Increased Surface Expression of CD11b/CD18 (Mac-1) Is Not Required for Stimulated Neutrophil Adherence to Cultured Endothelium

Nicholas B. Vedder and John M. Harlan*
*Departments of Surgery and *Medicine, University of Washington, Seattle, Washington*

Abstract

The mechanism whereby the human neutrophil membrane heterodimer, CD11b/CD18 (Mac-1, Mo1), mediates neutrophil adherence is not known. We studied the role of CD11b/CD18 surface expression in the promotion of neutrophil adhesiveness. We found that phorbol myristate acetate (PMA), calcium ionophore (A23187), and FMLP caused a three- to sevenfold increase in surface expression of both CD11b (α_M) and CD18 (β) as assayed by binding of MAbs 60.1 (anti-CD11b) and 60.3 (anti-CD18). Increased binding of MAbs was temporally associated with the promotion of neutrophil aggregation and adherence to cultured endothelial monolayers. Pretreatment of neutrophils with the anion channel-blocking agent, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), inhibited the increased surface expression of CD11b and CD18 after stimulation by PMA, A23187, or FMLP and resulted in nearly complete inhibition of neutrophil aggregation. However, pretreatment with DIDS did not diminish either PMA-, A23187-, or FMLP-stimulated neutrophil adherence to endothelial monolayers. We also observed that stimulation of granule-depleted neutrophil cytoplasts by PMA, A23187, or FMLP induced aggregation and adherence to endothelial monolayers without increasing surface expression of CD11b or CD18.

We conclude that the increased surface expression of CD11b/CD18 that occurs after stimulation is neither sufficient nor necessary for enhanced adherence to endothelium. Moreover, though both are CD11b/CD18-dependent, the mechanisms involved in neutrophil aggregation are different from those involved in neutrophil adherence to endothelium.

Introduction

Recent studies have identified a human neutrophil membrane glycoprotein complex required for numerous adhesion-dependent functions including aggregation, spreading on artificial substrates, chemotaxis, phagocytosis, and adherence to endothelium (1–12). This complex is composed of three heterodimeric subunits: LFA-1, Mac-1 (Mo1), and p150,95. Each subunit consists of a distinct heavy or α-chain polypeptide noncovalently linked to a light or β-chain polypeptide common to all three subunits (13, 14). The heavy chain polypeptide of the LFA-1 subunit (α_L) has been designated CD11a, that of the Mac-1 subunit (α_M) has been designated CD11b, and that of the p150,95 subunit (α_X) has been designated CD11c by the Third International Workshop on Leukocyte Differentiation Antigens (15). The common β-chain polypeptide has been designated CD18 (15).

Patients with a congenital deficiency of CD11/CD18 suffer from recurrent bacterial infections and an inability to suppurate (reviewed in reference 16). These patients' neutrophils demonstrate in vitro defects in adhesion-dependent functions that can be reproduced in normal neutrophils by the addition of MAbs directed to function-related epitopes of the glycoprotein complex (1–7). The CD11b/CD18 subunit has been shown to play an important role in neutrophil adherence to vascular endothelium, a critical early event in neutrophil emigration during the acute inflammatory response. The MAbs anti-CD11b (60.1) and anti-CD18 (60.3), recognizing respectively the CD11b and CD18 polypeptides (2), have been shown in vitro to prevent stimulated neutrophil aggregation and adherence to endothelial monolayers (1, 3, 5).

The mechanism by which CD11b/CD18 augments neutrophil adhesiveness in response to stimulation is not known. It has been shown that in unstimulated neutrophils, the CD11b polypeptide exists both on the cell surface and in far greater quantities within the secondary and/or tertiary granules. After stimulation, contents of these granules are translocated to the cell surface, resulting in a 3- to 10-fold increase in surface-associated CD11b (assayed as increased binding of CD11b-specific MAb) (12, 17–19). It has been suggested that this increase in surface expression may play an important role in the mechanism of enhanced neutrophil adhesiveness (12, 17–20). This hypothesis has not been critically examined, however.

To investigate the role of increased CD11b/CD18 surface expression in stimulated neutrophil adherence we employed the anion channel-blocking agent, DIDS[1] (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), which blocks neutrophil degranulation (21–24). We show that pretreatment of neutrophils with DIDS effectively blocks the increased surface expression of CD11b/CD18 in response to phorbol myristate acetate (PMA), A23187, or FMLP. Neutrophil aggregation after stimulation by these agents is also inhibited by pretreatment with DIDS. However, stimulated neutrophil adherence to endothelium is not inhibited but in fact is potentiated when increased surface expression of CD11b/CD18 is blocked by DIDS.

We also show that neutrophil cytoplasts, devoid of cytoplasmic granules, can be stimulated by PMA, A23187, or FMLP to increase their adherence to endothelium despite

Address all correspondence to Dr. John M. Harlan, Division of Hematology, ZA-34, Harborview Medical Center, 325 Ninth Ave., Seattle, WA 98104.

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1. *Abbreviations used in this paper:* BEC, bovine aortic endothelial cells; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; HBSS−, Hanks' balanced salt solution without calcium or magnesium; HEC, human umbilical vein endothelial cells; MPO, myeloperoxidase.

being incapable of increasing surface expression of CD11b or CD18.

These findings indicate that the increase in surface expression of CD11b and CD18 that occurs with neutrophil stimulation is neither sufficient nor necessary for enhanced adherence to endothelium.

Methods

Cell culture. Human umbilical vein endothelial cells (HEC) and bovine aortic endothelial cells (BEC) were prepared by collagenase treatment of vessels as described (25, 26). All cell lines were maintained in endotoxin-free RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 20% FCS with heparin (90 mg/ml) and endothelial cell growth factor as described by Thornton et al. (27). Endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. (28). HEC and BEC were harvested with 0.05% trypsin and 0.02% EDTA in HBSS (Gibco Laboratories, Gibco Div., Chagrin Falls, OH) without calcium or magnesium (HBSS−). The cells were then plated in 11-mm-diam wells in 48-well plates (Cluster 3548; Costar, Data Packaging Corp., Cambridge, MA) at 5×10^4 cells/well in RPMI 1640 with 20% FCS. Visually confluent monolayers were formed after overnight incubation.

Neutrophil isolation and labeling. Peripheral blood from healthy donors was obtained by venipuncture and collected in syringes containing heparin, 10 U/ml. Neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation followed by 3% dextran sedimentation and hypotonic saline lysis of erythrocytes (29). The resulting cells were > 95% neutrophils and > 95% viable by trypan blue exclusion. The cells were then suspended in HBSS− for use in aggregation experiments and for analysis by immunofluorescence flow cytometry. Cells for adherence experiments were suspended in PBS and labeled with ^{51}Cr as sodium chromate (New England Nuclear, Boston, MA) 1 μCi/10^6 cells for 60 min at 37°C (30). After labeling, the cells were washed three times in PBS. Immunofluorescence flow cytometry, aggregation, and adherence experiments were all performed simultaneously on neutrophils from the same preparation.

Preparation of neutrophil cytoplasts. Neutrophil cytoplasts were prepared according to Korchak et al. (31). Briefly, purified neutrophils were suspended in 12.5% Ficoll-70 in HBSS− with cytochalasin B, 5 μg/ml, and incubated at 37°C for 5 min. This suspension was then layered on a 37°C discontinuous Ficoll gradient (12.5; 16; 25% in HBSS− with 5 μg/ml cytochalasin B throughout) and centrifuged at 81,000 *g* for 30 min in a model L2-65B ultracentrifuge with an SW-28 swinging bucket rotor (Beckman Instruments, Palo Alto, CA). The cytoplast band was aspirated and washed three times in HBSS−. Cytoplasts were then used in the same immunofluorescence, aggregation, and adherence assays as normal neutrophils, using cytoplasts from the same preparation in all three assays simultaneously.

Immunofluorescence flow cytometry. Neutrophils were suspended in HBSS− (5×10^6 cells/ml) with or without DIDS (250 μM) at room temperature for 15 min. Then, 5×10^5 cells were placed into polypropylene tubes (12 × 75 mm; Falcon Labware, Becton, Dickinson and Co., Oxnard, CA). Cells were then stimulated with HBSS− (control), PMA (100 ng/ml final concentration), A23187 (10^{-5} M final concentration), or FMLP (10^{-5} M final concentration) for 30 min at 37°C in a shaking water bath. Unstimulated controls were placed directly on ice. Next, 50 μl of heat-inactivated adult bovine serum was added and the cells incubated at 4°C for 30 min. After this, a saturating concentration (40 μg/ml) of MAb or HBSS− (control) was added and the cells incubated for an additional 30 min at 4°C. After two washes in cold HBSS−, the cells were suspended in 25 μl of a saturating concentration of detecting antibody (FITC goat anti-mouse IgG; Tago Inc., Burlingame, CA) for 30 min at 4°C. After two final washes in cold HBSS−, the cells were fixed in 1% paraformaldehyde.

Analysis was performed using a fluorescence activated cell sorter (B-D FACS-440; Becton-Dickinson & Co., Sunnyvale, CA) with a 50-μm orifice. The results were displayed as single parameter histograms with the *x*-axis representing the $\log_{10}$ of the relative fluorescence intensity per cell and the *y*-axis representing the number of cells. Peak fluorescence for each histogram was also converted to a linear number for tabular display.

Neutrophil aggregation. Aggregometry was performed by the nephelometric technique of Craddock (32) using a model 330 platelet aggregometer (Chrono-Log Corp., Havertown, PA). Neutrophils were suspended in HBSS− at a concentration of 20×10^6/ml. The cells were treated with cytochalasin B (5 μg/ml) and either medium or DIDS (250 μM). After standing at room temperature for 15 min, 425-μl aliquots were transferred to silicone coated cuvettes and brought to 37°C. These were then placed in the aggregometer with a teflon stirring bar and stirred for 1 min after the addition of 25 μl of $CaCl_2/MgCl_2$ solution for a final concentration of 1 mM Ca^{++}, 0.5 mM Mg^{++}. Then, 50 μl of HBSS− (control), PMA (to final concentration 100 ng/ml), A23187 (to final concentration 10^{-5} M), or FMLP (to final concentration 10^{-5} M) was added and the change in light transmission was recorded over time. A 1:1 mixture of cells with HBSS− was used as a cell-poor calibration standard. The results were confirmed by examining neutrophil aggregation microscopically in 11-mm-diam wells using the same reagents, volumes, and concentrations.

Adherence assay. The purified ^{51}Cr-labeled neutrophils were suspended in endotoxin-free RPMI 1640 medium with 5% FCS at a concentration of 2×10^6 cells/ml (FCS prevented nonspecific detachment of endothelial cell monolayers from the tissue culture plastic). The cells were then treated with medium (control) or DIDS (250 μM) at room temperature for 15 min. The 48-well HEC or BEC plate was decanted and fresh medium was added: RPMI 1640 with 5% FCS with or without 250 μM DIDS. Then, 200 μl of the neutrophil suspension was added to each well followed immediately by 50 μl of medium (control), PMA (final concentration 100 ng/ml), A23187 (final concentration 10^{-5} M), or FMLP (final concentration 10^{-5} M). Plates were incubated for 30 min at 37°C in a 5% CO_2 incubator. Nonadherent neutrophils were removed with two well volume exchanges of PBS with 5% FCS. Adherent neutrophils were lysed with 1 N NH_4OH. The lysates were then counted in a gamma spectrophotometer (Micromedic ME Plus, Micromedic Systems Inc., Horsham, PA). Neutrophil adherence was calculated for each well and expressed as the percentage of the ^{51}Cr counts that remained adhered to the endothelial monolayer: % adherence = (^{51}Cr cpm in lysate)/(total ^{51}Cr cpm added) × 100.

Total ^{51}Cr cpm added was determined by counting 200-μl samples of the neutrophil suspension. The results were confirmed qualitatively by microscopic analysis. In experiments using unlabeled cells, results were quantified by counting adherent neutrophils in photomicrographs and by assaying for myeloperoxidase (MPO) activity as described by Lundquist and Josefsson (33).

MAb. Murine MAb, 60.1, is an IgG_1 antibody that recognizes a functional epitope on the CD11b polypeptide (2). Murine MAb, 60.3, is an IgG_{2a} antibody that recognizes a functional epitope on CD18 (2, 4). MAb, 60.5, is an IgG_{2a} antibody that recognizes an HLA class I framework antigen present on all peripheral leukocytes and human endothelial cells (4). MAbs 60.3 and 60.5 were purified on staphylococcal protein A columns and MAb 60.1 was purified by ammonium sulfate precipitation. MAbs 60.1, 60.3, and 60.5 were gifts of Dr. Patrick Beatty, Puget Sound Blood Center, Seattle, WA.

Reagents. DIDS, PMA, FMLP, heparin, dextran, Ficoll-70, and cytochalasin B were obtained from Sigma Chemical Co., St. Louis, MO. A23187 was obtained from Calbiochem-Behring Diagnostics, American Hoechst Corp., San Diego, CA. Ficoll-Hypaque was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ. FCS and adult bovine serum were obtained from HyClone Laboratories, Sterile Systems Inc., Logan, UT.

Statistics. Results were expressed as mean±SE and significance was determined by unpaired *t* test.

Results

DIDS blocks increased surface expression of CD11b/CD18 (Mac-1) in stimulated neutrophils. Stimulation of neutrophils with PMA, A23187, or FMLP resulted in a three- to sevenfold increase in the binding of MAb 60.1 (directed to the CD11b polypeptide) compared with unstimulated controls, as assayed by immunofluorescence flow cytometry. This increase was blocked to below control level by pretreatment of the cells with DIDS (Fig. 1 and Table I). PMA, A23187, or FMLP similarly increased binding of MAb 60.3 (directed to CD18) by three- to six-fold over unstimulated controls. Again, DIDS inhibited this increase by 92% for PMA, 76% for A23187, and by 88% for FMLP (Table I). DIDS alone had no effect on binding of MAbs 60.1 or 60.3 to unstimulated neutrophils (Table I).

The same results were obtained when stimulation and staining were performed in RPMI with 5% FCS. The presence of Mg^{++} in RPMI and in FCS, however, allowed neutrophils to aggregate when stimulated with PMA and resulted in flattened, though appropriately shifted, curves (data not shown). The effect of DIDS was reversible since washing the cells after treatment with DIDS allowed maximal binding of MAbs 60.1 and 60.3 after stimulation with A23187 to the level of non-DIDS-treated cells (data not shown). The binding of isotype-matched MAb 60.5, directed to a common HLA framework antigen, and present at a density similar to CD11b and CD18 in resting cells (4), remained constant with all treatments (Fig. 1).

DIDS blocks neutrophil aggregation. PMA, A23187, and FMLP all induced neutrophil aggregation as measured nephelometrically and by microscopic observation. Pretreatment of neutrophils with DIDS (250 μM) blocked aggregation in response to either PMA, A23187, or FMLP (Fig. 2 and Table I).

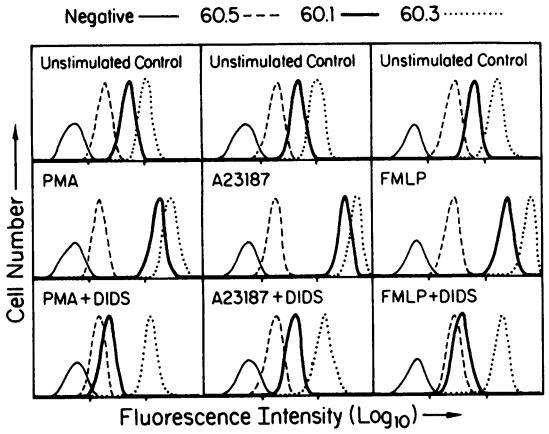

Figure 1. DIDS inhibits increased surface expression of CD11b and CD18. Neutrophils were pretreated with medium or medium containing DIDS (250 μM) then stimulated with HBSS− (control), PMA (100 ng/ml), A23187 (10^{-5} M), or FMLP (10^{-5} M). The surface binding of MAbs 60.1 and 60.3 were measured by indirect immunofluorescence and are compared with the binding of MAb 60.5 (anti-HLA class I framework antigen). The horizontal axis represents the amount of antibody binding per cell ($\log_{10}$) and the vertical axis represents the number of cells. The first curves (*Negative*) represent background nonspecific binding of detecting antibody to cells not exposed to any MAb. Similar results were obtained in five separate experiments.

Table I. Effect of DIDS on Surface Expression of CD11b/CD18, Aggregation, and Adherence to HEC

Stimulant	Fold increase in surface expression over unstimulated control		Aggregation ΔT over control	% Adherence to HEC
	CD11b	CD18		
Control	1.0±0.0*	1.0±0.0*	0±0*	2±0
Control + DIDS	1.0±0.2	1.1±0.1	0±0	5±1§
PMA (100 ng/ml)	4.6±0.9	3.5±0.5	70±3	41±2
PMA + DIDS	0.4±0.1§	1.2±0.1§	7±1‖	49±3‡
A23187 (10^{-5} M)	6.6±1.1	6.4±0.6	64±4	22±2
A23187 + DIDS	0.8±0.1§	2.2±0.1§	5±2‖	32±2‖
FMLP (10^{-5} M)	3.2±0.4	3.4±0.4	64±5	10±3
FMLP + DIDS	0.5±0.3§	1.3±0.1§	4±2‖	25±2‖

DIDS (250 μM) blocks the stimulated increase in surface expression of CD11b and CD18 and blocks neutrophil aggregation, yet potentiates stimulated neutrophil adherence to HEC. Increase in surface expression was calculated from histograms as in Fig. 1 by converting mean fluorescence for each curve to a linear format and expressing the result relative to unstimulated controls. Aggregation was calculated from tracings as in Fig. 2 using an arbitrary linear scale to express peak aggregation responses (ΔT) relative to unstimulated controls. Results above represent means±SE of five separate experiments for PMA and A23187; three separate experiments for FMLP.

* By definition.

‡ $P < 0.05$.

§ $P < 0.005$.

‖ $P < 0.0005$ (compared with stimulant without DIDS).

When aggregation was performed in RPMI with FCS, the inhibition effect of DIDS was still observed (data not shown). Non-cytochalasin B-treated cells demonstrated an attenuated aggregation response that was also blocked by DIDS (data not shown).

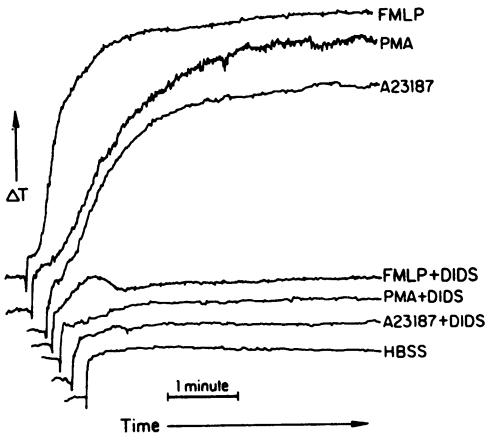

Figure 2. DIDS inhibits neutrophil aggregation induced by PMA, A23187, or FMLP. Aggregation was recorded as an increase in light transmission (ΔT) over time after addition of HBSS− (control), PMA (100 ng/ml), A23187 (10^{-5} M), or FMLP (10^{-5} M) to neutrophils suspended in HBSS or HBSS containing DIDS (250 μM). Addition of reagent is indicated by the initial small upward deflection representing the dilution artifact. Similar results were obtained in five separate experiments.

DIDS does not block neutrophil adherence to endothelium. Results of adherence experiments with HEC are shown in Table I. Unstimulated neutrophils demonstrated minimal adherence to HEC monolayers. PMA, A23187, and FMLP markedly increased neutrophil adherence to HEC. Pretreatment of neutrophils with DIDS (250 μM) did not diminish this stimulated adherence, but in fact potentiated adherence.

Similar results were obtained with BEC: in five experiments, unstimulated adherence was 3±0% without DIDS and 6±1% with DIDS; PMA-stimulated adherence in non–DIDS-treated neutrophils was 64±3 vs. 72±3% in DIDS-treated neutrophils ($P < 0.05$); A23187-stimulated adherence in non–DIDS-treated neutrophils was 26±2 vs. 50±4% in DIDS-treated neutrophils ($P < 0.0005$).

To control for the possibility that DIDS might be exerting a proadhesive effect via the endothelial cell, we examined neutrophil adherence to albumin-coated tissue culture plastic. These results were similar to those observed with BEC or HEC: in three experiments, unstimulated adherence was 4±1% without DIDS and 12±2% with DIDS; PMA-stimulated adherence in non–DIDS-treated neutrophils was 56±2% vs. 64±3% in DIDS-treated neutrophils ($P < 0.05$); A23187-stimulated adherence in non–DIDS-treated neutrophils was 44±4% vs. 53±4% in DIDS-treated neutrophils ($P = 0.13$); and FMLP-stimulated adherence in non–DIDS-treated neutrophils was 27±3% vs. 38±3% in DIDS-treated neutrophils ($P < 0.05$).

To demonstrate that adherence in the presence of DIDS was still occurring through a CD11b/CD18-dependent mechanism, we examined the effect of the MAb, 60.3, on adherence. Addition of MAb 60.3 (40 μg/ml) blocked both PMA- and A23187-stimulated neutrophil adherence with or without DIDS to below the level of unstimulated controls (for example: PMA + DIDS resulted in only 1% adherence to BEC in the presence of MAb 60.3, compared with 55±5% without MAb 60.3). Similar results were obtained with the MAb, 60.1. Addition of MAb 60.1 (40 μg/ml) blocked PMA-, A23187-, and FMLP-stimulated neutrophil adherence with or without DIDS to below the level of unstimulated controls (for example: PMA + DIDS resulted in only 1% adherence to BEC in the presence of MAb 60.1, compared with 55±5% without MAb 60.1).

To control for possible unaccounted translocation of CD11b or CD18 during ^{51}Cr labeling at 37°C (34) we examined adherence to BEC using neutrophils immediately after their isolation. Quantitative evaluation by microscopic inspection (counting adherent neutrophils in photomicrographs) confirmed the data obtained with ^{51}Cr-labeled cells. With non–DIDS-treated neutrophils PMA induced an 8.0-fold increase in adherence relative to unstimulated controls vs. a 10.0-fold increase with DIDS-treated cells. Similarly, A23187 induced a 4.7-fold increase in adherence relative to unstimulated controls with non–DIDS-treated neutrophils vs. a 4.9-fold increase with DIDS-treated cells. In another experiment, adherent neutrophils were quantified by assaying for MPO activity. PMA induced a 3.5±0.1-fold increase in MPO activity relative to unstimulated controls with non–DIDS-treated neutrophils vs. a 4.9±0.4-fold increase with DIDS-treated cells ($P < 0.005$). Similarly, A23187 induced a 3.1±0.1-fold increase in MPO activity relative to unstimulated controls with non–DIDS-treated neutrophils vs. a 4.2±0.2-fold increase with DIDS-treated cells ($P < 0.0005$).

Stimulated neutrophil cytoplasts aggregate and adhere to endothelium without increasing surface expression of CD11b or CD18. To further examine the role of CD11b/CD18 surface expression in neutrophil aggregation and adherence to endothelium, we prepared neutrophil cytoplasts that are devoid of cytoplasmic granules (31) and therefore incapable of increasing surface expression of CD11b (35, 36) or CD18. We found that unstimulated cytoplasts, like neutrophils, adhered minimally to HEC monolayers. However, with stimulation by either PMA, A23187, or FMLP, adherence was markedly increased (Table II). As with neutrophils, this increased adherence was blocked by coincubation with MAb 60.3 (Table II). We also observed that cytoplasts aggregated in response to

Table II. Effect of Stimulation on Neutrophil Cytoplast Surface Expression of CD11b/CD18, Adherence to HEC, and Aggregation

		Fold increase in surface expression over unstimulated control		% Adherence to HEC		Aggregation ΔT over control	
Stimulant	Experiment	CD11b	CD18	(−)MAb 60.3	(+)MAb 60.3	(−)MAb 60.3	(+)MAb 60.3
Control	1	0.0*	0.0*	7±1	5±0	0*	
	2	0.0*	0.0*	9±1	4±0	0*	
PMA (100 ng/ml)	1	0.8	1.0	17±2§	7±1	64	5
	2	1.0	0.8	22±6‡	7±2	75	17
A23187 (10^{-5} M)	1	1.4	1.0	14±1§	3±0		
	2	1.0	1.0	14±3‡	0±0		
FMLP (10^{-5} M)	1	1.3	1.1	12±1§	5±0		
	2	1.0	1.1	22±3§	15±2		

Stimulated neutrophil cytoplasts aggregate and adhere to endothelium but do not increase surface expression of CD11b or CD18. Stimulated cytoplast aggregation and adherence to endothelium are blocked by coincubation with MAb 60.3 (40 μg/ml). Surface expression was calculated as in Table I. Percent adherence is expressed as mean±SE of eight replicate wells (four replicate wells with MAb 60.3). Results of two separate experiments are shown. All assays in each experiment were performed with cytoplasts from the same preparation. * By definition.
‡ $P < 0.05$. § $P < 0.005$ (compared with unstimulated control).

PMA stimulation, as measured nephelometrically (Table II) and by microscopic examination. This aggregation response was also blocked by MAb 60.3 (Table II). Cytoplasts from the same preparation that aggregated and adhered to HEC, however, did not increase surface expression of CD11b or CD18 in response to the same stimuli under the same conditions (Table II).

Discussion

The central role of the CD11b/CD18 membrane glycoprotein subunit in adhesion-dependent neutrophil functions is well documented (1–12). MAbs directed against components of the subunit have been shown in vitro to interfere with neutrophil aggregation, spreading on plastic, antibody-dependent cellular cytotoxicity, chemotaxis, phagocytosis, and adherence to endothelium (1–7). It has been shown that cell surface binding of MAbs directed to CD11b or CD18 markedly increases in response to neutrophil stimulation (3, 12, 17, 18, 19). This is associated with a concomitant decrease in intracellular granule-associated CD11b, indicating that translocation from intracellular pools to the cell surface occurs in response to stimulation. It has therefore been suggested that increased surface expression of CD11b might be a mechanism that enhances surface adhesiveness (12, 17, 18, 19). Neutrophils from patients undergoing hemodialysis show this same increase in surface CD11b and it has been suggested that this may result in leukoaggregation and pulmonary sequestration of granulocytes, thereby accounting for the neutropenia seen in hemodialysis (20).

We found that treatment of neutrophils with the anion channel-blocking agent, DIDS, which is known to inhibit fusion of cytoplasmic granules with the plasma membrane (21–24), effectively blocked increased surface expression of CD11b and CD18 in response to stimulation. This suggests that translocation of CD11b and CD18 from the intracellular pools to the cell surface was similarly blocked. In fact, the amount of surface expression of the CD11b polypeptide (as assayed by MAb 60.1 binding) after stimulation at 37°C was below the level of unstimulated controls. This might be due to an accelerated turnover of receptor in which its replacement from the intracellular granule pool is blocked. The finding that CD18 expression (as assayed by MAb 60.3 binding) did not always parallel CD11b expression may be explained by the association of CD18 with not only CD11b (Mac-1), but with CD11a (LFA-1) and CD11c (p150,95) as well, whose expression may be regulated by different mechanisms. The effect of DIDS on granule release appears to be relatively specific as DIDS is known not to interfere with other neutrophil functions including superoxide generation and non–complement-mediated phagocytosis (21, 22, 24).

Our results show that when translocation of CD11b and CD18 to the surface of stimulated neutrophils is blocked by DIDS, neutrophil adherence to endothelium is not diminished but is in fact potentiated. This implies that quantitative surface recruitment of CD11b and CD18 from cytoplasmic granules is not the mechanism of enhanced neutrophil adherence to endothelium. The conclusion that increased surface expression of CD11b/CD18 is not causally related to increased adhesiveness is supported by the recent observations that granulocyte-monocyte colony stimulating factor increases the surface expression of CD11b (19, 37) but does not promote adherence to endothelium (37). Further support comes from our observations that neutrophil cytoplasts display increased adherence to endothelium when stimulated, which confirms the observations of Stroncek et al. (38), despite the fact that they do not increase surface expression of CD11b or CD18, which in turn confirms the observations of O'Shea et al. (35) and Petrequin et al. (36). The observation that stimulated cytoplasts adhered less avidly to endothelium compared with intact neutrophils is not surprising since the preparation is much longer and requires many additional steps. Diminished adherence of stimulated cytoplasts compared with intact neutrophils was also observed by Stroncek et al. (38).

These data all indicate that the quantitative increase in surface expression of CD11b and CD18 that follows stimulation is neither sufficient nor necessary to increase neutrophil adherence to endothelium. This suggests the possibility that increased neutrophil adherence after stimulation may be a result of some qualitative alteration of the glycoprotein on the cell surface, such as a conformational change due to phosphorylation, as has been shown to occur with CD18 in peripheral blood mononuclear leukocytes (39). The effect of DIDS in potentiating adherence to endothelium is similar to its observed potentiation of stimulated superoxide generation (21). The mechanism of this potentiation is not clear. In the case of adherence to endothelium, our results indicate that this effect is mediated through CD11b/CD18, yet is not a consequence of increased surface expression of CD11b/CD18, again suggesting the possibility of some qualitative "activation" of the surface glycoprotein(s). Such a qualitative change has been shown to occur with glycoprotein IIb/IIIa in platelets (40) and recent data suggest that IIb/IIIa and the CD11/CD18 complex are part of the same "family" of adherence-mediating cell surface glycoproteins (41).

In contrast to its effect on neutrophil adherence to endothelium, we observed that DIDS inhibited aggregation of stimulated neutrophils. One interpretation of this observation is that recruitment of CD11b and CD18 from an intracellular pool plays an important role in the regulation of neutrophil–neutrophil adhesiveness. However, we found, as others have reported, that neutrophil cytoplasts aggregate when stimulated (31), despite the fact that they do not increase surface expression of CD11b (35, 36). These observations and the recent studies of Philips et al. (42) suggest that, as with adherence to endothelium, increased surface expression of CD11b is not the mechanism responsible for aggregation. It is possible that the inhibition of aggregation by DIDS is due to its effects on cell function apart from granule secretion (e.g., ion fluxes, etc.).

The finding that DIDS prevents aggregation but not adherence to endothelium supports Dahinden and Fehr's conclusion that aggregation and adherence are separate phenomena (43). This is further supported by the recent observations that granulocyte-monocyte colony stimulating factor stimulates neutrophil aggregation (20), but does not stimulate adherence to endothelial monolayers (37), whereas tumor necrosis factor-α (TNF-α) and lipopolysaccharide enhance neutrophil adherence to endothelium (44) but do not promote neutrophil aggregation (Harlan, J., unpublished observation and reference 43).

The relative importance of the in vitro phenomena of aggregation and adherence as indicators of in vivo pathophysiol-

ogy is not clear. The recent in vivo demonstration, using intravital microscopy, that blocking chemotaxin-induced neutrophil adherence to microvascular endothelium with MAb 60.3 also prevents neutrophil accumulation and associated plasma leakage (45) indicates that adherence to endothelium is a critical step in the process leading to neutrophil-mediated inflammation. Elucidation of the mechanisms that govern neutrophil adherence to endothelium, therefore, may be crucial to our ability to study and to alter therapeutically neutrophil-mediated inflammation and tissue injury.

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