

## TRANSIENT ADHESION OF NEUTROPHILS TO ENDOTHELIUM

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Circulating polymorphonuclear leukocytes (PMN)<sup>1</sup> exhibit negligible binding affinity for unstimulated vascular endothelium (EC). After intravenous administration of a chemotactic factor such as C5a, PMN attach to EC as evidenced by a rapid (<1 min) neutropenia (1, 2), but they soon detach and normal levels of circulating PMN are restored in 15–20 min. Thus PMN possess the means of transiently making then breaking adhesions to EC. Transient adhesion of PMN to EC appears necessary for extravasation in response to a chemotactic signal. A gradient of chemotactic agent emanating from the surrounding tissues causes PMN first to bind to the luminal surface of the EC, then to break that adhesion as they move out of the vascular space.

There is strong evidence that members of the CD11/CD18 complex of leukocyte receptors mediate adhesion of stimulated PMN to unstimulated EC. mAbs against CD11/CD18 block adhesion of PMN to EC both in vitro (3, 4) and in vivo (5, 6). In addition, PMN from patients deficient in CD11/CD18 fail to adhere to EC both in vitro (3) and in vivo (7). The CD11/CD18 molecules are ideal candidates for mediating transient adhesion of PMN to EC in vivo, because their capacity to bind ligands in vitro can be transiently enabled. CD11b/CD18, the most abundant member of the CD11/CD18 complex on PMN, functions as a receptor for surface-bound C3bi (8). We have previously shown that the ability of this receptor to bind to erythrocytes coated with C3bi is dramatically stimulated by phorbol esters and that this stimulation is short in duration (9). Here we show that CD11/CD18-dependent adhesion of phorbol-treated PMN to EC increases and subsequently decreases with a time course identical with that shown for the effect of phorbol esters on the binding activity of CD11b/CD18 for C3bi. We further show that adhesion of PMN to EC may be transiently stimulated with the physiological mediators TNF or C5a.

Expression of CD11b/CD18 on the surface of PMN is increased upon stimulation with chemotactic factors (9–11). We found that cytoplasts, which are depleted of in-

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<sup>1</sup> *Abbreviations used in this paper:* EC, endothelial cells; PDB, phorbol dibutyrate; PMN, polymorphonuclear leukocytes.

tracellular pools of CD11b/CD18, exhibited the same transient adhesion to EC as whole PMN. Thus, changes in expression of CD18 are not necessary for either the initial increase or the subsequent decrease in CD18-dependent adhesion.

### Materials and Methods

**Reagents.** Recombinant human TNF was a gift of Dr. A. Cerami (The Rockefeller University). TNF was stored sterilely at 4°C before use. Human recombinant complement fragment C5a was a gift of Dr. M. Springer (Merck Research Laboratories, Rahway, NJ) and was dissolved in HAP buffer (Dulbecco's PBS containing 0.5 mg/ml human serum albumin, 3 mM glucose, and 0.3 U/ml aprotinin) that contained ( $10^{-4}$  M) Plumber's inhibitor (DL-2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid; CalBiochem-Behring Corp., San Diego, CA) to inhibit the activity of carboxypeptidases. Phorbol dibutyrate (PDB) was obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ). Human fibronectin was supplied from the New York Blood Center (New York, NY).

**Monoclonal Antibodies.** mAb OKM10 (IgG<sub>2b</sub>), directed against CD11b (8), was a gift of Dr. G. Goldstein, (Ortho Pharmaceuticals, Raritan, NJ); mAb TS1/22 (IgG1) against CD11a (12) and TS1/18 (IgG1) directed against CD18 (13) were supplied by Dr. T. A. Springer (Harvard Medical School, Boston, MA); mAb LeuM5 (IgG2b) directed against CD11c (14) was obtained from Dr. L. Lanier (Becton Dickinson and Co., Mountain View, CA). Anti-CD18 mAb IB4 (IgG2a) and Fab fragments of IB4 were as described (8). mAb 60.3 (IgG2a) directed against CD18 (15) was a gift from Dr. J. Harlan (University of Washington, Seattle, WA). Additional anti-CD18 reagents, mAbs H52, MHM23 (ascitic fluids) were provided by Dr. J. Hildreth (Johns Hopkins University, Baltimore, MD). mAb 3G8 (IgG1) directed against the low avidity Fc receptor of PMN (FcRIII, CD16) was as described (16).

**Cells.** Human PMN were purified from freshly drawn human blood on Ficoll-Hypaque gradients as described (17). Isolated PMN were >99% pure as assessed by Wright's stained cytocentrifuge preparations and >99% viable as assessed by exclusion of trypan blue. PMN were fluorescently labeled with a hydrophobic fluorescent compound (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI); Molecular Probes, Eugene, OR). Cells at  $4-8 \times 10^6$  cells/ml were incubated with 50 µg/ml DiI in HAP buffer for 10 min at 0°C, unbound dye was removed by three washes with HAP buffer, and labeled PMN were resuspended in Medium 199 (M199; Hazleton Research Products Inc., Denver, PA.) for the adhesion assay. Labeled PMN bound and phagocytosed IgG- and C3bi-coated erythrocytes as well as unlabeled PMN (data not shown), indicating that there was no loss of functional activity during the labeling. There was no visible transfer of DiI from PMN to EC during adhesion assays at coincubation times up to 60 min. Both spread and rounded PMN appeared very bright and were easily visible.

PMN were also isolated from two children of French Canadian origin who have been diagnosed as congenitally deficient in the CD11/CD18 complex (Detmers, P. A., S. D. Wright, E. Olsen-Egbert, R. Adamowski, Z. Chad, L. G. Kabbash, and Z. A. Cohn, manuscript in preparation). These cells were labeled with DiI as described above. EC were harvested from veins of human umbilical cords using collagenase as previously described (18). Primary EC cultures were maintained in M199 plus 20% human serum (heat inactivated). When EC cultures became confluent monolayers, EC were harvested from 35-mm tissue culture plates (Corning Glass Works, Corning, NY) by exposure to 0.025% trypsin plus 1 mM EDTA for 2-3 min. EC were resuspended in M199 plus 20% human serum and seeded onto Terasaki plates (Miles Laboratories, Naperville, IL) at a cell density of  $10^6$  cells/ml. The Terasaki plates were precoated with human fibronectin (50 µg/ml) for 15 min at 37°C. EC were cultured a further 2-3 d before use in adhesion assays. During this time a confluent monolayer formed with characteristic cobblestone morphology (Fig. 1). The cell junctions stained with silver nitrate (data not shown), and previous studies indicate that the EC monolayers stain positive for factor VIII-related antigen (18).

**Adhesion of PMN to EC.** DiI-labeled PMN ( $10 \mu\text{l}$  of  $10^6$  cells/ml) were added to twice-washed EC monolayers. Preliminary studies indicated that this concentration maintained

EC in excess of PMN, and yielded consistent and quantifiable adherent PMN without disrupting the EC during the assay. Adhesion was allowed to proceed for 15 min at 37°C, and unbound PMN were removed by three washes with M199. Residual adherent PMN on EC surfaces were counted manually on an inverted microscope (Diaphot-TMD; Nikon Inc., Garden City, NY) equipped for fluorescence using the filter IF535-550. Values of five replicates were averaged, and variations between replicates were small (<10%). The adhesion of maximally stimulated PMN was very consistent from experiment to experiment, but the adhesion of unstimulated PMN varied considerably. Thus, the enhancement of adhesion by stimulants varied from 3 to 10-fold. The background binding observed in the absence of stimulants was partially inhibitable with anti-CD18 mAb IB4.

In experiments where PMN were treated with PDB (300 ng/ml), TNF (0.66 nM), or C5a ( $10^{-8}$ M), DiI-labeled PMN were treated with agonists for various times at 37°C, washed three times with HAP buffer, and resuspended in M199 before the adhesion assay. Preliminary experiments showed these concentrations of agonists yielded optimal enhancement of adhesion of PMN to EC. Treatment of PMN with any of these agonists did not alter PMN viability, as judged by exclusion of trypan blue.

In experiments using mAbs to inhibit adhesion, PMN were treated with PDB for 15 min at 37°C, washed three times with HAP buffer, and incubated with various mAbs (10 µg/ml) for 20 min at 0°C before the adhesion assay. mAbs were present with the PMN-EC coculture throughout the assay.

**Preparation and Characterization of PMN Cytoplasts.** Cytoplasts were prepared by centrifuging cytochalasin B-treated PMN on a two-step Ficoll gradient exactly as described (19). Cytochalasin B was removed by washing the cytoplasts five times with incubation medium (138 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.6 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 5.5 mM glucose, and 0.5% (wt/vol) human albumin). The yield of the cytoplast preparation was >98%. Cytoplasts were either used immediately or frozen with 20% DMSO as described (19), and were used within a month.

To confirm that cytoplasts were free of granular constituents, the activities of several enzyme markers were assayed and compared with those of whole PMN (Table I).  $\beta$ -glucuronidase (20), myeloperoxidase (21), vitamin B<sub>12</sub>-binding protein (22), and alkaline phosphatase (23) were assayed by standard procedures. The cytoplasts had about one-third of the activity of alkaline phosphatase found in whole PMN (Table I), which is consistent with a report that cytoplasts have a surface area about one-third as great as that of whole cells (19). Enzymatic activity of granule constituents in our cytoplast preparation was <4% that of whole PMN, confirming previous reports (19) that cytoplasts are deficient in both specific and azurophilic granules. The absence of granules within cytoplasts was confirmed by electron microscopy (not shown).

**Expression of Cell Surface Antigens.** Expression of CD11b/CD18 on the surface of PMN and

TABLE I  
*Enzyme Marker Activities in PMN and Cytoplasts*

Enzymes	PMN	Cytoplasts	Percent activity*
Alkaline phosphatase (plasma membrane)	$4.1 \times 10^{-3}$ U/ml	$1.2 \times 10^{-3}$ U/ml	34
$\beta$ -glucuronidase (azurophil granule)	$173 \times 10^{-3}$ U/ml	$8 \times 10^{-3}$ U/ml	0.5
Myeloperoxidase (azurophil granule)	$92 \times 10^{-7}$ M	$3 \times 10^{-7}$ M	3
Vitamin B <sub>12</sub> -binding protein (specific granule)	5.9 ng/ml	0.1 ng/ml	2

PMN and cytoplasts were used at  $10^7$  cells/ml for the enzyme assays.

\* The enzyme activity in cytoplasts divided by that in PMN.

cytoplasts was measured by flow cytometry. PMN or cytoplasts were labeled with 5  $\mu\text{g/ml}$  FITC-conjugated OKM1 for 20 min at 0°C and analyzed with a Becton Dickinson FACScan cell analyzer. Nonspecific fluorescence was determined in parallel preparations incubated with 20-fold excess unlabeled OKM1. To measure the expression of CD11a/CD18, cells were incubated at 0°C for 20 min with 5  $\mu\text{g/ml}$  TS1/22, washed, and incubated with 10  $\mu\text{g/ml}$  FITC-conjugated goat anti-mouse IgG (HyClone Laboratories Inc.). The intensity of fluorescence was subsequently analyzed by FACS.

The expression of CD11b/CD18 on cytoplasts stimulated with PDB (300 ng/ml for 15 min at 37°C) was measured to determine if depletion of granules abolished intracellular storage pools of CD11b/CD18. Unlike intact PMN, cytoplasts did not exhibit any increase in the surface expression of CD11b/CD18 upon PDB stimulation (Table II).

## Results

*Adhesion of Stimulation PMN to EC Is CD11/CD18 Dependent.* DiI-labeled PMN were used to assay the adhesion of unstimulated and PDB-stimulated PMN to EC. Without stimulation, very few PMN bound to EC (Fig. 1 *a* and *b*). In contrast, when PMN were treated with PDB (300 ng/ml) for 15 min at 37°C, increased numbers of adherent PMN were observed (Fig. 1 *c* and *d*). Based on counts made before and after washing, ~50% of the added PMN bound to EC. PMN adhered as individuals rather than aggregates, and the integrity of the EC monolayers was not altered during the adhesion assay (Fig. 1 *a* and *c*).

The adhesion of PMN to EC observed in our assay is mediated entirely by the CD11/CD18 complex on PMN. Treating PDB-stimulated PMN with mAb IB4 against CD18 before the incubation with EC completely abrogated the adhesion of PMN to EC (Fig. 2). Fab fragments of IB4 also had potent inhibitory activities, indicating that the Fc portion of the mAb is not involved in the inhibition of PMN adhesion (Fig. 3). Several other anti-CD18 mAbs (H52, MHM23, 60.3, and TS1/18) had a similar potent inhibitory effect on binding of PDB-stimulated PMN to EC, but control mAbs against other determinants on PMN were without inhibitory effects (Fig. 3, and see below). Further evidence for the role of CD11/CD18 in adhesion was derived from studies on PMN from two patients with a genetic deficiency in CD11/CD18. These cells failed to adhere to EC, even after stimulation with PDB (Fig. 2). These results confirm the primacy of CD11/CD18 in adhesion to unstimulated EC and demonstrate that our EC do not exhibit CD11/CD18-independent adhesion characteristic of stimulated EC (4, 24).

*CD11a/CD18 and CD11b/CD18, but not CD11c/CD18 Mediate Phorbol-enhanced PMN Adhesion of PMN to EC.* To determine which individual members of the CD11/CD18 complex were responsible for the adhesion of phorbol-treated PMN to EC, adhesion

TABLE II  
FACS Analysis of CD11b/CD18 Expression on Cytoplasts

	Mean channel of fluorescence
Background	5
Unstimulated cytoplasts	777
PDB-stimulated cytoplasts	732

Cytoplasts ( $10^6$  cells/ml) were treated with PDB (300 ng/ml) for 15 min at 37°C, washed, and stained with FITC-OKM1 (10  $\mu\text{g/ml}$ ) for 20 min on ice. Fluorescence was analyzed by flow cytometry.

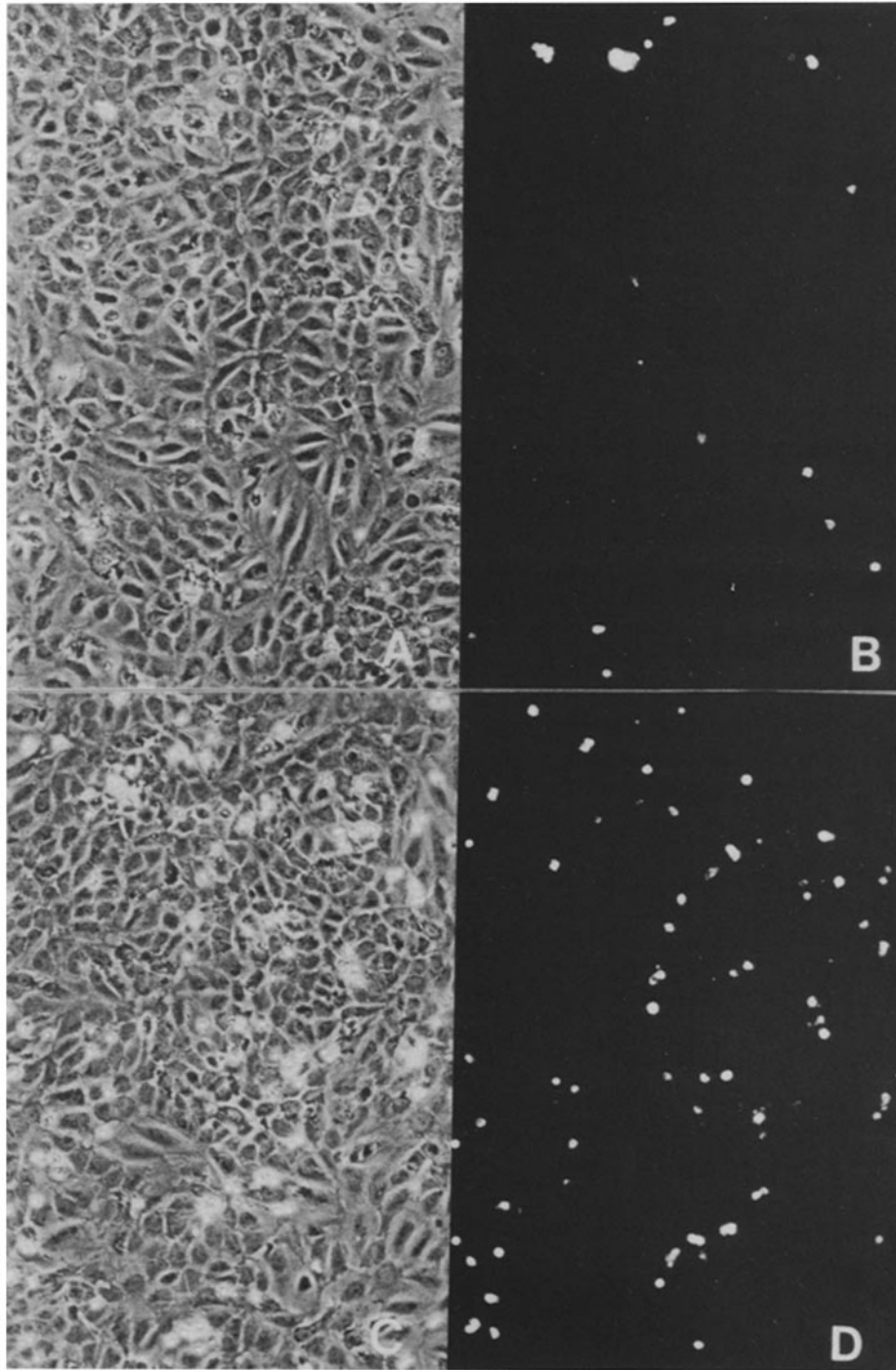


FIGURE 1. Adhesion of unstimulated PMN and PDB-stimulated PMN to EC. Unstimulated PMN (*A* and *B*) or PDB-stimulated PMN (*C* and *D*) were incubated for 15 min at 37°C with EC, then the monolayers were washed. (*A* and *C*) Phase contrast micrographs; (*B* and *D*) respective fluorescent images showing DiI fluorescence from adherent PMN.

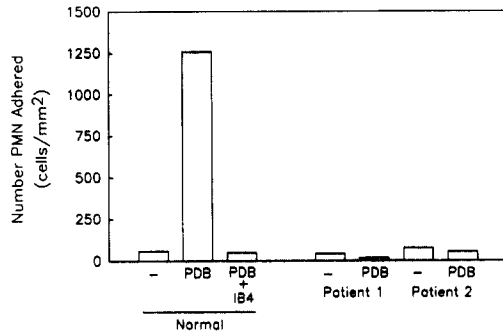


FIGURE 2. CD11/CD18-dependent adhesion of stimulated PMN to EC. The adhesion of PMN from a normal donor and two CD18-deficient patients was assayed as described in Materials and Methods. Where indicated, PMN were stimulated with 300 ng/ml PDB for 15 min at 37°C before the assay. PDB-treated PMN were treated with mAb IB4 as described in Materials and Methods. These data were confirmed by a second experiment that gave similar results.

was measured in the presence of mAbs against CD11a, CD11b, or CD11c (see Materials and Methods). mAb TS1/22 against CD11a and OKM10 against CD11b each inhibited binding of stimulated PMN by ~66% (Fig. 3). mAb LeuM5 against CD11c caused no inhibition (Fig. 3). The inhibition caused by mAbs was unlikely to be due to a steric effect since mAb 3G8, directed against FcRIII on PMN, had virtually no effect on adhesion (percent inhibition = 5%) (Fig. 3), and Fc receptors are more abundant on the cell surface than CD11b/CD18 or CD11a/CD18 (9).

The inhibition of binding caused by anti-CD11a and CD11b was partial, yet the inhibition by anti-CD18 was nearly complete (>90%), suggesting that CD11a/CD18 and CD11b/CD18 may both function in adhesion of stimulated PMN to EC. This was confirmed by the observation that a mixture of mAbs OKM10 and TS1/22 had an additive inhibitory effect on adhesion (percent inhibition = 93%) (Fig. 3). The combined inhibitory effect of anti-CD11a and CD11b mAbs was similar to that achieved by anti-CD18 mAbs (Fig. 3). These data suggest that CD11a/CD18 and CD11b/CD18

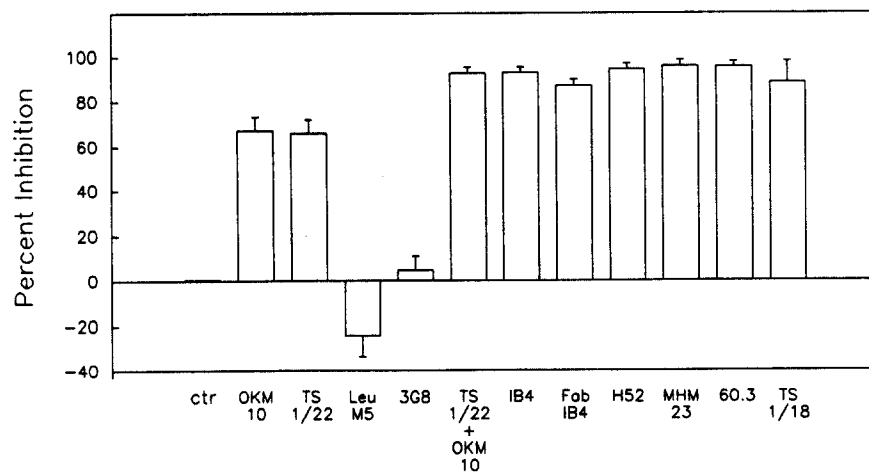


FIGURE 3. CD11a/CD18 and CD11b/CD18 but not CD11c/CD18, mediate phorbol-enhanced adhesion of PMN to EC. DiI-labeled PMN were stimulated with 300 ng/ml PDB for 15 min at 37°C, treated with mAb as described in Materials and Methods, and adhesion to EC was assayed. The data are means of five to seven experiments, and error bars indicate SEM.

each mediate adhesion of phorbol-stimulated PMN to EC, that each makes a comparable contribution, and that CD11c/CD18 makes very little if any contribution to adhesion.

*The Capacity of CD11/CD18 to Mediate Adhesion of PMN to EC Is Transiently Stimulated by Phorbol Esters.* We previously showed that the capacity of CD11b/CD18 on PMN to bind to erythrocytes coated with complement fragment C3bi (EC3bi) is transiently enhanced upon stimulation of the cells with phorbol esters (9). To determine if CD11/CD18-dependent binding of PMN to EC is regulated in a similar fashion, PMN were incubated for various intervals with PDB at 37°C, then assayed for adhesion to EC. The binding of PMN to EC increased sharply after 15 min of stimulation and then fell below control levels by 60 min (Fig. 4). The time course of these changes in binding activity was identical with that for the binding of EC3bi by phorbol-treated PMN (9). The loss of binding activity after 60 min of stimulation by PDB was not caused by cell death, as >95% of PMN excluded trypan blue after treatment with PDB for 60 min. Furthermore, the loss of adhesivity required active metabolism, since PMN incubated with PDB for 15 min at 37°C retained high binding activity to EC after a 1-h incubation at 4°C (data not shown).

*Transient Binding Activity Induced by Physiological Stimuli.* It has been reported that the physiological mediators TNF and C5a cause enhanced adhesion of PMN to EC (25, 26), and we next asked whether enhancement by these agents is also transient. Pretreatment of PMN with TNF (0.66 nM) caused a rise and subsequent fall in binding activity with a time course similar to that seen with PDB (Fig. 4). C5a ( $10^{-8}$  M) also caused a similar transient increase in binding activity (Fig. 5), but the peak occurred before 15 min. To accurately follow the time course of C5a-stimulated adhesivity, we added C5a ( $10^{-8}$  M) directly to cocultures of PMN and EC and incubated them for different times. C5a caused strong adhesion of PMN to EC as early as 2 min, and maximum adhesion was observed at 5 min. Adhesion of PMN to EC returned to basal levels by 15–20 min (Fig. 5). The adhesion stimulated by both C5a and TNF is CD11/CD18 dependent, since both were completely blocked by treating PMN with soluble mAb IB4 (data not shown).

*Detachment of PMN from EC.* Upon stimulation of cells with C5a, PMN first adhered to and then detached from the EC (Fig. 5). Since C5a-stimulated attachment

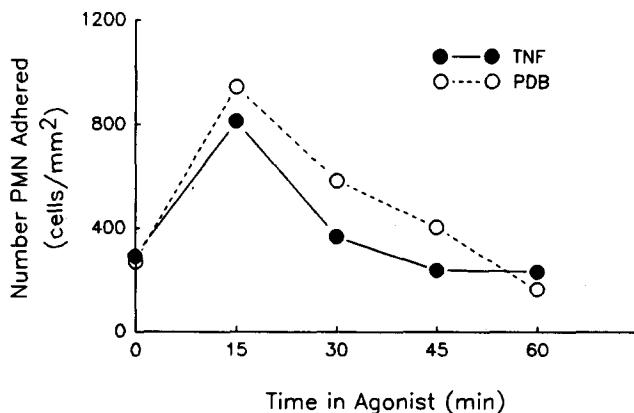


FIGURE 4. Transient enhancement of PMN adhesion to EC by phorbol or TNF. DiI-labeled PMN were stimulated with either PDB (300 ng/ml) or TNF (0.66 nM) for various times at 37°C. Agonists were removed by three washes with HAP before the adhesion assay. Control PMN were incubated for 15 min at 37°C in the absence of agonists. The experiment is representative of three separate experiments.

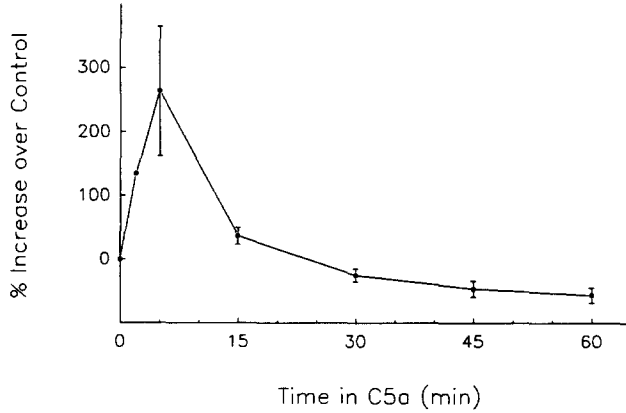


FIGURE 5. C5a stimulates transient adhesion of PMN to EC. DiI-labeled PMN were added to EC monolayers and incubated at 37°C for 5 min. C5a ( $10^{-8}$  M) was added and incubation was continued for the indicated times. Cocultures were washed three times with M199 to removed nonadherent PMN. Data points represent the results from four separate adhesion experiments. Error bars indicate SEM.

to EC was mediated by CD11/CD18, we presume the subsequent detachment was associated with release of ligand by these adhesive receptors. It is unlikely that release is due to an irreversible process such as proteolysis of receptors or ligands because adhesion could be rapidly reestablished by restimulation of the cells. Adhesion of PMN was initiated by stimulation of cells for 5 min with C5a ( $10^{-8}$  M). Nonadherent cells were removed by washing, and during a subsequent 15 min at 37°C, the PMN were observed to detach (Fig. 6). Restimulation of the detached cells for 5 min with PDB (300 ng/ml) or TNF (0.66 nM) caused rebinding of PMN to EC. In contrast, C5a restimulation did not cause the detached PMN to re-adhere to EC. The second enhancement in PMN adhesion was also CD11/CD18 dependent since it was blocked by mAb IB4 (data not shown).

*Regulation of Binding of PMN to EC Does Not Correlate with Changes in Expression of CD11a/CD18 and CD11b/CD18.* To determine whether the increase and subsequent decrease in CD11/CD18-dependent binding of PMN to EC was associated with quan-

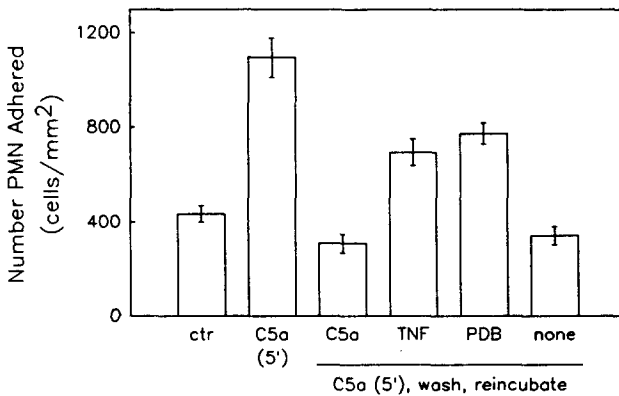


FIGURE 6. Restimulation of C5a-stimulated adhesion of PMN to EC. PMN were layered on EC and exposed to C5a ( $10^{-8}$  M) for 5 min at 37°C by which time the ability of PMN to adhere to EC had reached maximum (see Fig. 5). Unbound PMN were removed by three washes. The Terasaki plates containing adherent PMN on EC were incubated for an additional 15 min at 37°C, to allow adherent PMN to detach from EC. PDB (300 ng/ml), TNF (0.66 nM), or C5a ( $10^{-8}$  M) was then added to the PMN-EC

cocultures, and adhesion was allowed to continue for an additional 5 min at 37°C. Adherent PMN were scored after nonadherent PMN were removed by three washes with M199. The experiment shown here is representative of three separate experiments. Error bars indicate the standard deviation of the mean.



titative changes in the cell surface receptors, the expression of CD11a/CD18 and CD11b/CD18 on PMN stimulated with agonists for different lengths of time was measured by flow cytometry.

**CD11a/CD18.** Treatment of PMN with PDB (300 ng/ml) caused little change in the expression in CD11a/CD18 at any time from 0 to 60 min (Fig. 7). Similarly, neither TNF nor C5a caused changes in the expression of CD11a/CD18 on the cell surface (data not shown). These data confirm the observations of other investigators (9, 27) who showed that CD11a/CD18 expression is not changed upon stimulation of PMN. Phorbol-stimulated changes in adhesion of PMN to EC cannot, therefore, be accounted for by changes in the number of CD11a/CD18.

**CD11b/CD18.** PDB (300 ng/ml) caused a rapid 2.7-fold increase in the expression of CD11b/CD18 that reached maximum at 15 min and remained at above two-fold for 60 min (Fig. 7). While the time course of the increased expression was comparable to that for increased adhesion, this change in expression is insufficient to explain the transient changes in adhesivity. The 2–3-fold increase in CD11b/CD18 expression is much less than the 3–10-fold increase in adhesion observed after 15 min of stimulation. More importantly, expression of CD11b/CD18 remained high for 60 min, during which time the capacity of PMN to bind to EC fell to baseline levels.

PMN treated with TNF (0.66 nM) exhibited a rapid and sustained rise in the expression of CD11b/CD18, with a time course differing from that of the binding activity (Fig. 8). An increase in the expression of CD11b/CD18 was also observed when PMN were treated with C5a ( $10^{-8}$  M) (Fig. 8). Although the initial rate of increase was similar to that seen with TNF, the maximum expression by 10–15 min was lower than with TNF, and the amount of CD11b/CD18 on the cell surface subsequently declined to that observed before stimulation. For treatment with either TNF or C5a, the time course of change in the expression of CD11b/CD18 does not correlate well with that for changes in binding activity. These observations suggest that changes in the expression of CD11b/CD18 on the cell surface may contribute to the transient enhancement of binding activity but do not provide a complete explanation for this phenomenon.

**Cytoplasts Bind Transiently to EC.** To test the hypothesis that a mechanism unrelated to changes in the expression of CD11b/CD18 functions to regulate attachment of

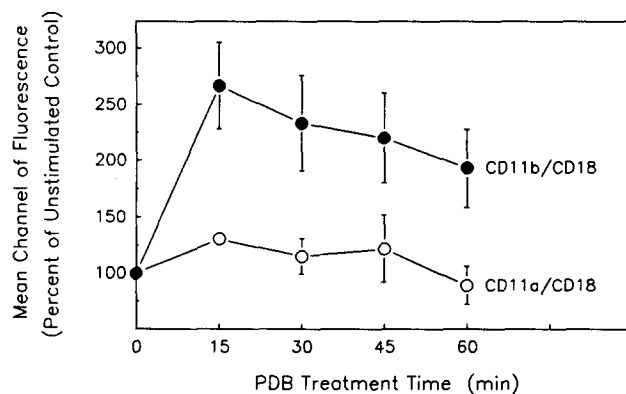


FIGURE 7. Expression of CD11a/CD18 and CD11b/CD18 on the cell surface of PMN upon stimulation with PDB. PMN were treated with PDB (300 ng/ml) at 37°C for the indicated times, then stained with FITC-mAb TS1/22 or OKM1 as described in Materials and Methods. Since each experiment had a different baseline mean channel of fluorescence, the percent change in mean channel of fluorescence was used to more accurately reflect the changes. Data shown represent the means of three experiments, and the error bars indicate the SEM.

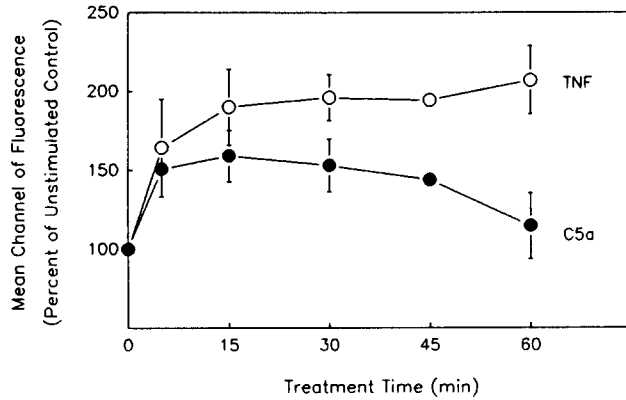


FIGURE 8. CD11b/CD18 expression induced by TNF and C5a. PMN were treated with TNF (0.66 nM) or C5a ( $10^{-8}$  M) for the indicated times at 37°C and stained with FITC-OKM1 as described in Materials and Methods. The data represent the means of three experiments, and the error bars indicate the SEM. Parallel studies on CD11a/CD18 showed that neither TNF nor C5a changed expression (<15% change from baseline at any time point).

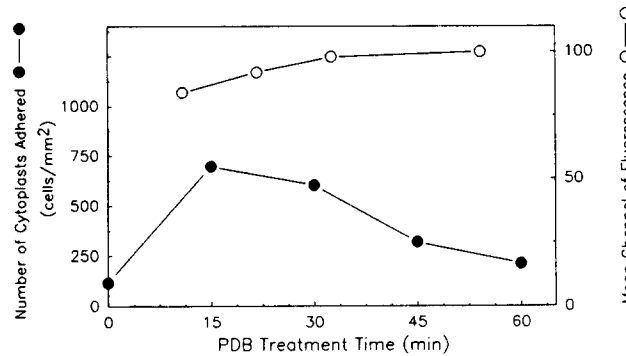


FIGURE 9. Transient binding of cytoplasts to EC is not accompanied by changes in the expression of CD11b/CD18. Cytoplasts were treated with 300 ng/ml PDB for the indicated times at 37°C, washed three times with HAP buffer, and added to EC monolayers for the adhesion assay. Expression of CD11b/CD18 on cytoplasts was measured by labeling PDB-treated cytoplasts with FITC-OKM1 as described in Materials and Methods and analyzing the cells by flow cytometry. Results shown are representative of three separate experiments.

PMN to EC, we employed cytoplasts, which are depleted of the intracellular granules that act as storage pools of CD11b/CD18. Flow cytometry was used to measure the expression of CD11b/CD18 on the surface of cytoplasts treated with PDB, and no significant increase was observed at times up to 60 min (Fig. 9). Resting cytoplasts bound poorly to EC, but stimulation for 15 min with PDB caused a fourfold enhancement in binding. Further incubation with PDB caused binding to return to baseline levels by 60 min. Thus transient changes in the adhesion capacity of CD11/CD18 may occur in the absence of alteration in the surface expression of these molecules and in the absence of granule contents.

### Discussion

*Adhesion of PMN to EC Is Mediated by Two Members of the CD11/CD18 Complex.* Essentially all of the adhesion of stimulated PMN to unstimulated EC we observed in our assay was dependent on CD11/CD18 molecules on the PMN. Anti-CD18 mAbs blocked adhesion completely, and PMN from CD11/CD18 deficient patients did not adhere (Fig. 2). However, other mechanisms for adherence of PMN to EC do exist.

Several laboratories have shown that stimulation of EC for several hours with either LPS, IL-1, or TNF results in strong adherence of PMN that does not require CD11/CD18 expression (24, 28, 29). We have also observed CD11/CD18-independent adhesion on EC stimulated with TNF for 3 h (unpublished observations). These observations confirm that our EC are sensitive to stimulation and our assay can detect adhesions mediated by other mechanisms, but that these other mechanisms are not operative in our experiments. Our EC thus resemble naive, unstimulated EC, and our system best models the rapid (1–15 min) binding of PMN to EC observed after the introduction of an inflammatory stimulus into tissues *in vivo*. The rapid time course of action of the stimuli used (PMA, TNF, C5a) is consonant with this view.

The adherence of maximally stimulated PMN appears to use CD11a/CD18 and CD11b/CD18 to an approximately equal degree. Antibodies against CD11a and CD11b inhibited binding to a similar extent (66%) and a combination of anti-CD11a and anti-CD11b completely blocked binding (Fig. 3). CD11c/CD18, on the other hand, did not appear to contribute to the adhesion measured here. It is not clear whether this reflects an intrinsic inability of CD11c/CD18 to mediate adhesion to EC or if it reflects the source of endothelial cells. While CD11a/CD18 is known to recognize the molecule ICAM-1 (30, 31), the identity of the ligands on EC recognized by CD11b/CD18 is not known. It is also not known whether CD11b/CD18 or CD11c/CD18 can recognize ICAM-1.

*Relationship of Transient Adhesion to PMN Function In Vivo.* The ability of CD11/CD18 to mediate binding to EC was transiently stimulated by PMA, TNF, and C5a. Maximal binding in response to C5a occurred after 5 min of stimulation but receded to baseline levels after 15 min (Fig. 5). This response mirrors the rapid, transient neutropenia observed in animals injected intravenously with C5a (1, 2). A slower time course of adhesion was observed for PMN treated with PMA and TNF, with maximal binding after 15 min and a return to baseline adhesion by 60 min. These times correspond well with the transient neutropenia observed in rabbits injected intravenously with TNF (32).

Transient adhesion of PMN to EC may represent an important aspect of transendothelial migration of PMN *in vivo*. PMN extravasting in response to a spatial gradient of chemoattractant diapedese by forming new adhesion at the leading front while simultaneously breaking adhesions in the uropod region. The enhanced binding of PMN to EC observed in the response to a temporal gradient of stimulant may be related to the formation of new adhesions at the leading edge of a migrating PMN, while loss of adhesivity after longer times of incubation in stimulant may correspond to detachment of the uropod from the substrate. The adhesion of a migrating cell is of necessity transient, and we suggest that the CD11/CD18 molecules may mediate this transient adhesion.

Previous studies from this laboratory (9) showed that phorbol esters cause a transient change in the capacity of CD11b/CD18 to bind to C3bi-coated erythrocytes with a time course and magnitude identical with that shown here for adhesion to EC. These observations thus suggest that the binding site on CD11b/CD18 for EC is regulated in the same way as the binding site for C3bi. Since both CD11a/CD18 and CD11b/CD18 contribute to adhesion, these observations also suggest that not only CD11b/CD18 but also CD11a/CD18 is regulated in a transient fashion by phorbol esters.

*Mechanism of Induced CD18 Function.* Stimuli that enhance adhesion of PMN also

cause an increase in the expression of CD11b/CD18 molecules on the cell surface (9, 10, 11; Figs. 7 and 8). However, several observations suggest that this change in CD11b/CD18 expression cannot fully explain the changes observed in adhesivity. Phorbol esters caused no increase in CD11a/CD18 and only a three-fold increase in CD11b/CD18 expression, but adhesion to EC increased as much as 10-fold. More importantly, expression of CD11b/CD18 remained high during the subsequent drop in adhesivity (Figs. 4 and 7). Thus, an additional mechanism unrelated to changes in surface expression may be at work to control CD11b/CD18-dependent adhesion. The existence of such a mechanism is confirmed by observations on cytoplasts. These cell fragments did not alter expression of CD11b/CD18 molecules in response to stimulation with phorbol because they lacked intracellular pools of receptor (Tables I and II, Fig. 9), yet they showed a rise and fall in adhesivity to EC in response to phorbol esters with a time course similar to that of whole cells. Thus changes in expression of CD18 are not necessary for either the initial rise or subsequent fall in CD18 function. We conclude that qualitative changes in existing receptors appear to be necessary and sufficient to control CD18-dependent adhesion. Alteration in surface expression of CD11b/CD18 may contribute to changes in adhesivity but a clear role for this phenomenon has yet to be determined.

Other studies have explored the initial rise in CD18-dependent adhesivity of PMN caused by chemotactic stimuli. Changes in the number of CD18 molecules on the cell surface were found not to correlate with enhanced aggregation of PMN (33), enhanced binding of C3bi-coated particles (9), or enhanced binding to endothelium (34). Our results confirm these findings, and extend them by showing that CD18-dependent adhesion of PMN to endothelium is transient, and that the decline in binding is not dependent upon changes in the number of CD18 molecules on the cell surface.

What is the nature of the qualitative change(s) in CD11/CD18? Proteolysis of cell surface receptors appears unlikely to participate since immunoprecipitation experiments showed no evidence of proteolysis of CD11/CD18 accompanying loss of receptor activity (Lo, S. K., and S. D. Wright, unpublished observation), and PMN treated with C5a until they were no longer adherent were able to readhere when stimulated subsequently with PMA or TNF (Fig. 6). The action of lytic enzymes contained within granules is also unlikely to contribute since granule-free cytoplasts show normal transient binding behavior. An alternative explanation is suggested by the recent work of Detmers et al. (35), who found that CD11b/CD18 aggregates in the plane of the membrane in response to stimuli that enhance its binding capacity, and that aggregation is a prerequisite for binding. The time course of enhanced binding of PMN to EC (Fig. 4) and the CD11b/CD18-dependent binding of C3bi-coated erythrocytes to PMN (9). It is thus possible that stimulation of PMN leads to aggregation of CD11/CD18 molecules, and that the aggregates mediate adhesion to EC.

### Summary

Fluorescently labeled polymorphonuclear leukocytes (PMN) were used to measure adhesion to human umbilical vein endothelial cells (EC) cultured *in vitro*. Stimulation of PMN with phorbol dibutyrate (PDB), TNF, or C5a caused an increase in adhesion followed by a return to prestimulation levels of adhesion of longer times of incubation. Maximal adhesion of PMN to EC occurred rapidly in response to

C5a (5 min) and more slowly with TNF or PDB (15 min). PMN stimulated to adhere with C5a detached from EC by 15 min.

PMN from CD11/CD18-deficient patients and PMN incubated with anti-CD18 mAbs failed to bind to EC despite maximal stimulation. Anti-CD11a/CD18 and anti-CD11b/CD18 each partially inhibited adhesion, and a combination of these two reagents completely blocked adhesion. The adhesion we measured was therefore completely dependent on CD11/CD18, and CD11a/CD18 and CD11b/CD18 each contributed to adhesion. Stimuli that enhanced adhesion of PMN to EC also enhanced expression of CD11b/CD18 on the cell surface, but the time course of expression correlated poorly with changes in adhesivity. To determine if changes in the expression of CD11b/CD18 are necessary for the changes in adhesivity, we used enucleate cytoplasts that did not increase expression of CD11b/CD18. Cytoplasts showed a normal rise and fall in adhesivity in response to PDB. We conclude that the transient adhesion of stimulated PMN to naive EC is regulated by changes in the nature of existing CD11/CD18 molecules on the PMN surface. Changes in expression of CD11b/CD18 may contribute to enhancement of adhesivity, but a definite role for this phenomenon has yet to be established.

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