Recognition of an Endothelial Determinant for CD18-dependent Human Neutrophil Adherence and Transendothelial Migration

C. W. Smith,* R. Rothlein,‡ B. J. Hughes,* M. M. Mariscalco,* H. E. Rudloff,§ F. C. Schmalstieg,§ and D. C. Anderson*||
*Speros P. Martel Laboratory of Leukocyte Biology, Departments of Pediatrics, and ||Microbiology and Immunology, and Cell Biology, Baylor College of Medicine, Houston, Texas 77054; ‡Department of Immunology/Cell Biology, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut 06877; §Department of Pediatrics, University of Texas Medical Branch, Galveston, Texas 77550

Abstract

Human neutrophil (PMN) attachment to human umbilical vein endothelial cells (HUVEC) was evaluated in vitro using two MAbs, R6-5-D6 and RR1/1, that recognize intercellular adhesion molecule-1 (ICAM-1), and one MAb, TS1/18, that recognizes CD18. Pretreatment of the HUVEC with anti–ICAM-1 MAbs produced > 50% inhibition of attachment to HUVEC, and IL-1 (0.5 U/ml)-or lipopolysaccharide (LPS) (10 ng/ml)-stimulated HUVEC, and > 99% inhibition of f-Met-Leu-Phe (0.5 nM) enhanced adherence. Anti–ICAM-1 MAbs also inhibited by > 85% the transendothelial migration induced by 4-h IL-1 (0.5 U/ml) and LPS (10 ng/ml) activation of the HUVEC. That these effects involved a CD18-dependent mechanism is supported by the following results: pretreatment of PMN with TS1/18 produced the same degree of inhibition of attachment and migration as seen with R6-5-D6. In addition, the use of both MAbs together did not further increase the inhibition of cell attachment to stimulated HUVEC. The attachment of PMN from patients with CD18 deficiency to stimulated HUVEC was not reduced by R6-5-D6, and both R6-5-D6 and TS1/18 revealed the same time course for appearance and disappearance of an adherence component on stimulated HUVEC not blocked by either MAb. These results demonstrate that attachment and transendothelial migration of PMN in vitro depend substantially on both CD18 on the PMN and ICAM-1 on the endothelial cell.

Introduction

Interaction with endothelial cells is fundamental to the localized infiltration of neutrophils and monocytes into extravascular inflammatory sites (1, 2) or the homing of lymphocytes to lymphoid organs (2–4). Recent experimental and clinical observations have begun to define the molecular determinants on the surface of leukocytes that contribute to the adhesive component of this interaction (5–15). The most convincing clinical evidence has accrued from studies of the heritable disorder (14, 15) termed leukocyte adherence deficiency disease (LAD)[1] (14). Characteristic features of patients with LAD including necrotic soft tissue lesions, impaired pus formation and wound healing, as well as abnormalities of adhesion-dependent leukocyte functions in vitro, are attributed to a severe or total deficiency of the glycoprotein, CD18 (14, 15).

The CD18 complex consists of three heterodimers, Mac-1 (iC3b receptor), lymphocyte function-associated antigen-1 (LFA-1) and p150,95, each of which consists of noncovalently associated α and β subunits with $\alpha_1\beta_1$ stoichiometry. They share an identical β subunit (M_r = 95,000 kD) and are distinguished immunologically by distinct α subunits whose relative molecular masses are 165,000, 177,000, and 150,000 kD for Mac-1 (αM, CD11b), LFA-1 (αL, CD11a), and p150,95 (αX, CD11c), respectively. That these heterodimers are involved in the adherence of leukocytes to endothelial cells is clearly demonstrated by the fact that leukocytes from patients with LAD exhibit profoundly reduced adherence to endothelial monolayers in vitro (16, 17) as well as by the absence of leukocytes in infected tissues of these patients (14, 15). Additional evidence for the involvement of these heterodimers comes from the use of subunit monoclonal antibodies. Endothelial adherence of lymphocytes and monocytes in vitro is inhibited by pretreatment of these leukocytes with MAbs reactive with LFA-1 or the β subunit. The enhanced adherence of neutrophils to endothelium in vitro caused by exposure of the neutrophils to chemotactic or degranulating stimuli is inhibited by pretreatment of the neutrophils with MAbs reactive with either Mac-1 or the β subunit (10, 16–20).

The endothelial surface factors with which the CD18 complex on neutrophils and monocytes is interacting appear to be induced by exposure of the endothelial cells to the inflammatory mediators, endotoxin (lipopolysaccharide, LPS), interleukin 1 (IL-1), and tumor necrosis factor-α (TNFα) (20, 21). An important recent finding regarding cytokine-induced adherence factors involves the identification of a ligand for LFA-1 on a variety of human cell types. A MAb (RR1/1) prepared against CD18-deficient human lymphoblasts has been identified which inhibits aggregation of normal lymphocytes (22). This MAb recognizes a normal cell surface molecule distinct from LFA-1 that is designated intercellular adhesion molecule 1 (ICAM-1). Recently reported studies (23) have shown that ICAM-1 participates in the adhesive interactions of multiple leukocyte cell types, including transformed B cells, myeloid cell lines, and T lymphocyte blasts. It was also found on nonlymphoid cells, including thymic epithelium, dendritic cells in germinal centers, and cultured fibroblasts and endothelial cells. Further characterizations of ICAM-1 have demonstrated

Address reprint requests to Dr. C. Wayne Smith, Texas Children's Hospital Clinical Care Center, 8080 N. Stadium Dr., Suite 2100, Houston, TX 77054.

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1. *Abbreviations used in this paper:* EIA, enzyme immunoassay; ICAM-1, intercellar adhesion molecule 1; IF, immunofluorescence; HUVEC, human umbilical vein endothelial cells; LAD, leukocyte adherence deficiency disease; LAF-1, leukocyte function-associated antigen-1; TNFα, tumor necrosis factor-α.

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its enhanced expression on vascular endothelium or dendritic cells in germinal centers of inflamed lymphoid tissues. ICAM-1 expression on dermal fibroblasts or endothelial cells is greatly enhanced by either interleukin 1 (IL-1) or interferon-γ (23, 24). Induction is dependent upon protein and mRNA synthesis and is reversible. Importantly, intercellular adhesion of lymphocytes to fibroblasts is enhanced by prior IL-1 exposure and is blocked by the monoclonal antibody reactive with ICAM-1 (RR1/1). Moreover, normal lymphocytes demonstrate an enhanced adherence to vascular endothelium preexposed to IL-1, TNF or interferon-γ, and this increased adhesive property is directly correlated with ICAM-1 expression. Thus, ICAM-1 or ICAM-1–like molecules (16) exist on vascular endothelium and other mesenchymal cell types which appear to represent selective ligands for LFA-1 (25, 26) and possibly other glycoproteins in the CD18 complex, and are under the influence of a variety of inflammatory mediators.

In this report, by utilizing a newly developed monoclonal antibody reactive with ICAM-1, R6-5-D6, we present the first direct evidence of a human endothelial cell determinant of major importance in the CD18-dependent human neutrophil adherence to endothelial cells. Our findings indicate that this determinant is necessary for the migration of human neutrophils through endothelial monolayers. Additionally, we describe some characteristics of a CD18- and ICAM-1-independent mechanism for attachment of neutrophils to endothelial cell monolayers that also appears to be involved in transendothelial migration.

Methods

Isolation of PMN. PMN obtained from healthy adult individuals and two patients with a severe form of CD18 deficiency (27) were purified from citrate-anticoagulated, dextran-sedimented venous blood samples over Ficoll-Hypaque gradients and were suspended in Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY), pH 7.4, containing 0.2% dextrose as described (28). PMN were maintained at 4°C in PBS for up to 4 h at a concentration of 10^7/ml.

MAbs. MAbs used in these studies included dilutions of ascites fluid, preparations of IgG, and Fab fragments. The anti-β MAb was TS1/18 (IgG1) a generous gift of Dr. T. Springer, Dana-Farber Cancer Institute, Boston (29). R6-5-D6 (IgG2a) was developed by fusing spleen cells from mice immunized with a battery of ICAM-1–bearing cell lines. Its profile of reactivity in lymphoblast aggregation assays and the molecular weight of its immunoprecipitated antigen on JY cells are identical to RR1/1 anti-ICAM-1 (22, 23). The anti-HLA framework MAb was W6/32 (IgG2a) (30, 31).

Preparation of human umbilical vein endothelial cells (HUVEC). HUVEC were harvested (32, 33) and characterized as to acLDL binding (34) and factor VIII expression (32) according to established techniques. Cells from 5–10 umbilical cords were pooled and plated in RPMI 1640 containing 10% fetal calf serum (FCS), antibiotics, heparin (0.1 mg/ml), and endothelial cell growth factor (0.05 mg/ml), and maintained for 3–4 d at 37°C, 5% CO_2-humidified atmosphere. Visually confluent monolayers on gelatin (0.1%)- and fibronectin (5 $\mu g/cm^2$)-coated 25-mm round glass coverslips were prepared from first-passage cells harvested with 0.05% trypsin and 0.02% EDTA in PBS. Monolayers in fibronectin (5 $\mu g/cm^2$)-coated 96-well microtiter plates were prepared from first- and second-passage cells and grown to confluence (1–3 d). HUVEC were pretreated with various concentrations of LPS (Sigma Chemical Co., St. Louis, MO. *Escherichia coli* 026:B6), or IL-1 (Genzyme Corp., Boston, MA; cell derived) for varying lengths of time.

Adherence assay. A visual adherence assay was utilized as previously described (35) with the exception that adherence to HUVEC monolayers was assessed instead of adherence to protein-coated glass. HUVEC monolayers on 25-mm round glass coverslips were washed by dipping three times in two changes of PBS and immediately inserted into the adherence chambers specifically made for use with an inverted microscope and phase-contrast optics. The chambers consisted of two metal plates designed to hold two 25-mm round cover glasses separated by a Sykes-Moore chamber O-ring (Bellco Glass, Inc., Vineland, NJ). Within this closed compartment PMN could be observed as they contacted the HUVEC monolayer. PMN suspended in PBS (10^6 cells/ml) or pretreated with low concentrations of fMLP (35–40) were injected into the chamber and allowed to settle onto the monolayer for a period of 500 s. The number of PMN in contact with the monolayer was determined by counting at least 10 microscopic fields (× 50 objective), and the chamber inverted for an additional 500 s. The percentage of cells remaining in contact with the monolayer was determined and is expressed in Results as percent adherence. In blocking experiments, HUVEC were pretreated with MAbs for 15 min then washed three times by dipping in two changes of PBS prior to evaluating adherence. PMN were pretreated for 5–15 min, and then injected into the adherence chamber in most instances without washing. It should be noted that the procedure used in this study does not utilize shear stress (41–43) as with techniques requiring a washing step (10, 16–21). Rather it simply assesses attachment.

The percentage of cells migrating through the monolayer was also determined using criteria previously developed by Beesley et al. (44). Using phase-contrast otics, PMN adherent to endothelium are round and appear refractile with a surrounding halo since they do not spread on the surface of endothelial cells (45). In contrast, the leukocytes become quite flattened after migrating to a position between the monolayer and the substratum (46), and they lose the refractile appearance (44). Intracellular granules are readily seen. The focal plane for these leukocytes is beneath the nuclei of the endothelial cells and that for the PMN adherent to the upper surface of the monolayer is unequivocally above. Other direct demonstrations of transendothelial migration were provided by sections of HUVEC monolayers embedded in epoxy resin. HUVEC monolayers grown on human amnion for 8 d (47, 48) were stimulated with 0.5 U/ml IL-1 for 4 h at 37°C, then washed by dipping in PBS. PMN suspended in PBS at 10^6/ml were allowed to contact the monolayers for 1,000 s before the membrane was submerged in 1% glutaraldehyde in PBS for 1 h, rinsed in PBS, dehydrated, and embedded. Sections were stained with 1% methylene blue, 1% Azure II in 1% sodium borate.

Assessment of MAb binding to cells. Immunofluorescence flow cytometry was performed as previously described (49) using MAbs and fluorescein isothiocyanate (FITC)-conjugated antibody to mouse IgG. Surface-stained cells were fixed in 1% paraformaldehyde and analyzed in a flow cytometer (FACScan, Becton, Dickson & Co., Sunnyvale, CA). Background fluorescence was determined after incubation with either nonimmune ascites or X63 IgG1 control antibody.

Fluorescence microscopy was performed on paraformaldehyde (1%, 15 min, room temperature) fixed cells, washed in PBS, and incubated 30 min in PBS containing 2% human serum albumin (HSA) and 1% glycine. The binding of MAbs was detected by use of a second antibody to mouse IgG conjugated with FITC or rhodamine isothiocyanate. Cells were examined using a Leitz Diaplan fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ). Background fluorescence was determined after incubation of cells with either nonimmune ascites or X63 IgG1 control antibody.

An enzyme immunoassay (EIA) was used to assess binding of MAbs to cell monolayers. Confluent HUVEC grown in fibronectin-coated (5 $\mu g/mm^2$) 96-well plates were fixed by addition of 1% paraformaldehyde in PBS for 15 min at room temperature. The wells were washed three times with PBS and incubated in 2% bovine serum albumin (BSA) for 30 min. After removal of BSA, MAb was added and incubated at 37°C for 1 h, washed three times in PBS, and then incubated for 1 h in goat anti-mouse IgG, IgM, IgA (Zymed, South San Francisco, CA) conjugated with alkaline phosphatase (1:500 dilution). After washing, substrate (*p*-nitrophenyl disodium phosphate, 1 mg/ml, in buffer, pH 9.8) was added and incubated for 30 min at room temperature. Plates were read at 405 nm in a Titertek (Flow General, Inc., McLean, VA) reader.

Immunoprecipitation. Immunoprecipitation experiments designed to examine the specificity of the monoclonal antibody, R6-5-D6, were performed. JY cells (22, 23) (2×10^7) were grown in RPMI 1640 and 20% (vol/vol) fetal calf serum at 37°C in a 7.5% CO_2 atmosphere. These cells were treated with 25 ng/ml of PMA for 8–12 h before radiolabeling to increase the expression of the ICAM-1 molecule. After washing twice in PBS, 4×10^6 cells were resuspended in 1 ml of PBS containing 0.625 U of lactoperoxidase and 0.125 U of glucose oxidase as previously described (50). 2 mCi of Na[^{125}I] were added and the cells were incubated at 37°C for 15 min, washed three times in PBS, and solubilized in lysis buffer (0.1 M Tris, 0.9% NaCl, 0.5% NP-40, 10 μg/ml aprotinin, 1.0 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mM EDTA, 50 mM Na vanadate, and 2 mM PMSF. Three aliquots of the lysate (200 μl, 5×10^6 cells) were treated with 20 μl of W6/32 ascites fluid for 1 h to remove nonspecific binding proteins. The antibody was removed with insoluble protein A (2 mg). Aliquot I (100 μl) was then treated with 5 μl of R6-5-D6 ascites fluid for 1 h. The immune complexes were removed with 1 mg of insoluble protein A. The bound immune complexes were washed three times with lysis buffer containing 0.1% SDS. The immunoprecipitation process was repeated three additional times with R6-5-D6 and finally once with RR1/1 ascites fluid. Aliquot II was treated in an identical manner except 5 μl of RR1/1 ascites fluid was employed. Similarly, the last immunoprecipitate was accomplished with R6-5-D6. Aliquot III was treated similarly except no antibody was added until the last cycle, when 5 μl of R6-5-D6 was used. SDS-PAGE and autoradiography (7.5% acrylamide) was performed as described for HUVEC.

HUVEC were grown to confluency in tissue culture flasks and incubated with 20 ng/ml LPS for 2 h. The monolayers were washed twice with RPMI 1640 deficient in both methionine and cysteine supplemented with 20% FCS. After a 1-h incubation in this deficient medium, 1 mCi of [^{35}S]cysteine and 0.5 mM unlabeled methionine was added for 10 min at 37°C. The monolayers were then rinsed and incubated in RPMI 1640 containing methionine and cysteine supplemented with 20% FCS for 1 h at 37°C. After washing the cells in RPMI 1640 three times, lysis buffer (borate buffered saline, 0.5% Triton X-100, 0.05% Tween, 1% BSA, and 2 mM PMSF, pH 8.0) was then added to solubilize the cells. Nuclei and cell debris were pelleted at 12,000 *g* for 10 min. The supernatant was then precleared by the addition of protein A bound to Sepharose CL-4B (Sigma Chemical Co.). After removal of the protein A-Sepharose beads, 5 μl of R6-5-D6 ascites fluid was added and the mixture allowed to incubate at 4°C for 30 min. Protein A bound to Sepharose CL-4B was then added to the mixture and tipped for 1 h at room temperature. The Sepharose beads were washed three times with 0.1% NP-40 and placed in SDS sample buffer containing 2.5% SDS and 5% (wt/vol) mercaptoethanol, and boiled for 2 min. SDS-PAGE (7.5% acrylamide) and autoradiography were performed as previously described (50). The supernatants were tested for residual antigen by incubating with RR1/1.

Data presentation. Results are presented as means±1 SD, and *n* = the number of separate experiments. Statistical assessments were made using analysis of variance and Dunnett's *t* test, or Student's *t* test.

Results

Time- and dose-dependent augmentation of adherence by inflammatory mediators. The baseline adherence of PMN to HUVEC varied with each preparation of HUVEC from a low of 3.5±1.0% to a high of 35.5±2.6% of the PMN adhering to the monolayer. The mean±1 SD for 24 preparations of HUVEC was 16.9±7.5%. Adherence was significantly increased following stimulation of the PMN by fMLP (Fig. 1). Statistically significant increases were obtained at 0.1 nM fMLP (Fig. 1) and maximal adherence (62.5±5.3%; $n = 8$; 10 nM fMLP) was approached at a concentration of 0.5 nM (53.8±9.8%; $n = 23$). IL-1 stimulation of the HUVEC significantly increased adherence of PMN at 0.05 U/ml with near maximal adherence at a concentration of 0.5 U/ml (Figure 1; 81.2±7.4%; $n = 21$). Near maximal adherence was attained at 10 ng/ml LPS (80.1±7.6%; $n = 25$). Each of the latter two stimuli promoted 100% adherence after a 4-h incubation with the HUVEC provided the concentrations exceeded 2 U/ml IL-1 or 30 ng/ml LPS. The kinetics of the effects of IL-1 and LPS on HUVEC mediated adherence were very similar (Fig. 2), each peaking at 3.5–4 h and diminishing by ~ 50% at 7 h.

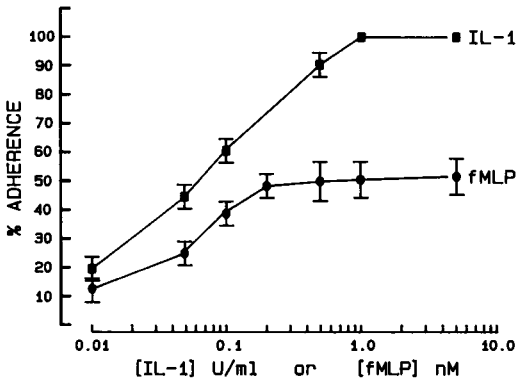

Figure 1. Effect of IL-1 and fMLP on adherence of human PMN to HUVEC. Effect of IL-1 was assessed in the following manner. HUVEC were incubated for 4 h, 37°C, in the presence of the indicated concentrations of IL-1 and then washed by dipping three times in two exchanges of PBS. The adherence of unstimulated PMN suspended in PBS was then determined using a visual assay performed at room temperature. The effect of fMLP was determined by suspending PMN in the indicated concentrations of fMLP and incubating for 5 min at room temperature before determining adherence to unstimulated HUVEC using a visual assay performed at room temperature. Error bars, ±SD; n = 4; $P < 0.01$ for all points beyond 0.05 nM fMLP and 0.01 U/ml IL-1.

Binding of MAbs to PMN and HUVEC: effects of fMLP, IL-1, and LPS stimulation. TS1/18 and W6/32 bound to PMN, and flow cytometry using a FITC-labeled anti-mouse antibody revealed that the MAb reactive with the β subunit (TS1/18) exhibited three- to fivefold enhanced binding after stimulation of the PMN with 10 nM fMLP. Fluorescence was maximum at 1:800 dilution of the ascites and at 5 μg/ml IgG. Binding of MAb reactive with the class I MHC antigen

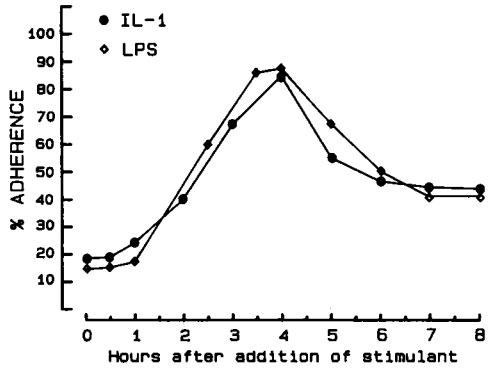

Figure 2. Comparison of the time courses for IL-1 and LPS stimulation of HUVEC adhesiveness. HUVEC monolayers were incubated at 37°C, in the presence of either LPS (10 ng/ml) or IL-1 (0.5 U/ml), then washed by dipping three times in two changes of PBS. Adherence of unstimulated PMN suspended in PBS was assessed at room temperature using the visual assay described in Methods. Each point is the mean of three determinations. 1 SD at each point was not greater than 12% of the mean.

(W6/32) did not increase after chemotactic stimulation. These observations are consistent with published results (31). R6-5-D6, RR1/1, and W6/32 bound to HUVEC. The EIA procedure using an alkaline phosphatase-labeled anti-mouse antibody revealed that exposure of HUVEC to 20 ng/ml LPS resulted in a four- to fivefold increase at 4 h and five- to sevenfold increase at 7 h in binding of R6-5-D6, and RR1/1. W6/32 binding was not increased at 4 h, and at 24 h the increase was < 20%.

Immunofluorescence (IF) microscopy revealed a uniform distribution of R6-5-D6 and RR1/1 over the surface of each endothelial cell after paraformaldehyde fixation. In the unstimulated monolayer, all cells exhibited fluorescence with about 20% obviously staining brighter than the average cell. After a 4-h stimulation with IL-1 (0.5 U/ml) or LPS (10 ng/ml) the overall intensity of the fluorescence was increased on all cells and ~ 50% were distinctly bright. Under maximum resolution (× 100 objective) the fluorescence was seen to be punctate with a uniform spacing of < 0.5 μm. IF microscopy of monolayers with attached PMN revealed no binding of R6-5-D6 or RR1/1 to PMN and an unstained or lightly stained area on the surface of the endothelial cell occupied by the neutrophil. The lack of binding to PMN was confirmed by flow cytometry in that the fluorescence intensity was not above the nonimmune ascites control. The distribution of TS1/18 was confined to the attached PMN with no staining of the HUVEC.

Immunoprecipitation of an endothelial cell glycoprotein by R6-5-D6. The reaction of R6-5-D6 with ICAM-1 was confirmed in co-immunoprecipitation experiments with RR1/1. Prior immunoprecipitation with either antibody greatly depleted the JY cell lysates of antigen reactive with the other antibody. As has been previously observed, the molecular weight of ICAM-1 obtained from JY cells is lower than that from endothelial cells and fibroblasts (23). SDS-PAGE of NP-40 lysates of HUVEC treated with LPS, pulse-labeled with [^{35}S]cysteine, and immunoprecipitated with MAb R6-5-D6 revealed a single band at M_r = 95,000 kD (Fig. 3). As with the JY cell lysates, immunoprecipitation with R6-5-D6 cleared the lysate of antigen reactive with RR1/1 (Fig. 3).

Effects of MAbs on adherence: pretreatment of PMN. Incubation of the PMN with TS1/18 revealed maximal inhibition of adherence at a 1:800 dilution of ascites fluid and at 5 μg/ml of the IgG preparation. As shown in Fig. 4, this MAb significantly reduced baseline adherence and completely blocked the enhancement caused by fMLP stimulation of the PMN. Experiments employing LPS and IL-1 stimulated HUVEC (Fig. 4) revealed that treatment of the PMN with TS1/18 yielded quantitatively different results. TS1/18 inhibited the LPS and IL-1 enhanced adherence by ~ 50%. As with unstimulated HUVEC, TS1/18 completely blocked fMLP augmented PMN adherence to LPS stimulated HUVEC (Fig. 4). Increasing the concentration of TS1/18 either as ascites fluid or as isolated IgG did not reduce further the LPS stimulated adherence. W6/32 was ineffective in altering adherence of unstimulated or stimulated PMN to either control or LPS-stimulated HUVEC monolayers.

Effects of MAbs on adherence: pretreatment of HUVEC. Preincubation of the HUVEC with the various MAbs gave qualitatively different results. As shown in Fig. 5, R6-5-D6 reduced baseline adherence and the enhancement caused by IL-1 or LPS stimulation. RR1/1 and R6-5-D6 were equally effective in this inhibition and when combined, no more effective than either alone. This effect of R6-5-D6 was maximal at a 1:800 dilution of the ascites fluid and at 4 μg/ml IgG fraction of ascites (percent inhibition at 12 μg/ml, 59.2, n = 3; at 4 μg/ml, 60.1, n = 6; and at 2 μg/ml, 38.3, n = 3). Fab fragments were maximally active at 48 μg/ml (percent inhibition at 48 μg/ml, 56.6, n = 3; at 24 μg/ml, 34.0, n = 3). When fMLP activated PMN were used (Fig. 6), R6-5-D6 effectively blocked the adherence increment caused by chemotactic stimulation. Furthermore, it blocked fMLP augmentation of PMN adherence to IL-1- and LPS-stimulated HUVEC. W6/32 was

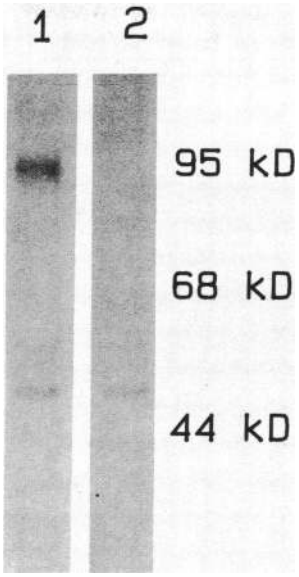

Figure 3. Immunoprecipitation of the protein recognized by R6-5-D6 from HUVEC. HUVEC were labeled with [^{35}S]cysteine and immunoprecipitated with R6-5-D6 (lane *1*) and sequentially with RR1/1 (lane *2*). The radioautograph demonstrates a prominent band at M_r = 95,000 kD after immunoprecipitation with R6-5-D6. As with the JY cell lysate, R6-5-D6 removed the antigen recognized by RR1/1.

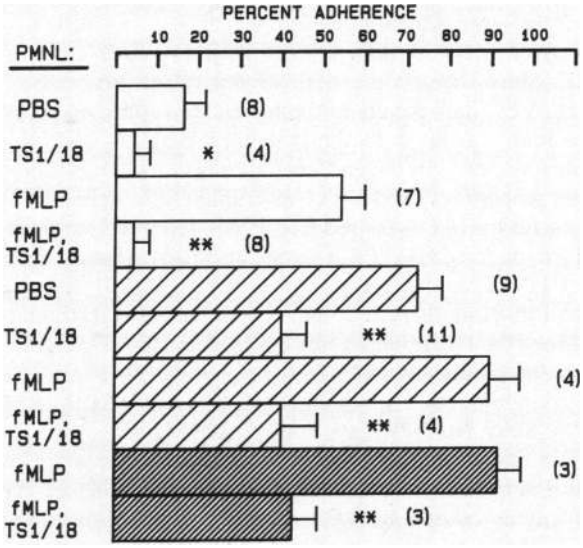

Figure 4. Effects of TS1/18 on the adherence of human PMN to HUVEC monolayers. PMN were preincubated for 5 min at room temperature in either PBS or PBS with 0.5 nM fMLP and 5 μg/ml TS1/18 as indicated. Adherence to control HUVEC (*open bars*), HUVEC stimulated for 4 h with 10 ng/ml LPS (*coarse hatched bars*), or HUVEC stimulated with 0.5 U/ml IL-1 (*fine hatched bars*) was determined at room temperature using the visual assay described in Methods. HUVEC monolayers were washed by dipping three times in two changes of PBS before use in the adherence assay; fMLP and TS1/18 were retained with the PMN suspension during the adherence assay. (), number of separate experiments; error bars, 1 SD; *P < 0.01; **P < 0.001.

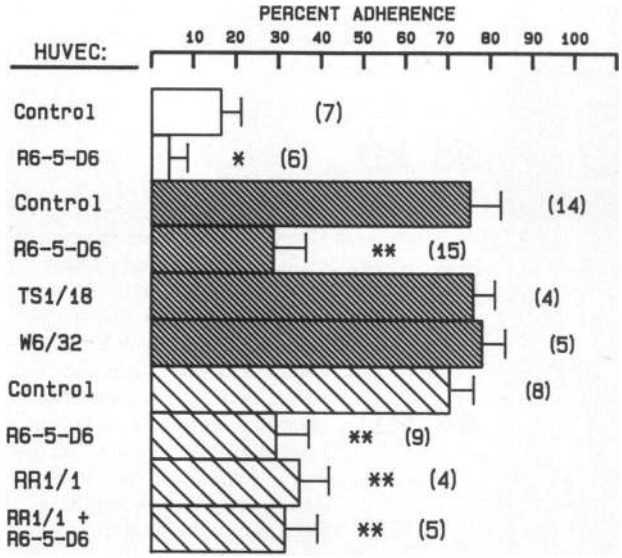

Figure 5. Effects of Mabs on IL-1- and LPS-enhanced HUVEC adhesiveness. HUVEC were incubated for 4 h, 37°C, in the presence of 0.5 U/ml IL-1 (*fine hatched bars*) or 10 ng/ml LPS (*coarse hatched bars*), washed by dipping three times in two changes of PBS and then exposed to the indicated MAbs for 15 min at 37°C, washed again, and inserted in adherence chambers. Adherence of unstimulated human PMN was assessed using the visual assay described in Methods performed at room temperature. TS1/18, 1:400 dilution of ascites; R6-5-D6, 1:400 dilution of ascites; W6/32, 1:200 ascites; RR1/1, 1:400 dilution of ascites. (), number of separate experiments; error bars, 1 SD; $^{**}P < 0.001$.

ineffective in altering adherence though it binds to HUVEC and is of the same isotype as R6-5-D6.

Effects of MAbs on adherence to paraformaldehyde-fixed HUVEC. Since the above experiments involved exposure of viable HUVEC monolayers to the MAbs for 15 min at 37°C, consideration was given to the possibility that R6-5-D6 was inducing a response in the endothelial cells that changed their adhesive properties. This seems very unlikely in light of the following results. R6-5-D6 reduced PMN adherence to paraformaldehyde-fixed HUVEC monolayers much the same as to viable HUVEC monolayers. There was high adherence of PMN to LPS-stimulated endothelial cells, and fMLP stimulation of the PMN augmented this adherence. R6-5-D6 significantly reduced adherence (Table I).

Direct comparison of inhibitory effects of TS1/18 and R6-5-D6. Since R6-5-D6 gave results qualitatively similar to TS1/18 in that it completely blocked the enhancement caused by fMLP stimulation of the PMN and partially blocked the enhancement caused by LPS or IL-1 stimulation of the HUVEC, direct comparison of these two MAbs on the same preparations of PMN and HUVEC was undertaken. Table II shows that the two MAbs gave almost identical results. Furthermore, in experiments where the PMN were blocked with TS1/18 and the HUVEC were blocked with R6-5-D6, inhibition of LPS-enhanced adherence was no greater than with either MAb alone. This comparison was further extended by the use of PMN from two patients deficient in the CD18 complex. Neither TS1/18 nor R6-5-D6 exhibited any inhibitory effect on the IL-1-enhanced adherence of the deficient PMN to normal HUVEC (Fig. 7). The same lack of effect was seen on adherence of CD18-deficient PMN to LPS-stimulated

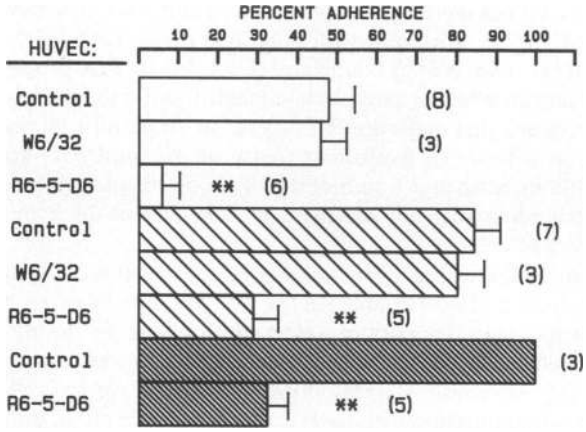

Figure 6. Effects of monoclonal antibodies, R6-5-D6 and W6/32, on fMLP-enhanced PMN adherence to HUVEC. HUVEC were incubated without (*open bars*) or with 10 ng/ml LPS (*coarse hatched bars*) or 0.5 U/ml IL-1 (*fine hatched bars*) for 4 h, 37°C, washed by dipping three times in two changes of PBS and then exposed to PBS alone (control) or PBS containing either W6/32 (1:200 dilution of ascites) or R6-5-D6 (1:400 dilution of ascites) for 15 min at 37°C. After washing the monolayer, the adherence of fMLP (0.5 nM)-preincubated (5 min, room temperature) PMN was assessed using a visual adherence assay performed at room temperature. fMLP was retained with the cells during the adherence assay. (), number of separate experiments; error bars, 1 SD; $^{**}P < 0.001$.

HUVEC (data not shown). A CD18-independent component to the LPS and IL-1 enhancement was clearly seen with the MAbs and CD18-deficient leukocytes.

Kinetics of CD18-dependent and CD18-independent adherence. The time course of LPS and IL-1 effects on R6-5-D6 binding to HUVEC was evaluated. The amount of R6-5-D6

Table I. Inhibition of PMN Adherence to Paraformaldehyde-fixed HUVEC by MAb R6-5-D6

Pretreatment			
PMN	HUVEC	*n*	Adherence
			%
PBS	LPS	12	61.8±5.0
PBS	LPS, R6-5-D6	9	20.0±4.5*
PBS	LPS, W6/32	2	54.5
fMLP	LPS	10	87.3±11.3
fMLP	LPS, R6-5-D6	10	26.7±16.3*
fMLP	LPS, W6/32	2	71.0
PBS	Control	4	18.5±1.8
fMLP	Control	4	50.0±4.9
fMLP	Control, R6-5-D6	4	8.0±1.2*

HUVEC were incubated without (control) and with 10 ng/ml LPS for 4 h at 37°C, washed in PBS and then fixed for 15 min at room temperature in 1% paraformaldehyde. The monolayers were again washed in PBS and then incubated for 15 min in PBS containing 1% BSA and 1% glycine. Monolayers were again washed and incubated for 30 min with PBS, R6-5-D6 (1:400 dilution of ascites), or W6/32 (1:200 dilution of ascites) after which adherence of control or fMLP (0.5 nM)-activated PMN was determined.

* $P < 0.01$ compared to condition without MAb. Percent adherence±1 SD.

Table II. Comparison of the Inhibitory Activity of MAbs R6-5-D6 and TS1/18 for PMN Adherence to HUVEC

Pretreatment			
PMN	HUVEC	*n*	Adherence
			%
PBS	Control	5	15.0±3.7
PBS	Control, R6-5-D6	4	7.0±1.4*
TS1/18	Control	4	6.3±2.2*
PBS	IL-1	9	79.4±9.6
PBS	IL-1, R6-5-D6	13	33.0±5.5*
TS1/18	IL-1	8	30.4±5.3*
TS1/18	IL-1, R6-5-D6	4	31.3±6.2*

HUVEC were incubated without (control) and with IL-1 (0.5 U/ml) for 4 h at 37°C, then exposed to R6-5-D6 (1:400 dilution of ascites) or PBS for 15 min. The monolayers were then washed and the adherence of PMN preincubated in either PBS or TS1/18 (1:400 dilution of ascites) was assessed. TS1/18 was retained with the cells during the adherence assay.

* $P < 0.01$, compared to condition without MAb.

bound to HUVEC increased markedly over the first 3 h and remained high over the 8 h of observation as revealed by the EIA procedure (Fig. 8). This was in contrast to the changes seen in adherence of unstimulated PMN over the same time period (Fig. 2). The diminishing adherence seen after 4 h stimulation of HUVEC with IL-1 or LPS apparently could not be accounted for by reductions in the amount of the endothelial surface factor recognized by R6-5-D6. Therefore, the contribution of the CD18-independent determinants of PMN adherence was evaluated. Adherence of PMN pretreated with saturating concentrations of TS1/18 to LPS (Fig. 8) or IL-1 (data not shown) stimulated HUVEC peaked at 4 h, and fell by 70% at 6 h and by 79.8% at 8 h. The CD18 contribution to adherence over this time, as reflected by the difference in the adherence of TS1/18-treated and untreated PMN, fell by 9.5% at 6 h and by 26.0% at 8 h. In a separate set of experiments utilizing cells from two patients with CD18 deficiency, the time course of the CD18-independent adherence was clearly the same as that revealed by TS1/18 inhibition (Fig. 9). Thus, the largest contribution to the diminishing adherence after 4 h appeared to be the CD18-independent factor(s). CD18-deficient PMN and R6-5-D6 pretreatment of stimulated HUVEC revealed adherence changes over time comparable to those seen with TS1/18 pretreatment of PMN (Table III).

Increasing concentrations of LPS or IL-1 resulted in increasing the CD18-independent component of PMN adherence to HUVEC (Table IV). When the stimulus to the endothelial cells was sufficient to promote adherence of 99% of the PMN in the absence of added chemotactic factors, the contribution of CD18 to this adherence was only about 25%.

Requirements for PMN migration through HUVEC monolayers. As has been observed by others (37, 44, 46, 51, 52), a portion of the PMN adherent to the HUVEC moved through the monolayer and migrated between the HUVEC and the substratum. These neutrophils assumed the bipolar configuration typical of motile PMN, though in marked contrast to the cells attached to the upper surface of the monolayer, they appeared greatly flattened within this space. Movement through the monolayer was quantitated using phase contrast observations of the monolayer *en face.* This was possible because of the morphologic change and the distinct difference in focal planes of the cells on the surface of the monolayer versus those beneath (Fig. 10 *A*). It was often possible to discern the uropod of migrating cells protruding through the monolayer before the cell fully traversed the thickness of the monolayer (Fig. 10 *A*). Cross sections of monolayers confirmed this transendothelial migration and revealed protruding uropods (Fig. 10 *B*).

The percent PMN exhibiting this behavior was very low

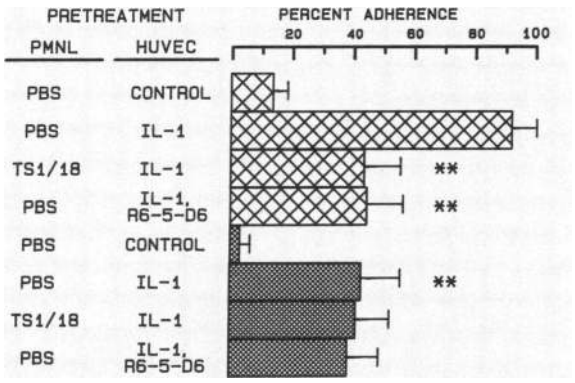

Figure 7. Adherence of CD18-deficient PMN to HUVEC. Effects of monoclonal antibodies R6-5-D6 and TS1/18. HUVEC were incubated with and without IL-1 (0.5 U/ml) for 4 h, 37°C, washed by dipping three times in two changes of PBS and then exposed to R6-5-D6 (1:400 dilution of ascites) for 15 min at 37°C. After washing, the adherence of normal (*coarse grid bars*) or deficient PMN (*fine grid bars*) pretreated in PBS or TS1/18 (1:400 dilution of ascites) was assessed using a visual adherence assay performed at room temperature. TS1/18 was retained with the cells during the adherence assay. Results of four separate experiments with PMN from two patients with a genetic absence of cell surface CD18. Error bars, ±1 SD.

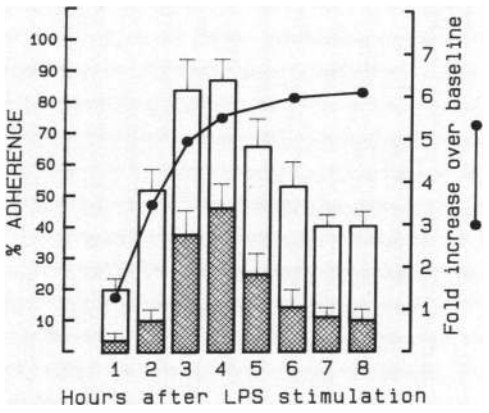

Figure 8. Changes over time in R6-5-D6 binding and adherence of PMN to HUVEC monolayers. HUVEC monolayers were exposed to LPS (10 ng/ml) for the times indicated then washed by dipping three times in two changes of PBS. Adherence of PMN preincubated (5 min, room temperature) in TS1/18 (1:400 dilution of ascites) or PBS was assessed using a visual assay at room temperature. TS1/18 was retained with the cells during the adherence assay. R6-5-D6 binding was assessed using an EIA procedure (results shown from a representative experiment). Error bars, ±1 SD; $n = 4$. *Open bars* plus the *fine grid bars* indicate the adherence of PMN suspended in PBS; the *fine grid bars* indicate the adherence remaining after preincubation of PMN in TS1/18.

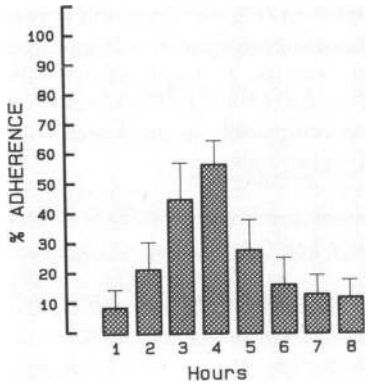

Figure 9. Changes over time in adherence of CD18-deficient PMN to LPS stimulated HUVEC. HUVEC monolayers were exposed to 10 ng/ml LPS for the time indicated, washed by dipping three times in two changes of PBS, and inserted into adherence chambers. Adherence of PMN from two patients with a genetic absence of CD18 was determined at room temperature using the visual assay described in Methods. Results are from four separate experiments. Error bars, 1 SD.

under baseline conditions (< 1%; $n = 45$) or when PMN were exposed to fMLP (Fig. 11). However, when the HUVEC were stimulated for 4 h with IL-1 or LPS, the percentage migrating through the monolayer increased significantly (33.3±10.3%; $n = 25$; 10 ng/ml LPS; Table IV; Fig. 11). When the adherence assay was performed at 37°C, this behavior on LPS stimulated monolayers was exhibited by a significantly greater percentage of the cells (78.4±4.2%; $n = 8$; $P < 0.01$), though the percent adherence was increased less by this increase in temperature (Fig. 12). In contrast to the results with 4-h stimulated HUVEC, migration through the monolayer was rarely found if the HUVEC were used 8 h after stimulation with either LPS or IL-1. R6-5-D6 and TS1/18 were both equally effective in

Table III. Time-dependent Changes in PMN Adherence to LPS-treated HUVEC Monolayers and the Inhibitory Activity of MAbs TS1/18 and R6-5-D6

Pretreatment			
PMN	HUVEC	*n*	Adherence
			%
PBS	LPS, 4 h	5	88.2±7.0
TS1/18	LPS, 4 h	5	46.2±4.9
PBS	LPS, 4 h, R6-5-D6	4	46.8±5.9
CD18-deficient	LPS, 4 h	4	50.5±6.4
PBS	LPS, 6 h	5	52.0±10.7
TS1/18	LPS, 6 h	9	13.7±3.8
PBS	LPS, 6 h, R6-5-D6	4	23.7±4.7
CD18-deficient	LPS, 6 h	4	13.0±1.2
PBS	LPS, 8 h	4	40.4±3.1
TS1/18	LPS, 8 h	4	9.3±2.6
PBS	LPS, 8 h, R6-5-D6	4	10.1±3.3
CD18-deficient	LPS, 8 h	4	11.3±2.6

HUVEC were incubated with LPS (10 ng/ml) for time indicated, washed by dipping three times in two changes of PBS, and then exposed to PBS or R6-5-D6 IgG (12 μg/ml) for 15 min. The HUVEC monolayers were washed again. Adherence of adult PMN suspended in PBS or TS1/18 (1:400 dilution of ascites), or CD18-deficient PMN was determined using a visual assay at room temperature. Percent adherence±1 SD.

Table IV. Effect of Increasing Concentrations of LPS and IL-1 on the CD18-independent Adherence of Human PMN to HUVEC

		PBS		TS1/18	
HUVEC	*n*	Adherence	Migration	Adherence	Migration
		%			
LPS, 0.3	4	16±4	1±1	6±1	0
LPS, 1.0	4	39±2	4±2	10±2	0
LPS, 3.0	4	55±4	11±3	23±3	0
LPS, 10.0	4	76±6	33±1	43±7	1±1
LPS, 30.0	4	90±5	35±3	58±6	1±1
LPS, 100.0	4	99±1	44±4	77±7	2±1
IL-1, 0.1	3	36±2	1±1	5±2	0
IL-1, 0.3	3	54±8	8±4	16±1	0
IL-1, 1.0	3	95±7	39±2	55±6	1±1
IL-1, 3.0	3	99±3	43±4	73±4	2±2

HUVEC were exposed to the concentrations of LPS (ng/ml) or IL-1 (U/ml) indicated for 4 h at 37°C, washed by dipping in two changes of PBS and inserted into adherence chambers. The adherence of PMN suspended in either PBS or PBS containing 10 μg/ml TS1/18 IgG was determined at room temperature using the visual assay described in Methods. Transendothelial migration was determined at 1,000 s using the visual assay described in Methods performed at room temperature. Means±SD given.

blocking the migration of PMN through the 4-h stimulated HUVEC monolayer. With the adherence assay performed at room temperature, the percentage of cells migrating through IL-1 stimulated (0.5 U/ml) monolayers was 30.6±12.6% ($n = 17$) compared to 1.6±1.6% ($n = 17$; $P < 0.01$) when PMN were preincubated in TS1/18 (1:400 dilution of ascites), or 2.5±1.7% ($n = 6$; $P < 0.01$) when the HUVEC monolayer was preincubated with R6-5-D6 (1:400 dilution of ascites). When adherence and migration were heightened by increasing the concentrations of the stimulus to the endothelial cells, TS1/18 was very effective in blocking migration (Table IV). W6/32 was without inhibitory effect (% migrating cells, 36.0±11.5%) on HUVEC stimulated with LPS (10 ng/ml, 4 h, 37°C). R6-5-D6 IgG and Fab, and RR1/1 IgG preparations were evaluated at 37°C. Each produced significant inhibition of migration and adherence (Fig. 12). Inhibition of migration by R6-5-D6 was much greater than inhibition of adherence, both at 37°C and at room temperature. The percent inhibition of adherence at 37°C by R6-5-D6 Fab and IgG preparations was 51 and 60, respectively, while the percent inhibition of migration was 81 and 86. At room temperature, percent inhibition of adherence by R6-5-D6 IgG was 59, and percent inhibition of migration was 92. The importance of the CD18 complex in transendothelial migration in vitro was further demonstrated by the results with CD18-deficient cells. As shown in Fig. 12, they did not migrate at room temperature and migrated very little at 37°C.

Discussion

The human endothelial determinant recognized by the MAb, R6-5-D6, appears to be of major importance in the CD18-dependent adherence of human neutrophils to HUVEC in vitro. This conclusion obviously depends on a direct, reliable documentation of CD18-dependent PMN adherence to HUVEC.

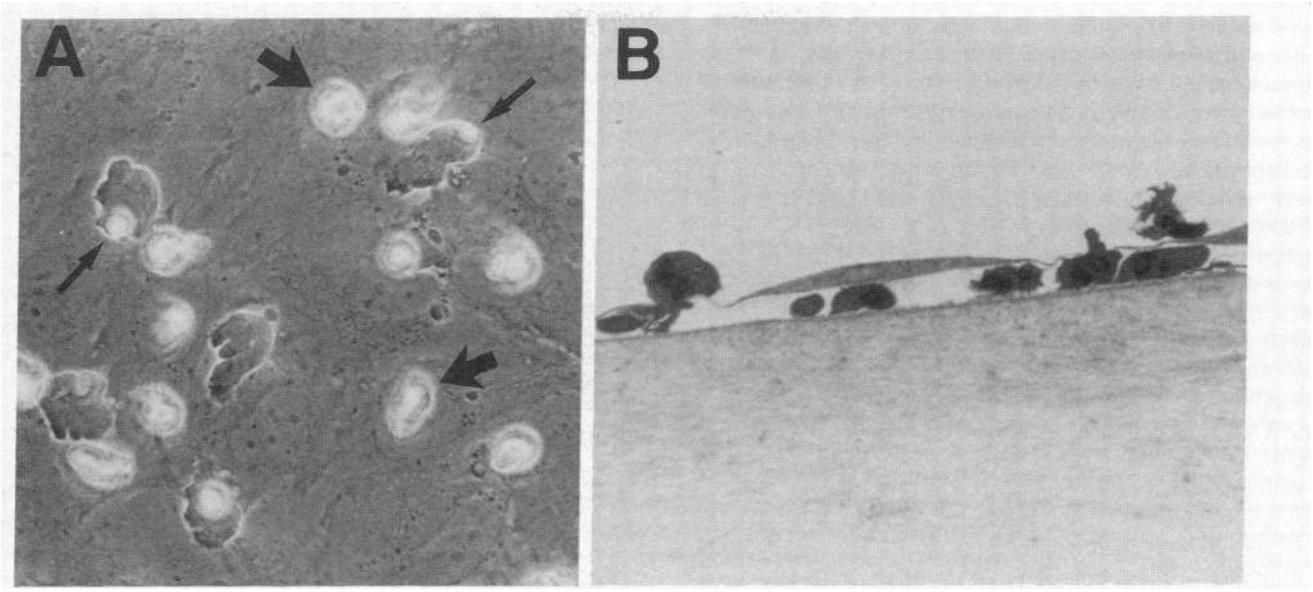

Figure 10. Transendothelial migration of human PMN. (*A*) Phase-contrast image of 4-h LPS (10 ng/ml)-stimulated HUVEC monolayer with attached and migrating PMN observed *en face* at the focal plane of PMN beneath the monolayer 1,000 s after injecting PMN into the assay chamber. The chamber was incubated at room temperature. Uropods of migrating cells protruding through the monolayer are visible (*small arrows*). Note that PMN attached to the upper surface of the HUVEC monolayer (*large arrows*) are distinctly out of focus. (*B*) Cross section of 4-h LPS (10 ng/ml)-stimulated HUVEC monolayer exposed at room temperature to PMN for 1,000 s before being fixed in 1% glutaraldehyde and embedded in epoxy resin. PMN are seen above and below the HUVEC, and the uropod of a migrating cell is still protruding through the monolayer.

Such dependence has been clearly shown in three types of experiments utilizing radiolabeled human neutrophils and HUVEC (10, 16) or human microvascular endothelium (17, 18). (*a*) A MAb reactive with CD18 (60.3) partially inhibited adherence of human PMN to unstimulated HUVEC and HUVEC stimulated with IL-1, LPS, and rTNF-α (16) and thrombin (19, 53). (*b*) PMN from patients deficient in CD18 exhibited low levels of adherence and there was no enhancement after stimulation with chemotactic factors (16, 17). (*c*) The augmented adherence after exposure of normal PMN to a

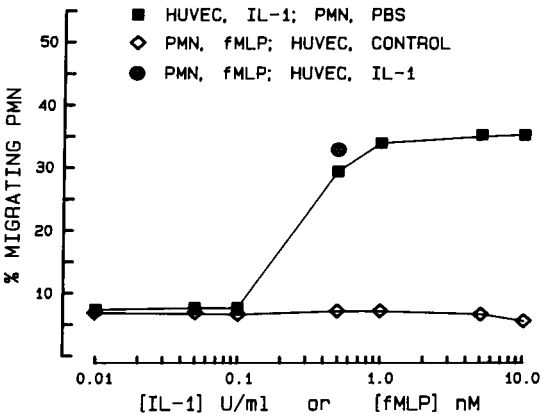

Figure 11. Effects of IL-1 and fMLP on the migration of PMN through HUVEC monolayers. The effect of IL-1 was determined by incubating HUVEC monolayers for 4 h, 37°C, in the presence of the indicated concentration of IL-1 and then washed by dipping three times in two changes of PBS. The effect of fMLP on migration through unstimulated HUVEC was determined after 15 min preincubation of the PMN with the indicated concentration of fMLP at room temperature. The percentage of cells migrating through the monolayer and between the endothelial cells and the substratum was determined at room temperature 1,000 s after allowing PMN to settle onto the monolayer.

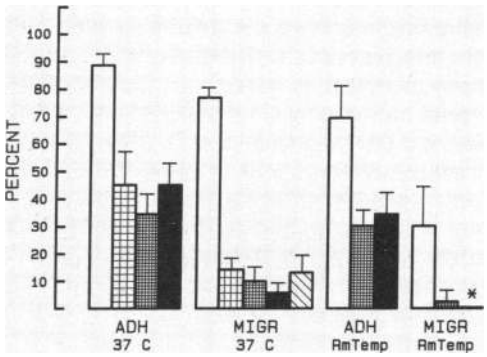

Figure 12. Inhibition of PMN migration through LPS stimulated HUVEC by R6-5-D6 IgG and Fab preparations, and comparison with CD18-deficient PMN. HUVEC incubated for 4 h, 37°C, with 10 ng/ml LPS and 15 min with the MAb or PBS before being washed and mounted in the adherence chamber. R6-5-D6 IgG and Fab at the concentration used in the preincubation (8 and 48 μg/ml, respectively) were added to the leukocyte suspension before injection into the chamber. The entire adherence procedure was carried out at 37°C or room temperature (*Rm Temp*). $P < 0.01$ ($n = 4$) for each experimental condition compared to normal adult cells without MAb. (*MIGR*) % migration ±1 SD of total cells originally coming in contact with the monolayer that migrate between the endothelial cells and substratum within 1,000 s after contacting the monolayer. (ADH) % adherence ±1 SD (*Open bars*) Adult PMN without Mab; (*coarse screen*), Fab preparation of R6-5-D6; (*fine screen*) IgG preparation of R6-5-D6; (*shaded bars*) CD18-deficient PMN; (*fine hatched*) IgG preparation of RR1/1. *No migration seen with CD18-deficient PMN.

secretagogue (16) or the chemotactic factors, C5a, fMLP, LTB_4, and platelet-activating factor (17, 18) was markedly inhibited by anti-β MAbs (60.3 and TS1/18) and 60.1, a MAb reactive with CD11b (16, 54). In the present study, the results clearly confirm the observations made by these investigators. The anti-β MAb, TS1/18, significantly reduced the attachment of unstimulated human PMN to unstimulated HUVEC and to HUVEC stimulated with IL-1 and LPS, and it completely blocked the enhanced adherence induced by fMLP stimulation of PMN. The most convincing evidence of CD18 dependence was the fact that PMN from two patients with total deficiency of surface CD18 (27) exhibited much lower than normal adherence to HUVEC and their adherence was not affected by exposure of the PMN to the anti-β MAb, TS1/18. Thus CD18-dependence can be experimentally documented in vitro as a portion of the adherence mechanisms stimulated in the endothelial cell by mediators such as IL-1, and as the principle adherence mechanism stimulated in the PMN by secretagogues and chemotactic factors.

Support for the conclusion that the MAb, R6-5-D6, recognizes an endothelial antigen critical to CD18-dependent PMN adherence to human endothelial cells can be found in the following observations: Upon binding to unstimulated endothelial monolayers, R6-5-D6 blocked adherence of control and fMLP-stimulated human PMN to the same degree as the anti-β MAb bound to PMN. Upon binding to LPS- or IL-1-stimulated HUVEC, R6-5-D6 reduced adherence of control and fMLP-stimulated human PMN to the same degree as the anti-β MAbs bound to PMN, and there was no additional reduction in adherence when both R6-5-D6 and anti-β MAb were used together in the same experiment. Furthermore, the level of PMN adherence to LPS and IL-1 stimulated HUVEC after binding of R6-5-D6 to HUVEC or binding of TS1/18 to PMN was the same as the level of adherence of CD18-deficient PMN. R6-5-D6 binding to LPS- and IL-1-stimulated HUVEC did not reduce the adherence of CD18-deficient PMN demonstrating that those mechanisms present on CD18-deficient PMN do not depend on the antigen recognized by R6-5-D6.

The existence of a CD18-independent component to the PMN–HUVEC adherence is revealed by results from several experiments: Anti-β MAb (TS1/18) at saturating concentrations lead to only a 25–70% inhibition of PMN adherence to stimulated HUVEC depending on the level of stimulus to the endothelium. In contrast, 99–100% inhibition of fMLP-stimulated adherence was attained by this MAb. As CD18-deficient PMN provide a convincing argument for the role of the β subunit in adherence of PMN to HUVEC, they also provide a convincing argument for a CD18-independent mechanism. Deficient PMN gave the same pattern as anti-β MAbs, i.e., very low adherence to control HUVEC and 25–50% of normal cell adherence to LPS-activated HUVEC. These findings are consistent with the observations of Pohlman et al. (16) using 60.3 and radiolabeled normal and CD18-deficient PMN in vitro, and Arfors et al. (13) using 60.3 in vivo, where infiltration of PMN into inflammatory sites was inhibited but one measure of margination, rolling along vessel walls, was not. In the present study, the CD18-independent component stimulated in the endothelial cell by LPS and IL-1 in vitro was maximum at 4 h after stimulation and diminished by > 80% at 7 h. The appearance and disappearance of an adherence component not blocked by R6-5-D6 corresponded remarkably with the CD18-independent component revealed by TS1/18-blocked PMN or CD18-deficient PMN. This finding provides additional support for the conclusion that R6-5-D6 selectively blocks the CD18-dependent adherence mechanism.

Characterization of the CD18-independent adherence is lacking. Its kinetics in vitro are consistent with expression of the human endothelial activation antigen detected by MAbs, H4/18 (55, 56), and H18/7 (57), in that this antigen peaks at 4 h after stimulation and diminishes thereafter (24, 56). Also, the same stimuli induce its expression (e.g., IL-1 and LPS). Support for the possibility that the endothelial activation antigen plays a role in PMN adherence comes from the observation that one of the MAbs (H18/7) partially inhibits neutrophil adherence to stimulated HUVEC (57). The relationship of this mechanism to the CD18-independent adherence observed after stimulation of HUVEC with α-thrombin or LTC_4 (19) is unknown. The kinetics differ. The α-thrombin–induced effect peaks within 10 min and the LTC_4, within 30 min (19).

The antigen recognized by R6-5-D6 clearly differs from the endothelial activation antigen. Immunoprecipitation studies with either H4/18 or H18/7 resulted in two polypeptides (M_r = 115,000 and 100,000 kD) (57) while R6-5-D6 immunoprecipitates a single polypeptide (M_r = 95,000 kD). However, R6-5-D6 appears to recognize the same glycoprotein as RR1/1, i.e., ICAM-1. That R6-5-D6 was selected using the same screening protocol as RR1/1 (22), the time courses of increased binding of R6-5-D6 and RR1/1 (23) after activation of HUVEC with IL-1 and LPS are the same, and both MAbs immunoprecipitate a single peptide (22) of the same molecular mass support this conclusion. More direct evidence that R6-5-D6 recognizes ICAM-1 comes from the sequential immunoprecipitation experiment (Fig. 3). Additionally, the observations that RR1/1 inhibits adherence and transendothelial migration to the same degree as R6-5-D6, and the combination of both MAbs is no more effective than either alone supports this conclusion. In unpublished experiments, Dr. Steven D. Marlin (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) has found that although R6-5-D6 and RR1/1 bind to ICAM-1, they bind to different epitopes. Thus, current evidence indicates that ICAM-1 is involved in CD18-dependent adherence of PMN while the endothelial activation antigen involves another adherence mechanism on the PMN.

Migration of PMN beneath endothelial cell monolayers grown on protein-coated glass or plastic substrata has been repeatedly observed (37, 44, 46, 51, 52). This migration occurs in the absence of added chemotactic stimuli and cannot be explained in terms of the relative adhesiveness of the endothelium and substratum (44). Since the adherence assay utilized in the present study allowed direct determination of the percentage of migrating PMN, this phenomenon could be monitored with each experimental manipulation. Migration of unstimulated PMN to a position between the HUVEC monolayer and the substratum occurred in a high percentage of PMN only when the HUVEC monolayer was stimulated with LPS or IL-1. This percentage increased substantially when the adherence assay was performed at 37°C compared to the results at room temperature. It is of interest to note that a chemokinetic stimulus (i.e., fMLP present in the adherence chamber at a uniform concentration) did not promote this behavior in the PMN. The migration of unstimulated normal PMN beneath LPS- or IL-1-stimulated HUVEC monolayers was profoundly inhibited by the presence of TS1/18 with the cells throughout the observation period. This result plus the fact that CD18-deficient PMN exhibited extremely low levels of transendothelial migration implicate a CD18-dependent

mechanism. This is consistent with previous work showing inhibition of adherence-dependent migration on protein-coated surfaces by TS1/18 (31) as well as extremely low adherence-dependent migration of CD18-deficient PMN (14, 15). R6-5-D6 was equal to TS1/18 in blocking PMN migration beneath the stimulated HUVEC monolayers. In all of these experimental conditions, a MAb control (W6/32) of the same isotype as R6-5-D6 failed to produce any change in adherence or migration of PMN though it bound to the HUVEC surface in greater amounts than R6-5-D6. The observations stated above indicate that the endothelial cells can play an active role in the transendothelial migration of PMN in vitro, and that ICAM-1-dependent adherence is critical to this phenomenon.

While the results in this report clearly indicate that endothelial ICAM-1 is necessary for CD18-dependent PMN attachment and transendothelial migration, there is evidence that ICAM-1 may not be sufficient for these functions. For example, IL-1 and interferon-γ both increase surface expression of ICAM-1 on human endothelial cells in vitro as recognized by the MAb RR1/1 (22, 23), but interferon-γ has not been found to stimulate increased attachment of PMN (21) in a assay utilizing radiolabeled PMN. Results in the present report indicate that at both 4 and 8 h after stimulation of HUVEC with either LPS or IL-1, surface ICAM-1 is abundant (i.e., R6-5-D6 binding is high as seen with EIA and IF) while CD18-dependent adherence is 26% less at 8 h than the peak at 4 h, and transendothelial migration, a prominent behavior at 4 h, is rarely seen at 8 h. Conversely, the adherence mechanisms remaining on CD18 deficient PMN are also insufficient for peak attachment and transendothelial migration. Thus, various experimental conditions in vitro indicate that both CD18-independent and CD18-dependent mechanisms are required for optimum function.

The extent to which ICAM-1 is necessary for adherence and emigration of PMN in vivo remains to be determined. The fundamental importance of CD18-dependent adherence mechanisms to accumulation of PMN at inflammatory sites is clearly demonstrated in the LAD patients (14, 15). The results in vitro indicate that ICAM-1 may play a major role in the CD18-dependent adherence and emigration of PMN in vivo.

Acknowledgments

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References

1. Colditz, I. G. 1985. Margination and emigration of leukocytes. *Surv. Synth. Pathol. Res.* 4:44–68.

2. Harlan, J. M. 1985. Leukocyte-endothelial cell interactions. *Blood.* 65:513–525.

3. Jalkanen, S., N. Wu, R. F. Bartgatze, and E. C. Butcher. 1987. Human lymphocyte and lymphoma homing receptors. *Annu. Rev. Med.* 38:467–476.

4. Woodruff, J. J., and L. M. Clarke. 1987. Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. *Ann. Rev. Immunol.* 5:201–222.

5. Yednock, T. A., E. C. Butcher, L. M. Stoolman, and S. D. Rosen. 1987. Receptors involved in lymphocyte homing: relationship between a carbohydrate-binding receptor and the Mel-14 antigen. *J. Cell Biol.* 104:725–731.

6. Colditz, I. G., and H. Z. Movat. 1984. Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxis and chemotaxinigens. *J. Immunol.* 133:2168–2177.

7. Mentzer, S. J., M. A. V. Crimmins, S. J. Burakoff, and D. V. Faller. 1987. Alpha and beta subunits of the LFA-1 membrane molecule are involved in human monocyte-endothelial cell adhesion. *J. Cell. Physiol.* 130:410–415.

8. Mentzer, S. J., S. J. Burakoff, and D. V. Faller. 1986. Adhesion of T lymphocyte to human endothelial cells is regulated by the LFA-1 membrane molecule. *J. Cell. Physiol.* 126:285–290.

9. Haskard, D. O., D. Cavender, P. G. Beatty, T. A. Springer, and M. Ziff. 1986. T lymphocyte adhesion to endothelial cells: Mechanisms demonstrated by anti-LFA-a monoclonal antibodies. *J. Immunol.* 137:2901–2906.

10. Harlan, J. M., P. D. Killen, F. M. Senecal, B. R. Schwartz, E. K. Yee, R. F. Taylor, P. G. Beatty, T. H. Price, and H. D. Ochs. 1985. The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium *in vitro*. *Blood.* 66:167–178.

11. Lewinsohn, D. M., R. F. Bargatze, and E. C. Butcher. 1987. Leukocyte-endothelial cell recognition: evidence of common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. *J. Immunol.* 138:4313–4321.

12. Ismail, G., M. L. Morganroth, R. F. Todd, and L. A. Boxer. 1987. Prevention of pulmonary injury in isolated perfused rat lungs by activated human neutrophils preincubated with anti-Mol monoclonal antibody. *Blood.* 69:1167–1174.

13. Arfors, K. E., C. L. Lundberg, L. Lindbom, K. Lundberg, P. G. Beatty, and J. M. Harlan. 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood.* 69:338–340.

14. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1 and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175–194.

15. Todd III, R. F., and D. R. Freyer. 1988. The CD11/CD18 leukocyte glycoprotein deficiency. *Hematol./Oncol. Clin. North Am.* 2:13–28.

16. Pohlman, T. H., K. A. Stanness, P. G. Beatty, H. D. Ochs, and J. M. Harlan. 1986. An endothelial cell surface factors(s) induced *in vitro* by lipopolysaccharide, interleukin-1, and tumor necrosis factor increases neutrophil adherence by a CDw18-dependent mechanism. *J. Immunol.* 136:4548–4553.

17. Tonnesen, M. G., D. C. Anderson, T. A. Springer, A. Knedler, N. Avdi, and P. M. Henson. 1986. Mac-1 glycoprotein family mediates adherence of neutrophils to endothelial cells stimulated by chemotactic peptides. *Clin. Res.* 34:419a. (Abstr.)

18. Tonnesen, M. G., D. C. Anderson, T. A. Springer, A. Knedler, N. Avdi, and P. M. Henson. 1986. Mac-1 glycoprotein family mediates adherence of neutrophils to endothelial cells stimulated by leukotriene B_4 and platelet activating factor. *Fed. Proc.* 45:379a. (Abstr.)

19. Zimmerman, G. A., and T. M. McIntyre. 1988. Neutrophil adherence to human endothelium in vitro occurs by CDw18 (Mol, Mac-1/LFA-1/GP150,95) glycoprotein-dependent and -independent mechanisms. *J. Clin. Invest.* 81:531–537.

20. Gamble, J. R., J. M. Harlan, S. J. Klebanoff, A. F. Lopez, and M. A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA.* 82:8667–8674.

21. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone, Jr. 1985. Interleukin-1 activation of vascular endothelium: effects on precoagulant activity and leukocyte adhesion. *Am. J. Pathol.* 121:393–403.

22. Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer. 1986. An intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* 137:1–5.

23. Dustin, M. L., R. Rothlein, A. K. Bhan, C. A. Dinarello, and T. A. Springer. 1986. Induction by IL-1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* 137:245–254.

24. Pober, J. S., M. A. Gimbrone, L. A. Lapierre, D. L. Mendrick,

W. Fiers, R. Rothlein, and T. A. Springer. 1986. Activation of human endothelium by lymphokines: overlapping patterns of antigenic modulation by interleukin 1, tumor necrosis factor and immune interferon. *J. Immunol.* 137:1893–1896.

25. Makgoba, M. W., M. E. Sanders, G. E. Ginther Luce, M. L. Dustin, T. A. Springer, E. A. Clark, P. Mannoni, and S. Shaw. 1988. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature (Lond.).* 331:86–88.

26. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell.* 51:813–819.

27. Anderson, D. C., F. C. Schmalstieg, A. S. Goldman, W. T. Shearer, and T. A. Springer. 1985. The severe and moderate phenotypes of heritable Mac-1, LFA-1, p150,95 deficiency: their quantitative definition and relation to leukocyte dysfunction and clinical features. *J. Infect. Dis.* 152:668–689.

28. Anderson, D. C., M. L. Mace, R. R. Martin, and C. W. Smith. 1981. Recurrent infection in glycogenesis type 1b: Abnormal neutrophil motility related to impaired redistribution of adhesion sites. *J. Infect. Dis.* 143:447–459.

29. Sanchez-Madrid, F., J. Nagy, E. Robbins, P. Simon, and T. A. Springer. 1983. A human leukocyte differentiation antigen family with distinct alpha subunits and a common beta subunit: the lymphocyte function associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. *J. Exp. Med.* 158:1785–1803.

30. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, and C. Milstein. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell.* 14:9–21.

31. Anderson, D. C., L. J. Miller, F. C. Schmalstieg, R. Rothlein, and T. A. Springer. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific monoclonal antibodies. *J. Immunol.* 137:15–27.

32. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745–2757.

33. Gimbrone, M. A. 1975. Culture of vascular endothelium. *Prog. Hemostasis Thromb.* 3:1–28.

34. Voyta, J. C., D. P. Via, C. E. Butlerfield, and B. R. Zetter. 1984. Identification and isolation of endothelial cells based on their increased uptake of acetylated low density lipoprotein. *J. Cell Biol.* 99:2034–2040.

35. Smith, C. W., J. C. Hollers, R. A. Patrick, and C. Hassett. 1979. Motility and adhesiveness in human neutrophils: effects of chemotactic factors. *J. Clin. Invest.* 63:221–229.

36. Charo, I. F., C. Yuen, H. D. Perez, and I. M. Goldstein. 1986. Chemotactic peptides modulate adherence of human polymorphonuclear leukocytes to monolayers of cultured endothelial cells. *J. Immunol.* 136:3412–3419.

37. Tonnesen, M. G., L. A. Smedley, and P. M. Henson. 1984. Neutrophil-endothelial cell interactions: modulation of neutrophil adhesiveness induced by complement fragments C5a and $C5a_{desArg}$ and formyl-methionyl-leucyl-phenylalanine in vitro. *J. Clin. Invest.* 74:1581–1592.

38. Smith, C. W., and J. C. Hollers. 1980. Motility and adhesiveness in human neutrophils: redistribution of chemotactic factor induced adhesion sites. *J. Clin. Invest.* 65:804–812.

39. Anderson, D. C., G. S. Krishna, B. J. Hughes, M. L. Mace, C. W. Smith, and B. L. Nichols. 1983. Impaired polymorphonuclear leukocyte motility in malnourished infants: relationship to functional abnormalities of cell adherence. *J. Lab. Clin. Med.* 101:881–895.

40. Anderson, D. C., B. J. Hughes, and C. W. Smith. 1981. Abnormal mobility of neonatal polymorphonuclear leukocytes: relationship to impaired redistribution of surface adhesion sites by chemotactic factor or colchicine. *J. Clin. Invest.* 68:863–874.

41. Schmid-Schoenbein, G. W., Y. C. Fung, and B. W. Zweifach. 1975. Vascular endothelium-leukocyte interaction: sticking shear force in venules. *Circ. Res.* 36:173–184.

42. Schmid-Schoenbein, G. W., S. Usami, R. Skalak, and S. Chien. 1980. The interaction of leukocytes and erythrocytes in capillary and post-capillary vessels. *Microvasc. Res.* 19:45–70.

43. Forrester, J. V., and J. M. Lackie. 1984. Adhesion of neutrophil leukocytes under conditions of flow. *J. Cell Sci.* 70:93–110.

44. Beesley, J. E., J. D. Pearson, A. Hutchings, J. S. Carleton, and J. L. Gordon. 1979. Granulocyte migration through endothelium in culture. *J. Cell Sci.* 38:237–248.

45. Hoover, R. L., M. Robinson, and M. J. Karnovsky. 1987. Adhesion of polymorphonuclear leukocytes to endothelium enhances the efficiency of detoxification of oxygen-free radicals. *Am. J. Pathol.* 126:258–268.

46. Buchanan, M. R., C. A. Crowley, R. E. Rosin, M. A. Gimbrone, and B. M. Babior. 1982. Studies on the interaction between GP-180-deficient neutrophils and vascular endothelium. *Blood.* 60:160–165.

47. Furie, M. B., E. V. Cramer, B. L. Naprstek, and S. C. Silverstein. 1984. Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. *J. Cell Biol.* 98:1033–1041.

48. Furie, M. B., B. L. Naprstek, and S. C. Silverstein. 1987. Migration of neutrophils across monolayers of cultured microvascular endothelial cells. *J. Cell Sci.* 88:161–175.

49. Anderson, D. C., F. C. Schmalstieg, S. Kohl, M. A. Arnaout, B. J. Hughes, M. F. Tosi, G. J. Buffone, B. R. Brinkley, W. D. Dickey, J. S. Abramson, T. A. Springer, L. A. Boxer, J. M. Hollers, and C. W. Smith. 1984. Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): common relationship to diminished cell adherence. *J. Clin. Invest.* 74:536–551.

50. Schmalstieg, F. C., H. E. Rudloff, and D. C. Anderson. 1986. Binding of adhesive protein complex (LFA-1/Mac-1/p150,95) to concanavalin A. *J. Leukocyte Biol.* 39:193–203.

51. Gimbrone, M. A., A. F. Brock, and A. I. Schafer. 1984. Leukotriene B_4 stimulates polymorphonuclear leukocyte adhesion to cultured vascular endothelial cells. *J. Clin. Invest.* 74:1552–1555.

52. Armstrong, P. B., and J. M. Lackie. 1975. Studies on intercellular invasion in vitro using rabbit peritoneal neutrophil granulocytes (PMNs) I. Role of contact inhibition in locomotion. *J. Cell Biol.* 65:439–462.

53. Bizios, R., L. C. Lai, J. A. Cooper, P. J. Del Vecchio, and A. B. Malik. 1988. Thrombin-induced adherence of neutrophils to cultured endothelial monolayers: increased endothelial adhesiveness. *J. Cell. Physiol.* 134:275–280.

54. Wallis, W. J., P. G. Beatty, H. D. Ochs, and J. M. Harlan. 1985. Human monocyte adherence to cultured vascular endothelium: monoclonal antibody-defined mechanisms. *J. Immunol.* 135(4): 2323–2330.

55. Cotran, R. S., M. A. Gimbrone, M. P. Bevilacqua, D. L. Mendrick, and J. S. Pober. 1986. Induction and detection of a human endothelial activation antigen in vivo. *J. Exp. Med.* 164:661–666.

56. Pober, J. S., L. A. Lapierre, A. H. Stolpen, T. A. Brock, T. A. Springer, W. Fiers, M. P. Bevilacqua, D. L. Mendrick, and M. A. Gimbrone, Jr. 1987. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *J. Immunol.* 138:3319–3324.

57. Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone, Jr. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 84:9238–9242.