and migration to inflammatory sites in humans *in vivo*. In this study, we report that human neutrophil adherence *in vitro* to cultured human microvascular endothelial cells, as well as to umbilical vein endothelial cells, when stimulated by chemotactic peptides (FMLP and C5a) and lipid mediators (LTB<sub>4</sub> and PAF), is dependent on neutrophil surface expression of the Mac-1, LFA-1, p150,95 glycoprotein family.

## Methods

**Preparation of reagents.** As reported previously (30, 31), all reagents and plasticware were tested for the presence of lipopolysaccharides (LPS), and only those containing < 0.1 ng/ml LPS as determined by the limulus amoebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA) were used in adherence studies.

The assay buffer employed was Krebs-Ringer phosphate buffered saline, with 0.2% dextrose (KRPD). For assays involving lipid mediators, the assay buffer was supplemented with 0.01% human serum albumin (HSA; Cutter Laboratories, Emeryville, CA). Fresh assay buffer was prepared on each day of experimentation. The chemotactic peptide FMLP (Vega Biochemicals, Tucson, AZ) was kept frozen at -20°C in dimethyl sulfoxide (DMSO; Fisher Scientific Co, Fair Lawn, NJ) at 10<sup>-3</sup> M and diluted in assay buffer before use.

The biologically active chemotactic fragment of the fifth component of complement (C5a) was purified from human serum by a procedure modified from that of Fernandez and Hugli (32), as reported previously (14, 33).

LTB<sub>4</sub> was generously provided by Dr. Robert Murphy (University of Colorado), kept frozen at -20°C in KRPD with 0.1% HSA at 2 × 10<sup>-6</sup> M for a maximum of 2 mo, and diluted in assay buffer before use.

PAF (Avanti Polar Lipids, Inc., Birmingham, AL) and Lyso-PAF (Bachem AG, Bubendorf, Switzerland) were kept frozen at -20°C in KRPD with 0.25% HSA at 10<sup>-4</sup> M for a maximum of 6 mo and diluted in assay buffer before use.

**Isolation and culture of human endothelial cells.** Human microvascular endothelial cells were isolated from fresh, healthy, omental adipose tissue (obtained after informed consent) according to the method of Kern et al. (34) and cultured as previously described (31). Briefly, human omental fat was minced finely, digested in 200 U/ml collagenase, and then filtered through 250 μm mesh to remove undigested fat and through 30 μm mesh to separate endothelial clumps from single stromal cells and erythrocytes. Further isolation of endothelial cell clumps was accomplished by gravity sedimentation through 5% BSA. The essentially pure endothelial cells were then plated in MCDB 131 supplemented with 5% FCS (Irvine Scientific, Santa Ana, CA), 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone (both from Sigma Chemical Co., St. Louis, MO), which is a selective culture system for endothelial cells (35). Primary cultures formed confluent monolayers within 7 d and were characterized by demonstration of the typical cobblestone morphology of endothelial cells, the expression of Factor VIII-related antigen and the uptake of acetylated low density lipoprotein using methods described by Kern (34) and Knedler (35).

Human umbilical vein endothelial cells were harvested by collagenase digestion according to the method of Gimbrone et al. (36) and grown as previously described (14) in supplemented MCDB 131.

Endothelial cells were used in first or second passage only for adherence assays.

**Isolation and radiolabeling of neutrophils.** Blood samples were drawn from normal donors and from patients with leukocyte adhesion deficiency (patients 1, 4, 6, and 9) after informed consent was obtained, as previously reported (19, 26). Peripheral blood neutrophils were isolated as previously described (30, 31) using plasma-Percoll gradients and avoiding LPS exposure. In brief, fresh citrated venous blood was centrifuged; the platelet-rich plasma was aspirated and re-centrifuged to produce platelet-poor plasma (PPP); and the cell pellet was resuspended in 0.5% dextran to remove contaminating erythrocytes by gravity sedimentation. The leukocytes were then separated by centrifugation through 42%/51% discontinuous PPP-Percoll gradients. The neutrophils at the interface of the 42% and 51% Percoll layers were harvested, washed, and resuspended in KRPD. This preparation method yielded neutrophils that were > 95% pure and > 99% viable by trypan blue exclusion.

Human neutrophils (2 × 10<sup>7</sup>/ml) were radiolabeled by incubation with 10 μCi of <sup>111</sup>In (indium chloride solution; New England Nuclear, Boston, MA) per ml of cells and 4 × 10<sup>-4</sup> M tropolone (Sigma) for 5 min at 25°C followed by thorough washing in KRPD.

**Culture and characterization of HL-60 cells.** HL-60 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 containing 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with 20% heat-inactivated FCS and 2 mM L-glutamine (all from Hazleton Research Products, Denver, PA); passaged twice weekly; and maintained at a concentration of 1.5–6 × 10<sup>5</sup> cells/ml at 37°C in a 5% CO<sub>2</sub> incubator. The doubling time of the HL-60 cells was ~ 24 h (37), and the viability > 98%. Cultures were tested monthly for the presence of mycoplasma and were consistently negative.

Before their use in adherence assays, cultures of HL-60 cells were characterized in the following manner: (a) maintenance of immature promyelocytic morphology (37, 38) was confirmed on each experimental day by Diff-Quik (American Scientific Products, McGraw Park, IL) staining of cytospin preparations, typically showing < 5% contamination with more mature cells; (b) failure to secrete myeloperoxidase (MPO) (39), despite an intracellular content of MPO approximately twice that of mature neutrophils, was confirmed by assessment of MPO release (40) after stimulation with cytochalasin B (5 μg/ml)

and FMLP ( $10^{-7}$  M); (c) inability to generate oxygen radicals (37, 39, 41) was qualitatively confirmed by documenting absence of nitroblue tetrazolium (NBT; Sigma) reduction in the presence of phorbol myristate acetate (PMA, 200 ng/ml; Sigma) (41).

On each day of experimentation, the HL-60 cells were centrifuged and resuspended in assay buffer at  $20 \times 10^6$  cells/ml before use.

**Preparation of protein-coated plastic wells.** M199 tissue culture medium (Hazleton) supplemented with 10% FCS was dispensed in 100- $\mu$ l vol into the wells of microtiter tissue culture plates (Costar, Cambridge, MA), and the plates placed in a 37°C tissue culture incubator for at least 2 h and then washed before use in adherence assays.

**Adherence and antibody inhibition assay.** Adherence of neutrophils to monolayers of endothelial cells or to protein-coated plastic was assayed as previously described (14) with minor modification. Briefly, human microvascular or umbilical vein endothelial cells were grown to confluence in microtiter tissue culture wells in supplemented MCDB 131. The monolayers were washed twice before assay and maintained in serum free assay buffer. Triplicate or quadruplicate wells were prepared for each experimental variable to be tested.

After gentle suction aspiration of the assay buffer,  $1.5 \times 10^5$   $^{111}$ In-labeled neutrophils in assay buffer were added to each well followed by an appropriate concentration of stimulus dissolved in assay buffer or assay buffer alone. After incubation for 15 min at 37°C in a 5% CO<sub>2</sub> humidified tissue culture incubator, the adherent neutrophils were fixed by the addition of 2% glutaraldehyde in PBS, and the nonadherent cells were removed by gently washing twice with assay buffer. The adherent cells were then harvested by thorough swabbing with cotton-tipped applicators, and the applicators then counted in a gamma counter (Beckman Instruments, Inc., Fullerton, CA). Results were expressed as percent adherence = (cpm harvested)/(cpm added)  $\times$  100.

For time course experiments, neutrophils were allowed to presettle in the assay wells for 10 min before the addition of stimulus, as previously described (14).

For antibody inhibition experiments, unless otherwise indicated, a saturating concentration of antibody (as determined by immunofluorescence flow cytometry) diluted in assay buffer was added to the  $^{111}$ In-labeled neutrophils in the assay wells, at 37°C, 5 min prior to the addition of stimulus.

**Monoclonal antibodies.** The TS1/18 monoclonal antibody that recognizes an antigen on the common beta subunit of Mac-1, LFA-1 and p150,95 (20) was used as the IgG1 fraction, purified from ascites fluid as previously described (42). Anti-Leu-4, IgG1 fraction, (Becton Dickinson, Mountain View, CA) was used as a nonbinding control monoclonal antibody. NCD-1, IgG1 fraction, which binds to stimulated human neutrophils and specifically inhibits chemotaxis and enzyme secretion (43), was a gift from Dr. Susan Walker (National Jewish Center for Immunology and Respiratory Medicine) and was used as a positive binding control monoclonal antibody.

**Immunofluorescence flow cytometry.** For labeling,  $1 \times 10^6$  neutrophils in assay buffer were incubated with or without an appropriate concentration of stimulus for variable periods of time at 37°C before the addition of an excess of ice-cold blocking buffer (KRPD with 5% bovine serum albumin (fraction V; Sigma)). After an additional wash with cold blocking buffer, cells were incubated on ice with the desired concentration of monoclonal antibody for 10 min, washed twice to remove unbound antibody and then incubated with fluorescein-conjugated (FITC) goat anti-mouse antiserum, IgG fraction at 1.2 mg/ml (Cooper Biomedical, West Chester, PA) for 5 min at 4°C. After two final washes, the cells were suspended in KRPD, kept light protected on ice, and assayed within 1 h of preparation.

Analysis of monoclonal antibody binding to resting and stimulated cells by immunofluorescence flow cytometry was performed using either a cytofluorograph (Ortho Diagnostics, Westwood, MA) equipped with an Ortho 2150 computer or a Epics C system flow cytometer (Coulter Instruments, Hialeah, FL). The fluorescence signal was log-amplified in three decades with the photomultiplier gain setting selected in each experiment such that the fluorescence histogram of cells stained with the second antibody alone was bell-shaped in distribution

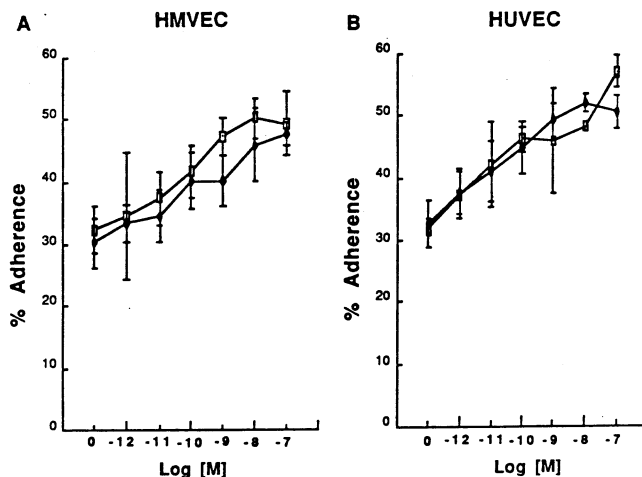
from the origin. Mean values from the logarithmic histograms were corrected for nonspecific binding of second antibody, converted to linear values, and expressed as relative fluorescence intensity (RFI) according to the following formula: Relative fluorescence intensity (RFI) = (antilog  $B - A$ ) / conversion factor.  $B$  is the mean value obtained with addition of primary and secondary antibody;  $A$  that with addition of secondary antibody only. The conversion factor for the Ortho was 333.33 and for the Epics was 341.3.

## Results

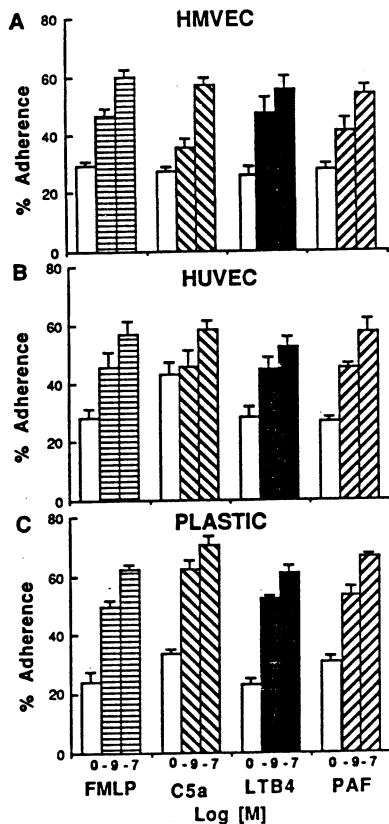
### Neutrophil adherence to endothelial cell monolayers stimulated by lipid mediators

Both LTB<sub>4</sub> and PAF (in concentrations ranging from  $10^{-12}$  to  $10^{-7}$  M) increased human neutrophil adherence in a dose-dependent manner to monolayers of human microvascular (HMVEC) or umbilical vein (HUVEC) endothelial cells (Fig. 1). Mean baseline spontaneous adhesion (buffer alone) was 30–32.5% and mean stimulated adherence, at maximal doses of mediator, ranged from 48 to 57%, although in some experiments up to 65–70% of the neutrophils were induced to adhere to endothelial cells. Mean neutrophil responses were similar for both mediators on both types of endothelium (as well as on protein-coated plastic, data not shown), although considerable donor-to-donor variability was seen when individual experiments were compared, analogous to that reported previously for chemotactic peptides (14). Under the same experimental conditions, Lyso-PAF, the biologically inactive precursor/metabolite of PAF, failed to enhance neutrophil adherence, even at as high a dose as  $10^{-6}$  M.

The degree of neutrophil adherence to all three substrata (monolayers of HMVEC and HUVEC as well as protein-coated plastic) induced by the lipid mediators LTB<sub>4</sub> and PAF was comparable to that induced by the chemotactic peptides FMLP and C5a to these surfaces. Fig. 2 illustrates the results obtained for all four mediators on the three substrata at two stimulatory concentrations:  $10^{-9}$  and  $10^{-7}$  M.

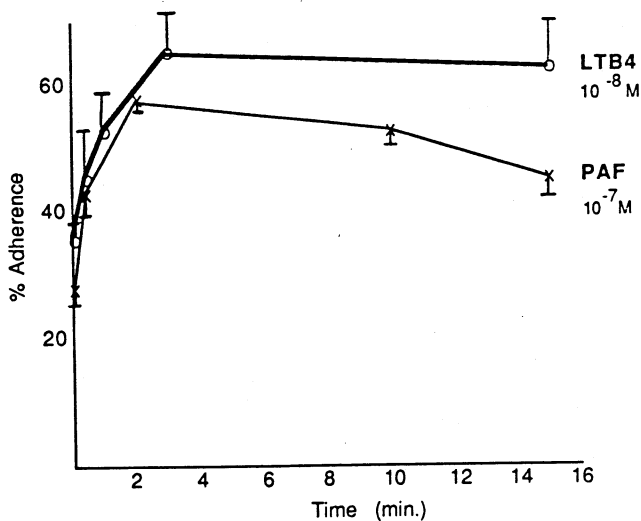


**Figure 1.** The effect of lipid mediators on adherence of neutrophils to endothelial cells. Human neutrophils were allowed to adhere to cultured monolayers of human microvascular endothelial cells (A) or human umbilical vein endothelial cells (B) for 15 min at 37°C in the presence of increasing doses of either LTB<sub>4</sub> (□) or PAF (◆). Each curve represents the mean  $\pm$  1 SEM (or  $\pm$  1 SD) of values from two to four separate experiments, each performed in quadruplicate.



**Figure 2.** The effect of chemotactic peptides and lipid mediators on adherence of neutrophils to endothelial cells and to plastic. Adherence of human neutrophils to human microvascular endothelial cells (A), human umbilical vein endothelial cells (B), or protein-coated plastic (C) was determined in the presence of FMLP (□) C5a (▢), LTB<sub>4</sub> (■), PAF (▨), or buffer alone (□). Data represent the mean ± 1 SEM of values from three to seven separate experiments, each performed in quadruplicate.

Time course experiments (Fig. 3), in which the time delay required for neutrophils to settle onto the surface of the assay wells was averted by permitting the cells to presettle for 10 min at 37°C before the addition of stimulus, demonstrated that the onset of neutrophil adherence induced by both LTB<sub>4</sub> and PAF occurred within 30 s. Maximal adherence was attained within



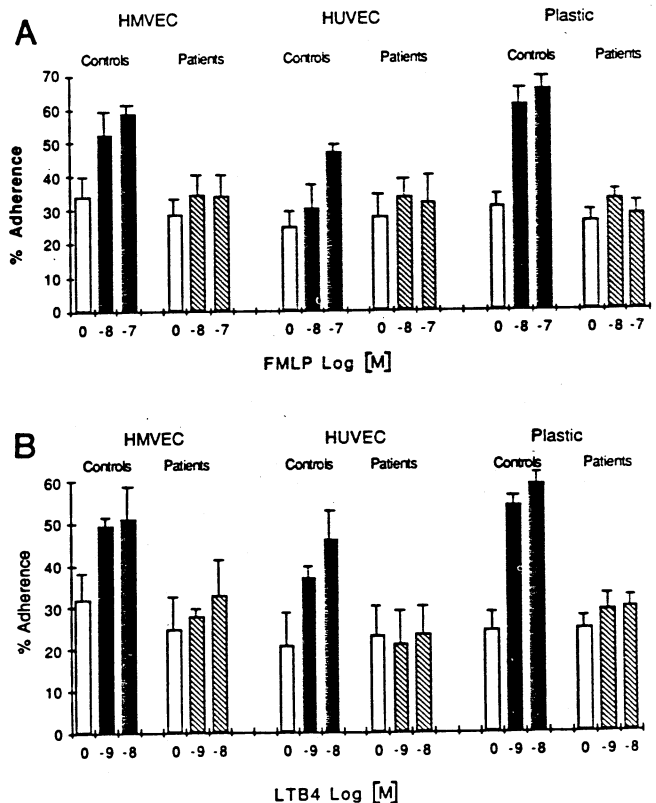
**Figure 3.** Time course of lipid mediator-stimulated neutrophil adherence. Neutrophils were allowed to settle in assay wells for 10 min at 37°C before the addition of stimulus (LTB<sub>4</sub>, 10<sup>-8</sup> M [O] or PAF, 10<sup>-7</sup> M [X]). The assay was stopped at the indicated times of incubation, from 30 s to 15 min, by the addition of glutaraldehyde. Data are expressed as the mean ± 1 SD of quadruplicate determinations.

2–3 min and persisted for 15 min. The same rapid onset of stimulated adherence occurred with the chemotactic peptides as stimuli, as previously reported (14).

#### Role of the Mac-1, LFA-1, p150,95 glycoprotein family in neutrophil adherence to endothelium

**Patient studies.** To assess whether stimulated neutrophil adherence to human endothelial cells was dependent upon the Mac-1, LFA-1, p150,95 glycoprotein family, neutrophils isolated from the blood of patients with leukocyte adhesion deficiency were incubated with chemotactic peptides and lipid mediators in the presence of cultured endothelial cell monolayers. As illustrated in Fig. 4, patient neutrophils demonstrated markedly diminished adherence to HMVEC and HUVEC as well as to protein-coated plastic when stimulated with either FMLP or LTB<sub>4</sub> compared to the response of neutrophils from normal controls. A similar diminished adherence response occurred with C5a and PAF (data not shown). Spontaneous adherence in the absence of stimulus was similar for patient and control neutrophils in this assay system.

**Analysis of cell surface antigens.** In agreement with previous reports (21, 26, 44, 45), both the chemotactic peptides, FMLP and C5a, and the lipid mediators, LTB<sub>4</sub> and PAF, en-

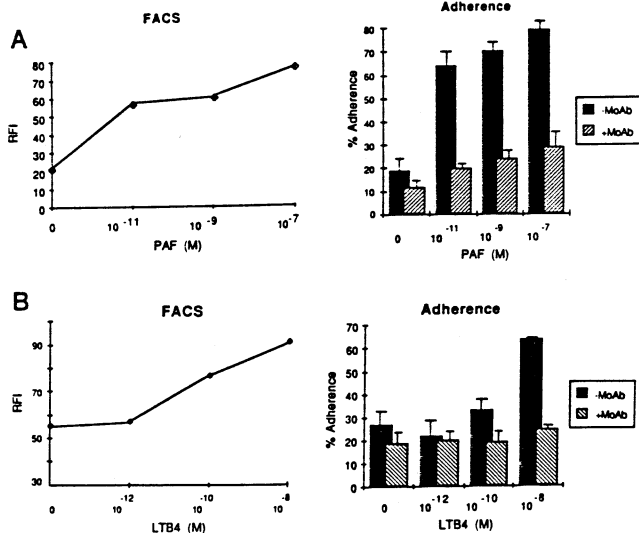


**Figure 4.** Comparison of adherence of patient and control neutrophils to endothelial cells and to plastic. Adherence of patient (□) and control (■) neutrophils to human microvascular endothelial cells (HMVEC), human umbilical vein endothelial cells (HUVEC), or protein-coated plastic was determined under baseline conditions (□) and after stimulation with FMLP (A) or LTB<sub>4</sub> (B). Four different patients were studied. Data shown represent the mean ± 1 SEM (or ± 1 SD) of values from one to six separate experiments, each performed in triplicate.

hanced expression of the Mac-1, LFA-1, p150,95 glycoprotein family on the surface of normal neutrophils as determined by increased binding of a monoclonal antibody to the common beta subunit (TS1/18), and expressed as RFI. Data for LTB<sub>4</sub> and PAF are shown in Fig. 5. Results with peptide were comparable (data not shown). The baseline RFI values (representing homogeneous cell populations) for neutrophils isolated at room temperature (according to our usual procedure), to which antibody but no stimulus had been added, showed considerable preparation-to-preparation and donor-to-donor variability, ranging from 10 to 120. For example, baseline RFI values of neutrophils isolated in parallel on the same day from five normal donors and subjected to immunofluorescence flow cytometry varied up to fivefold (data not shown). Stimulation with optimal concentrations of either chemotactic peptides or lipid mediators typically resulted in two- to fourfold increases over baseline expression. Neutrophil RFI values for anti-Leu-4, the nonbinding negative control monoclonal antibody, never exceeded 2.0 under either baseline or stimulated conditions.

The concentration range of stimulus that resulted in increased expression of the glycoprotein family on the neutrophil surface correlated with the concentration range that induced enhanced neutrophil adherence (Fig. 5).

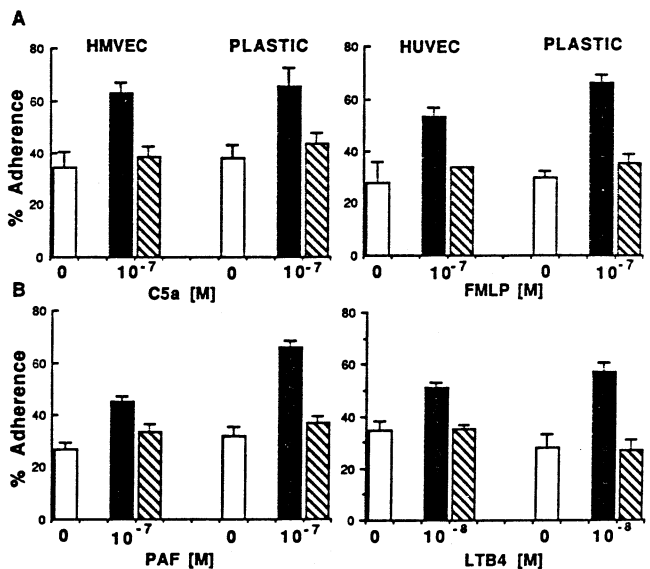
**Specific inhibition of neutrophil adherence.** To determine whether expression of the Mac-1 glycoprotein family on the neutrophil surface was necessary for stimulated neutrophil adherence to human endothelial cells, monoclonal antibody (TS1/18), specific for the common beta subunit, was added to the adherence assay, either 5 min or immediately before the addition of chemotactic stimulus. Fig. 5 illustrates marked inhibition by monoclonal antibody of lipid mediator-stimu-



**Figure 5.** Neutrophil surface expression of the glycoprotein family, and monoclonal antibody inhibition of adherence of control neutrophils stimulated by lipid mediators. Surface expression of the glycoprotein common beta subunit was determined by immunofluorescence flow cytometry (FACS) using TS1/18 (20  $\mu$ g/ml) and calculated as RFI. Neutrophil adherence to plastic stimulated by increasing doses of PAF (A) or LTB<sub>4</sub> (B) was assessed in the absence (■) or presence (□) of TS1/18 (20  $\mu$ g/ml). Adherence data represent the mean  $\pm$  1 SD of triplicate determinations.

lated neutrophil adherence to protein-coated plastic. In these experiments, both immunofluorescence flow cytometry, which documented enhanced neutrophil glycoprotein surface expression, and antibody inhibition of stimulated neutrophil adherence were performed concomitantly on cells isolated from a common normal donor. TS1/18 also inhibited stimulated neutrophil adherence to endothelial monolayers. This effect was dose dependent with 34% inhibition at 2.5  $\mu$ g/ml increasing to 79% inhibition at 20  $\mu$ g/ml TS1/18 (data not shown). The means of multiple experiments (Fig. 6) demonstrate that enhanced neutrophil adherence to either microvascular or umbilical vein endothelium or protein-coated plastic stimulated by all four mediators was inhibited up to 100% by the addition of 20–30  $\mu$ g/ml of TS1/18. Of note is the fact that, in the presence of antibody and stimulus, adherence was inhibited to baseline level (that which occurred in buffer alone) not to zero. No inhibition of adherence occurred when NCD-1, the positive binding control monoclonal antibody, was added to the adherence assay at concentrations up to 40  $\mu$ g/ml in the presence of stimulus.

**Comparison of neutrophils with HL-60 cells.** To confirm that neutrophil surface expression of adhesive glycoproteins is essential for stimulated adherence to human endothelium, we compared the responses of normal neutrophils with those of undifferentiated HL-60 cells. HL-60 cells, a human promyelocytic leukemia cell line, have been reported to express constitutively on their surface the LFA-1 alpha subunit ( $\alpha$  L) but not the Mac-1 alpha subunit ( $\alpha$  M) and little or none of the p150,95 alpha subunit ( $\alpha$  X) of the glycoprotein family (46, 47). No increase in surface expression occurs when HL-60 cells are stimulated briefly with PMA (46, 47). Consistent with

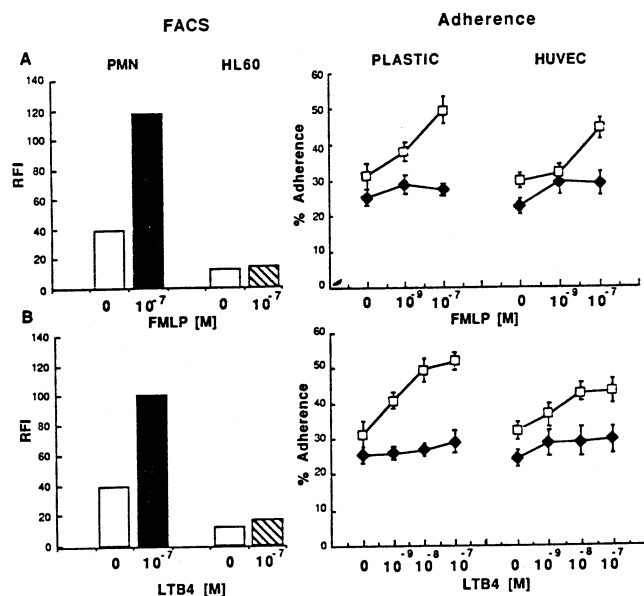


**Figure 6.** Monoclonal antibody inhibition of neutrophil adherence to endothelial cells and to plastic stimulated by chemotactic peptides and lipid mediators. Neutrophil adherence to human microvascular (HMVEC) or umbilical vein (HUVEC) endothelial cells or to protein-coated plastic, stimulated by chemotactic peptides (C5a, FMLP) (A) and lipid mediators (PAF, LTB<sub>4</sub>) (B), was determined in the absence (■) or presence (□) of monoclonal antibody TS1/18 (20–30  $\mu$ g/ml). Spontaneous adherence in buffer alone (□) is also shown. Data represent the mean  $\pm$  1 SEM (or  $\pm$  1 SD) of values from two to seven separate experiments, each performed in triplicate.

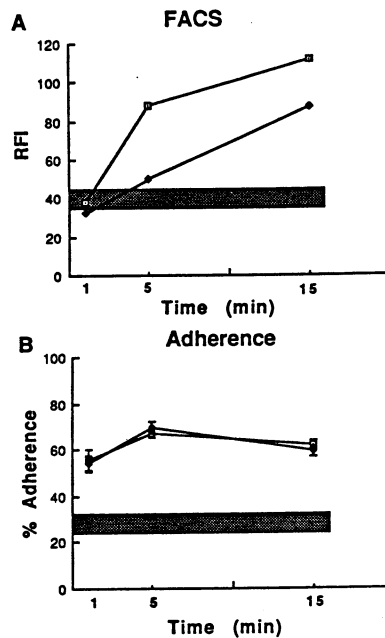
these reports, in our system surface expression of the glycoprotein family, as assessed by binding of TS1/18, was low but detectable on HL-60 cells in buffer alone and did not increase in response to incubation with either FMLP or LTB<sub>4</sub> (Fig. 7). In contrast, neutrophil surface glycoprotein expression in buffer alone was approximately twice that of the HL-60 cells and increased substantially upon stimulation, as described above. Likewise, HL-60 cells failed to increase adherence to protein-coated plastic or to HUVEC monolayers when incubated with FMLP or LTB<sub>4</sub>, in contrast to the typical enhanced adherence response of stimulated neutrophils.

*Time course of stimulated surface glycoprotein expression and stimulated adherence.* Although stimulation of neutrophils with either chemotactic peptides or lipid mediators resulted in both increased surface expression of the glycoprotein family and in enhanced adherence to substrata, it was not clear whether a causal relationship might exist between the two, e.g., did upregulation of surface expression precede adherence in time and did the degree of surface expression correlate with the degree of adherence?

To begin to address this question, the time course of stimulated surface glycoprotein expression was compared directly with the time course of stimulated adherence. Both time course experiments were performed concomitantly using neutrophils isolated at room temperature from a normal donor according to our usual procedure. Fig. 8 illustrates a representative experiment. Glycoprotein surface expression stimulated by maximal concentrations of either FMLP or LTB<sub>4</sub> increased gradually during the 15-min assay period. At 1 min, RFI values for stimulated neutrophils did not exceed values deter-



**Figure 7.** Neutrophil and HL-60 cell surface expression of the glycoprotein family, and adherence of neutrophils and HL-60 cells stimulated by chemotactic peptide or lipid mediator. Surface expression of the glycoprotein common subunit was determined by immunofluorescence flow cytometry (FACS) using TS1/18 (30  $\mu\text{g}/\text{ml}$ ). Neutrophil ( $\square$ ) and HL-60 cell ( $\blacklozenge$ ) adherence to plastic and to human umbilical vein endothelial cells (HUVEC) was determined in the presence of increasing doses of either FMLP (A) or LTB<sub>4</sub> (B). Adherence data represent the mean  $\pm$  1 SEM of values from four separate experiments, each performed in quadruplicate.



**Figure 8.** Time course of neutrophil surface expression of the glycoprotein family and adherence stimulated by chemotactic peptide or lipid mediator. Surface expression of the glycoprotein common beta subunit was determined by immunofluorescence flow cytometry (FACS) using TS1/18 (30  $\mu\text{g}/\text{ml}$ ) (A). For the adherence assay performed concomitantly, neutrophils were allowed to settle in assay wells for 10 min at 37°C before the addition of stimulus (B). Either FMLP ( $10^{-7}$  M,  $\square$ ) or LTB<sub>4</sub> ( $10^{-7}$  M,  $\blacklozenge$ ) were added for the times indicated prior to cessation of the interactions. Hatched bars indicate range of values for nonstimulated cells. Adherence data represent the mean  $\pm$  1 SD of triplicate determinations.

mined for neutrophils in buffer alone. In contrast, onset of enhanced neutrophil adherence occurred within 1 min. Maximal response was attained within 5 min and the effect persisted for 15 min.

## Discussion

In this study, we have demonstrated that chemotactic peptides, FMLP and C5a, and lipid mediators, LTB<sub>4</sub> and PAF, increased adherence of neutrophils in vitro to human microvascular endothelial cells. These mediators, which are believed to participate in the inflammatory response in vivo, have been previously shown to increase neutrophil adherence to a variety of substrata including large vessel endothelial cells in vitro (9–15) and to enhance interaction of leukocytes with the microvasculature in vivo (48–51). Our observations document that an adhesive interaction between neutrophils and human microvascular endothelial cells may indeed be directly induced by physiologically relevant chemotactic stimuli. This is significant in view of the commonly held belief that endothelial cells may vary in phenotype and function depending on their species of origin, on their in situ location, and on the size and nature of the vessel which they line (31, 52–54). Although the microvascular endothelial cells used in our studies were isolated from human omental fat, a site which does not often participate clinically in inflammatory events, we have determined that neutrophils do adhere to and migrate through the microvasculature of the omentum in vivo when a C5a preparation is introduced into the abdominal cavity of a rabbit (our unpublished observations). The rapid onset (maximal response within 2 min) of adherence stimulated by the lipid mediators was comparable to that previously reported by us for chemotactic peptides (14) and presumably is necessary to achieve appropriate neutrophil localization near a site of tissue

injury *in vivo*. A similarly rapid time course for stimulated neutrophil adherence to the microvasculature of the canine lung *in vivo* in response to infusion of a C5 fragment preparation has been observed by fluorescence videomicroscopy (55).

The essential role of the Mac-1, LFA-1, p150,95 glycoprotein family in the enhancement of stimulated neutrophil adherence to endothelium has been confirmed and extended by this report. It has now been well documented that neutrophils from patients with leukocyte adhesion deficiency demonstrate severe impairment of adherence-dependent cell functions *in vitro* (18, 23). Since this disorder is characterized by recurrent soft tissue bacterial infections with impaired pus formation, it has been postulated that the failure of patient neutrophils to be recruited to sites of tissue injury might be the result of a basic defect in the ability of the neutrophils, once stimulated, to adhere to the microvascular endothelium (24, 26). In support of this concept, neither PMA nor calcium ionophore (A23187) increased adherence of neutrophils from two patients to cultured large vessel endothelial cell monolayers (24). In this study, neutrophils isolated from four unrelated patients demonstrated markedly diminished adherence to cultured human microvascular as well as umbilical vein endothelial cells in response to stimulation with FMLP, C5a, LTB<sub>4</sub>, and PAF compared with normal control neutrophils.

The ability of monoclonal antibody TS1/18, specific for the common beta subunit, to inhibit enhanced adherence of normal neutrophils to cultured human microvascular endothelial cells stimulated by FMLP, C5a, LTB<sub>4</sub>, or PAF further defines the essential role of the Mac-1, LFA-1, p150,95 glycoprotein family. The effect was antibody dose-dependent and at optimal concentrations resulted in up to 100% inhibition of stimulated adherence. Our studies using TS1/18 are in agreement with those of previous reports (24, 25) in which a different monoclonal antibody to the beta subunit, 60.3, completely inhibited normal neutrophil adherence to large vessel endothelial cells stimulated by PMA, A23187, FMLP, LTB<sub>4</sub>, PAF, or TNF. Inhibition by antibodies to the common beta subunit, however, does not reveal which of the three glycoproteins are involved in adherence nor which antigenic site is the most critical. Comparative studies with a panel of monoclonal antibodies that recognize different epitopes in the glycoprotein family will be necessary to help elucidate the responsible glycoprotein(s) and the molecular basis for adherence.

Of interest is the fact that patient neutrophils, in the absence of added stimulus, adhere to endothelial cells to a degree comparable to the spontaneous adhesion of normal control neutrophils (24, 56, and this report). Buchanan and colleagues (56) also found that the rise in neutrophil count in response to the *in vivo* administration of epinephrine (considered to be an indicator of the presence of a marginated neutrophil pool) was normal in one patient. In addition, spontaneous (nonstimulated) adherence of normal neutrophils was not inhibited by TS1/18 (this report) or by 60.3 (24). Taken together, these observations suggest that margination, believed to be a reversible, spontaneous interaction of nonstimulated neutrophils with the vascular endothelium, occurs independently of the glycoprotein family, but that surface expression of the adhesive glycoproteins is essential for neutrophils, once stimulated with inflammatory mediators, to increase adherence to the microvascular endothelium before diapedesis.

Inflammatory mediators (specifically FMLP, C5a, and LTB<sub>4</sub>) as well as PMA and A23187 have clearly been shown to

induce 2- to 14-fold increases in expression of the glycoprotein family on the surface of normal human granulocytes (21, 26, 44, 45, 57–60). Current evidence indicates that this upregulation results from mobilization of preformed glycoproteins from an intracellular vesicular compartment(s), perhaps the specific granule and/or the gelatinase-containing organelles, to the plasma membrane (45, 58–59, 61). Absolute quantitation of stimulus-induced increases in cell surface expression has been difficult due to the fact that cell preparative procedures can lead to temperature-dependent increases in surface expression (44, 45, 59). It was to be expected, therefore, that our neutrophil isolation technique and the conditions of our adherence assay, involving a 15-min incubation at 37°C, would result in measurable expression of the glycoprotein family on the neutrophil surface in the absence of added stimuli. As assessed by binding of TS1/18, such expression involving the entire population of cells indeed occurred, but to a variable extent, depending in part upon the individual donor and the day of isolation. Of significance, however, was the documentation that further upregulation occurred upon neutrophil stimulation with chemotactic factors, and was dose dependent, with two- to fourfold increases over baseline at optimal concentrations of stimulus. In addition, the concentration range of stimulus which resulted in enhanced surface expression correlated with the concentration range that induced increased neutrophil adherence. Berger et al. (59) previously documented a temperature-dependent increase in the availability of the C3bi complement receptor (CR3, Mac-1) on the surface of an entire population of isolated nonstimulated human neutrophils, the extent of which varied among individuals and among preparations, but could be enhanced threefold by the addition of FMLP.

Nevertheless, evidence is slowly accumulating to question whether stimulus-induced upregulation and increased surface expression are required for enhanced adherence. Although temperature-dependent upregulation of surface glycoproteins occurs during routine isolation of neutrophils, unstimulated neutrophils do not aggregate (65) or avidly adhere to endothelial cells (this report), and the spontaneous endothelial adhesion which occurs cannot be inhibited by TS1/18 (see above). Preliminary reports also suggest that stimulus-induced upregulation of surface glycoprotein expression and stimulus-induced adherence-dependent cell functions can be dissociated. We report here lack of correlation in the time courses of upregulation and adherence. Maximal stimulated neutrophil adherence occurred within 1 to 2 min, while cell surface glycoprotein expression increased gradually over 15 min, and at 1–2 min did not exceed the level detectable on unstimulated neutrophils. A comparable dissimilarity has been noted in the time courses of stimulated neutrophil aggregation and glycoprotein upregulation (65). In addition, the anion channel-blocking agent DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), which is known to inhibit neutrophil degranulation, reduced the increased glycoprotein surface expression occurring after neutrophil stimulation and inhibited stimulated neutrophil aggregation but not stimulated neutrophil adherence to endothelium (60). These data, taken together, support the concept that upregulation may not be the only regulatory event, but that once adhesive glycoprotein molecules are expressed on the cell surface, a subsequent modification(s), perhaps a conformational or biochemical change, an interaction with another membrane component or possibly an alter-

ation of density distribution of the glycoproteins within the membrane (66) may be required to produce the actual adhesive site. Investigations directed at probing the functional domains of the membrane adhesive glycoproteins have begun (42, 62, 67) and may prove to be instructive in this regard.

Recently it has been reported that a human promyelocytic leukemia cell line, HL-60, in the undifferentiated state has detectable levels of the LFA-1 alpha subunit but not the Mac-1 alpha subunit and little or none of the p150,95 alpha subunit of the glycoprotein family expressed on the cell membrane. Brief stimulation with PMA failed to induce more surface glycoprotein expression (46, 47). In our studies, neither chemotactic peptide nor lipid mediator stimulation of HL-60 cells increased surface glycoprotein expression or enhanced adherence to endothelial cells. Thus as with patient neutrophils, stimulated adherence failed to occur in the absence of sufficient cell surface glycoprotein expression.

Considerable evidence now documents the involvement of the Mac-1, LFA-1, p150,95 family of adhesive glycoproteins in the adherence of neutrophils to endothelium in the presence of a variety of mediators of inflammation, possibly mediated by several different mechanisms. Chemotactic factors stimulate neutrophils to increase adherence to a similar degree to a wide variety of biologic substrata, both living and inert, including human umbilical vein endothelial cells, human dermal fibroblasts, human umbilical vein smooth muscle cells, protein-coated plastic (14) and HMVEC (this report), which appears to occur by a common mechanism requiring expression of the glycoprotein family on the neutrophil surface. The relative nonspecificity with regard to biologic substrata supports the concept that chemotactic factors act primarily on the neutrophil to effect enhanced adherence, as previously proposed (14). In contrast, IL-1, TNF, thrombin and LTC<sub>4</sub> induce cultured human umbilical vein endothelial cells to increase their adhesivity for neutrophils by mechanisms dependent only in part on the glycoprotein family since monoclonal antibodies to the beta subunit also inhibit such adherence, but not to the same degree as inhibition of chemotactic factor-stimulated neutrophil adherence to nonactivated endothelial cells (25, 63, 64, our unpublished observations).

Thus, in this report, we demonstrate that both chemotactic peptides and lipid mediators enhance neutrophil adherence to HMVEC, as well as to HUVEC, in a dose-dependent manner with a rapid time course. Stimulated neutrophil adherence to human endothelial cells is dependent on cell surface expression of the Mac-1, LFA-1, p150,95 glycoprotein family based on the inability of neutrophils from patients with leukocyte adhesion deficiency, as well as HL-60 cells, to be stimulated to adhere, and the ability of a monoclonal antibody specific for the common beta subunit to inhibit stimulated adherence of normal neutrophils. However, since the mechanism(s) involved are as yet unknown, much remains to be explored to further our understanding of the fascinating but complex interaction of circulating neutrophils and the microvascular endothelium during the induction of the acute inflammatory response.

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