Impaired Transendothelial Migration by Neonatal Neutrophils: Abnormalities of Mac-1 (CD11b/CD18)-Dependent Adherence Reactions

By Donald C. Anderson, Robert Rothlein, Steven D. Marlin, Sharon S. Krater, and C. Wayne Smith

In order to evaluate the functions of lymphocyte function antigen-1 (LFA-1) (CD11a/CD18) and Mac-1 (CD11b/CD18) on neonatal neutrophils, we examined neutrophil adhesion to and migration through human umbilical vein endothelial cell (HUVEC) monolayers in vitro. Transendothelial migration of adult neutrophils was greatly enhanced by preincubation of HUVEC with interleukin-1 (IL-1). This migration was significantly inhibited by monoclonal antibodies (MoAbs) against LFA-1 (CD11a) and Mac-1 (CD11b) subunits. Migration of neonatal neutrophils was markedly diminished compared to adult neutrophils, and MoAbs against LFA-1 further reduced migration. In contrast, anti-Mac-1 MoAb was not inhibitory. Adhesion of adult neutrophils was significantly enhanced by preincubation of HUVEC with IL-1, and was significantly inhibited by MoAbs against LFA-1. Adhesion of neonatal neutrophils was near adult levels and comparably inhibited by anti-LFA-1 MoAb. In addition, adhesion of neonatal and adult neutrophils to purified ICAM-1 in artificial planar membranes was comparable and almost completely inhibited by anti-LFA-1 MoAb. Chemotactic stimulation induced Mac-1-dependent adhesion of adult neutrophils to endothelial cells, purified intercellular adherence molecule-1 (ICAM-1) and protein-coated glass. In marked contrast, adhesion of neonatal neutrophils to these substrates was not significantly increased by chemotactic stimulation. These findings indicate that diminished transendothelial migration by neonatal neutrophils is related to abnormal interactions of Mac-1 with ICAM-1 and possibly other endothelial ligands. These functional deficits may contribute to impaired inflammation and infectious susceptibility in human neonates.

© 1990 by The American Society of Hematology.

THE SUSCEPTIBILITY of human neonates to soft tissue and systemic infections has been attributed to developmental delays in both specific and nonspecific defense systems. Among these infections, well-documented abnormalities in some neutrophil chemotactic functions in vitro have been proposed to underlie inflammatory deficits in vivo. Studies in neonatal animals have demonstrated diminished or delayed emigration of blood neutrophils to extravascular inflammatory sites. Schuit observed markedly diminished peritoneal exudates in neonatal as compared with adult rats inoculated with chemotactic reagents. Martin et al. observed impaired pulmonary clearance of group B Streptococcus, Staphylococcus aureus, and Pseudomonas aeruginosa in association with diminished neutrophil accumulation in experimentally-infected lungs of neonatal rodents. Impaired and age-dependent migration of neutrophils into the lungs of neonatal primates in response to bronchial alveolar lavage has also been reported. These experimental observations suggest that similar abnormalities may exist and underlie deficits of inflammatory host defenses in human neonates.

Several lines of experimental evidence indicate that inflammatory reactions involve specific interactions of circulating leukocytes with adhesion molecules on the luminal surface of vascular endothelial cells. The localization and extravascular emigration of neutrophils may require the recognition of at least two cytokine-responsive endothelial molecules, including the intercellular adherence molecule-1 (ICAM-1) and the endothelial adherence molecule-1 (ELAM-1). Receptors for ICAM-1 on neutrophil surfaces include the lymphocyte function antigen-1 (LFA-1) or CD11a/CD18 and Mac-1 (CD11b/CD18) heterodimers of the 82 or leukocyte integrin family (termed CD11/CD18). A major requirement for CD11/CD18-adherence proteins in acute inflammatory emigration has been convincingly shown in patients genetically deficient in this glycoprotein complex. Patients with this autosomal recessive trait (termed leukocyte adhesion deficiency or LAD) are susceptible to recurrent, necrotic, and often fatal soft tissue infections, primarily as a result of impaired extravascular infiltration of blood neutrophils in inflamed tissues. Monoclonal antibodies (MoAb) raised to CD11b/CD18 subunits or to ICAM-1 elicit profound anti-inflammatory effects when systemically administered in animal models of acute inflammation. Moreover, LAD neutrophils fail to undergo transendothelial migration in vitro, and anti-ICAM-1 or anti-CD11/CD18 MoAb impede neutrophil adhesion to and migration through monolayers of human umbilical vein endothelial cells (HUVEC) or microvascular endothelium.

Previous studies to define the molecular basis for impaired chemotactic functions of neonatal neutrophils have identified quantitative and functional abnormalities of Mac-1 (CD11b/CD18). Chemotactically stimulated neonatal neutrophils do not normally increase the surface expression of this determinant and fail to demonstrate Mac-1-dependent hyperadherence to protein-coated artificial substrates. Enhanced surface expression of Mac-1, as well as qualitative activation of this determinant by chemotactic factors, may both contrib-

From the Departments of Pediatrics and Cell Biology, Baylor College of Medicine, and the Speros P. Martel Leukocyte Biology Laboratory, Texas Children's Hospital, Houston; and the Department of Immunology, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT.

Address reprint requests to Donald C. Anderson, MD, Texas Children's Hospital Clinical Care Center, Leukocyte Biology Laboratory, 8080 N Stadium Dr, Suite 2100, Houston, TX 77054.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.
ute to the sharp increase in the adherence of chemotactically stimulated neutrophils to HUVEC in vitro.11,12,14 Evidence for functional activation of Mac-1 has been recently reported by Smith et al.,14 who showed that Mac-1 on chemotactically stimulated but not unstimulated neutrophils binds to purified ICAM-1; this qualitative alteration appears to facilitate cooperative interactions of Mac-1 and LFA-1 with ICAM-1 in the process of transendothelial migration in vitro. Thus, the present studies were designed to evaluate the hypothesis that abnormalities in Mac-1 will impair the interactions of neonatal neutrophils with endothelial cells. We describe here significantly reduced transendothelial migration by neonatal neutrophils. This result appears to reflect functional deficits of Mac-1 dependent cell adhesion because the functions of LFA-1 on these cells are comparable with those of adult cells. These studies provide further evidence that abnormalities of Mac-1 may contribute to the increased susceptibility of human neonates to bacterial infections.

MATERIALS AND METHODS

Blood samples. Neonates selected for study were products of a full-term uncomplicated pregnancy, labor, and delivery during which no general anesthesia or intravenous analgesia was administered. Their Agar scores at 1 or 5 minutes were ≥8, and their physical examinations at 48 hours were normal. Venous blood was drawn from the placentas of neonates immediately after birth; sedimentation of these samples in dextran was immediately initiated in the delivery suite. Alternatively, blood samples were obtained by phlebotomy from peripheral veins of neonates over 48 hours old, healthy adults, or from patients with LAD or their heterozygote parents.11 Informed consent was obtained from parents or LAD patients studied, and from adult donors.

Isolation of neutrophils. Blood samples were anticoagulated with citrate phosphate dextrose (Abbott, Chicago, IL) (0.14 mL/mL blood) and sedimented in 1% (wt/vol in 0.87% NaCl) Dextran Leukocyte-rich plasma was layered on Ficoll-Hypaque gradients and neutrophils were recovered, washed, and resuspended in ice-cold Dulbecco's Phosphate Buffered Saline (PBS; GIBCO Laboratories, Grand Island, NY) pH 7.4, containing 0.2% dextrose.26 Final leukocyte suspensions contained greater than 90% neutrophils and were maintained at 4°C before use in adhesion assays or MoAb binding studies.

MoAbs. MoAbs used in these studies included preparations of immunoglobulin G (IgG) and F(ab')2 fragments. Those MoAbs specific for CD11b included TS1/18 (IgG1), from Dr T. Springer, Harvard,11 and R15.7 (IgG1).35 The anti-CD11b MoAb 904 (IgG1) was obtained from Coulter, Inc (Hialeah, FL). An anti-CD11a MoAb, R3.1 (IgG1), was developed as previously described.14 R6.5 (IgG2a) was developed by fusing spleen cells from mice immunized with a battery of ICAM-1 bearing cell lines as described.11,12 The anti-HLA framework MoAb W6/32 (IgG2a)35 and the anti-neutrophil MoAb 4A5 (IgG1) were used as controls in adhesion/migration assays; each of these MoAbs has been shown previously to elicit no inhibitory effects in these assays.14 All MoAbs binding to neutrophils were titrated using flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) to determine the concentration that saturated surface binding sites for cells stimulated with f-Met-Leu-Phe (10 nmol/L, 37°C, 15 minutes) were maintained at 4°C before use in adherence assays or MoAb binding studies.

Preparation of endothelial cell monolayers, ICAM-1, and keyhole limpet hemocyanin (KLH) substrates. HUVEC were harvested and characterized as to acLDL binding and factor VIII expression according to established techniques. Cells from 5 to 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 to 4 days at 37°C, 5% CO₂ humidified atmosphere. Visual confluent monolayers on gelatin (0.1%) and fibronectin (5 μg/cm²)-coated 25-mm round glass coverslips were prepared and first-passage cells harvested with 0.05% trypsine and 0.02% EDTA in PBS. HUVEC were pretreated in selected experiments with interleukin-1 (IL-1) (Genzyme Corp, Boston, MA; cell derived) for varying lengths of time.13 ICAM-1 was purified from detergent lysates of SK-Hep-2 cells by immunoaffinity chromatography essentially as previously described14,17 using the R6.5 MoAb. Artificial lipid vesicles containing ICAM-1 or the control protein glycoporphin (Sigma Chemical Co, St Louis, MO) were prepared as previously described.14 Glass coverslips were prepared by placing 20 μL of vesicle suspension in the center of a 25-mm round coverslip and incubating at 37°C for 45 minutes.14 Coverslips spotted with lipid vesicles containing ICAM-1 were washed in PBS and mounted in adherence chambers before performing neutrophil adhesion studies. KLH substrates were prepared by spotting glass coverslips with KLH (Sigma) (0.5 mg/mL, 20 minutes at 37°C) and then washed twice in PBS before incorporation in adherence chambers.36

Adherence assays. A visual adherence assay previously described in detail11,12 was used employing artificial substrates or endothelial monolayers. Glass coverslips coated with KLH, ICAM-1 (prepared as above), or HUVEC monolayers (prepared as above) were incorporated into adherence chambers assembled with two metal plates designed to hold two 25-mm round coverslips separated by a Sykes-Moore chamber O-ring. Within this closed compartment, neutrophils were injected and observed to settle onto the protein coated glass or HUVEC monolayer for a period of 500 to 1,000 seconds at 21°C or 37°C. The number of cells in contact with protein-coated glass or endothelial monolayer was determined by counting at least 10 microscopic fields (50 × objective), and the chamber was inverted for an additional 500 seconds. The percentage of cells remaining in contact with the substrate was determined and expressed as percent adherence. In studies to define the contribution of selected neutrophil or endothelial determinants to adherence, cell suspensions were preincubated as follows: HUVEC monolayers were pretreated with MoAbs for 15 minutes at 37°C and then washed three times in PBS before incorporation in adherence chambers. Neutrophil suspensions were incubated for 5 to 10 minutes and then injected directly into chambers.

The percentage of cells migrating through endothelial monolayers was also determined using previously developed criteria.13 As observed by phase contrast optics, neutrophils adherent to the apical surface of the endothelium are round and refractile. Those cells undergoing transendothelial migration are easily distinguished by their flattened and nonrefractile appearance observed at a focal plane beneath the nuclei of the endothelial cells and between the cell monolayer and the glass surface.

Immunofluorescence flow cytometry. Indirect immunofluorescence of unstimulated or chemotactically stimulated intact neutrophils was performed by using subunit specific MoAbs directed at Mac-1α and fluorescein isothiocyanate (FITC)-conjugated antibody to mouse IgG. Surface-stained cells were fixed in 1% paraformaldehyde and analyzed in a Becton Dickinson FACScan as previously described.11 Linear specific fluorescence intensity (mean fluorescence channel of histogram) was calculated by subtraction of background fluorescence after labeling with IgG1 control antibody.
RESULTS

Comparative studies of transendothelial migration by neonatal or healthy adult neutrophils: assessments of the contributions of CD11/CD18 glycoproteins. As shown in Fig 1, adult neutrophils demonstrated the expected dose-dependent increase in transendothelial migration following incubation of HUVEC with IL-1 for 4 and 8 hours. Significantly elevated values were obtained when using IL-1 concentrations ≥0.05 U/mL for 4 hours, and peak values were evident at IL-1 concentrations of 1.0 U/mL. Preincubation of adult cells with anti-CD18 MoAb (TS1/18) decreased this level of migration by 77%. When assessed under the same conditions, migration by neonatal cells was significantly less than that of control cells for all IL-1 concentrations ≥0.1 U/mL (P < .01 for each). Peak migration (15% ± 6%) was observed when HUVEC were preincubated with an IL-1 concentration of 0.1 U/mL, while slightly lower values were evident when using higher concentrations. Anti-CD18 MoAb only decreased the percentage of migrating neonatal cells from 12% ± 4% to 9% ± 4% on the 4-hour IL-1-stimulated HUVEC monolayer, but almost totally inhibited migration through the HUVEC monolayers stimulated with IL-1 for 8 hours.

Our recent studies have defined cooperative interactions of Mac-1 and LFA-1 that facilitate neutrophil migration through endothelial cell monolayers prestimulated to elicit surface expression of ICAM-1. Thus, studies were performed to assess the importance of each of these integrins with respect to diminished migration by neonatal cells (Fig 2). HUVEC were preincubated with IL-1 (1.0 U/mL, 4 hours), and in contrast to the studies presented above, the assay was performed at 37°C to increase the percentage of migrating cells. Under these conditions, migration by neonatal cells was less than 50% of levels observed with adult neutrophils. To assess the relative contributions of LFA-1 and Mac-1, subunit specific MoAbs were present throughout the migration assay. As previously reported, anti-CD11b MoAb, when used alone, was substantially more inhibitory than anti-CD11b MoAb when employed alone, and inhibition by the combination of these MoAbs was significantly greater than with either MoAb alone. Migration by neonatal cells was significantly inhibited by anti-CD11a MoAb,
but anti-CD11b MoAb was without effect (Fig 2). Furthermore, the degree of inhibition observed when neonatal cells were preincubated with a combination of these MoAbs was not significantly different than that observed when incubated with anti-CD11a MoAb alone \((P = .4)\). In these studies, the control antibody, 4A5, did not influence the level of migration of neonatal or adult cells. These findings suggest abnormalities of Mac-1 but not LFA-1 underlie diminished migration by neonatal neutrophils.

**Comparative assessments of neonatal and adult neutrophil adherence to HUVEC.** The possibility that these findings reflected diminished neutrophil adhesion to one or more endothelial ligands was initially evaluated in IL-1 dose-response studies in which HUVEC monolayers were preincubated for 4 hours to maximally elicit CD18-dependent as well as CD18-independent endothelial determinants. As shown in Fig 3A, dose-dependent augmentation of neutrophil adherence was observed for both test groups, but significantly less adhesion of neonatal cells as compared with control cells was evident for IL-1 concentrations \(\geq 0.05 \text{ U/mL} \ (P < .001 \text{ for each})\). To determine if this diminished adherence was related to abnormalities of CD11/CD18 proteins, incubation-inhibition experiments were performed using anti-CD18 MoAb (TS1/18). As shown (Fig 3A), the net inhibitory effect of this MoAb was significantly less when preincubated with neonatal suspensions as compared with adult control cells (net inhibition of cells adhering; \(-13\% \pm 7\% \ [neonates], -49\% \pm 12\% \ [adult]; P < .001\)). Additionally, residual adherence of neonatal cells in the presence of MoAb (37\% \pm 6\%) was also diminished compared with adult control cells (51\% \pm 5\%), findings indicating deficits of CD18-independent adhesion.

Additional comparative adhesion protocols were performed using HUVEC substrates preincubated with varying concentrations of IL-1 for 8 hours, conditions more selective for CD18-dependent neutrophil adhesion (Fig 3B). Under these conditions, comparable dose-dependent augmentation of neutrophil adhesion was observed among a large number of neonatal and adult donors tested. While mean levels of adhesion by neonatal cells were somewhat less than control values at some concentration points, these differences were not statistically significant \((P \approx .05 \text{ for each})\). Moreover, comparable inhibition of adhesion by anti-CD18 MoAb was also observed (net inhibition of cells adhering; \(-36\% \pm 8\% \ [neonates], -47\% \pm 9\% \ [adult]; P > .05\)). These findings of normal adhesion are in striking contrast to findings of diminished transendothelial migration by neonatal neutrophils when studied under almost identical experimental conditions (see Fig 1B).

**Influence of chemotactic stimuli on neutrophil adhesion.** Augmentation of neutrophil adhesion to unstimulated HUVEC by chemotactic factors has been previously demonstrated to reflect quantitative upregulation and/or functional activation of Mac-1 on the neutrophil surface.\(^{14,19}\) To evaluate these relationships in neonatal neutrophils, two sets of comparative adhesion protocols were performed. In dose-response studies using f-Met-Leu-Phe (fMLP) and unstimulated endothelial substrates, neonatal neutrophils demonstrated profoundly diminished responses (Fig 4). Dose-dependent augmentation of adhesion was observed for control neutrophils over the entire fMLP concentration range and levels of adhesion elicited by 1 to 10 nmol/L fMLP exposure were approximately threefold those of unstimulated control cells. In contrast, stimulated adhesion of neonatal suspensions was significantly diminished compared with control over the entire concentration range \((P < .001 \text{ for each})\), and essentially no augmentation over baseline was elicited by concentrations of fMLP less than 5 nmol/L.

In a separate protocol, adhesion of fMLP-stimulated neonatal or control neutrophils was evaluated on HUVEC exposed to IL-1 (1.0 \text{ U/mL}) for 8 hours (Fig 5). This condition was chosen because it selects for CD18-dependent
IMPAIRED NEONATAL NEUTROPHIL MIGRATION

---

Fig 4. Effects of chemotactic stimulation on the adhesion of neonatal or adult neutrophils to unstimulated endothelial cells. Neutrophils were exposed to the concentration of fMLP indicated for 300 seconds at 37°C before contacting endothelial cell monolayers in adhesion chambers. Results shown represent the mean ± SD values of determinations on six neonates and six adult cell suspensions tested and are expressed as the percent of neutrophils adherent to the monolayers.

**Fig 5.** Comparative effects of anti-CD11a, CD11b, and CD18 MoAbs on adherence of neutrophils to IL-1-stimulated endothelial cells. Endothelial cell monolayers were preincubated with IL-1 for 8 hours at 37°C and washed before assay. Neonatal or adult neutrophils were incubated with 904 (anti-CD11b) (10 μg/mL), R3.1 (anti-CD11a) (10 μg/mL), or R15.7 (anti-CD18) (10 μg/mL) MoAb in the presence of fMLP (10 nmol/L) for 5 minutes before contacting the monolayer. Adherence was evaluated as described in Materials and Methods. Results shown represent mean ± SD determinations for studies of five neonatal (fine hatched bars) and four healthy adult (course hatched bars) cell suspensions; (*) P < .01 compared with unstimulated neonatal or adult suspensions; (**) P < .01 compared with stimulated adult neutrophils; (*** P < .01 compared with stimulated adult or neonatal suspensions containing anti-CD11b.

---

Endothelial ligands, and CD18-independent mechanisms are greatly reduced. As shown, fMLP failed to enhance the adherence of neonatal cells under these conditions. Mean group values for fMLP-stimulated adult neutrophils (81% ± 11%) were significantly greater than those for unstimulated adult (41% ± 8%) or neonatal (42% ± 7%) neutrophils, and significantly greater than mean determinations for fMLP-stimulated neonatal cells (44% ± 6%) (P < .001 for each). As previously reported, the increment of control cell adhesion elicited by fMLP was completely inhibited by anti-Mac-1 (CD11b) MoAb. In striking contrast, this MoAb failed to inhibit adhesion of neonatal neutrophils in the presence of fMLP. Anti-LFA-1 (CD11a) MoAb significantly inhibited neutrophil adhesion among both test groups, but in contrast to findings with adult neutrophils, this MoAb was as inhibitory as anti-CD18 MoAb when tested with neonatal suspensions. These observations indicate that chemotactic stimulation of neonatal neutrophils fails to elicit Mac-1 (CD11b/CD18)-dependent adhesion to endothelial cells, and they suggest that adherence interactions of neonatal cells under these experimental conditions are facilitated primarily through an LFA-1-dependent mechanism.

Adhesion of neutrophils to purified ICAM-1 substrates.

Results summarized in Fig 6 and Table 1 were performed to further evaluate the functional activity of Mac-1 when expressed on chemotactically stimulated adherent neonatal neutrophils. As shown in Fig 6, adherence of unstimulated neonatal neutrophils to artificial planar membranes containing ICAM-1 was comparable with that of healthy adult neutrophils. That LFA-1 is the principle determinant in this process was shown by the inhibitory effect of anti-LFA-1 MoAb and a lack of effect by anti-CD11b MoAb among both test groups. Chemotactic stimulation significantly enhanced adherence of adult control neutrophils but not neonatal neutrophils (see Fig 6 and Table 1); increments of adherence demonstrated by control cells exposed to fMLP were totally abrogated by anti-CD11b MoAb. This MoAb was not inhibitory when reacted with chemotactically stimulated neonatal neutrophils (Table 1). Furthermore, the combination of anti-CD11a and CD11b MoAb was significantly more inhibitory than when either of these MoAbs were reacted.
The inhibitory effect of CD1la was no greater than that of the combination of MoAbs. These data indicate that fMLP fails to promote CD18-dependent adhesion, ie, stimulation of the protein substrate.

For neonatal neutrophils, a process normally required for adherence to purified ICAM-1 substrates or endothelial cells expressing ICAM-1.

**Adhesion of neutrophils to KLH substrates.** Additional comparative assessments of fMLP-stimulated neutrophil adhesion were performed using glass coverslips coated with KLH. Neutrophil adhesion to this protein has been previously demonstrated to be Mac-1 but not LFA-1-dependent. As shown in Table 2, fMLP (10 nmol/L, 300 seconds, 37°C) significantly elevated the adhesion of healthy adult (but not neonatal) neutrophils to KLH; anti-CD11b and anti-CD18 MoAb abrogated the increment of adhesion elicited by fMLP. However, these MoAbs failed to significantly decrease adhesion exhibited by chemotactically stimulated neonatal neutrophil suspensions, and anti-CD11a MoAb were not inhibitory with respect to cell suspensions of either test group.

Comparisons of the migratory properties of neonatal neutrophils and neutrophils of heterozygotes for LAD. The findings presented above indicate that abnormalities of Mac-1-dependent adhesion underlie diminished transendothelial migration by neonatal neutrophils. Although diminished levels of Mac-1 on chemotactically stimulated neonatal cells have been previously reported, results of comparative studies shown in Table 3 suggest that functional deficits of Mac-1 on neonatal cells contribute to their diminished migration. As shown, neutrophils of patients with severe phenotype LAD demonstrated a profoundly diminished capacity to migrate through HUVEC monolayers. Importantly, neutrophils of individuals heterozygous for LAD consistently demonstrated normal migration, even though these cells expressed only 54% ± 7% of normal levels of Mac-1 when stimulated with chemotactic concentrations of fMLP. As shown in Table 3, neonatal cell suspensions expressed somewhat higher levels of surface Mac-1 when studied under these conditions (62% ± 9% of normal), but demonstrated diminished levels of transendothelial migration compared with adult control and with LAD heterozygote cells.

**Table 1. Neutrophil Adhesion to Purified ICAM-1 in Planar Membranes**

<table>
<thead>
<tr>
<th>Neutrophil Incubation Conditions</th>
<th>Level of Neutrophil Adhesion</th>
<th>Neutrophil Adhesion</th>
<th>Neutrophil Adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>45 ± 5</td>
<td>42 ± 7</td>
<td></td>
</tr>
<tr>
<td>Anti-CD11b</td>
<td>42 ± 6</td>
<td>39 ± 7</td>
<td></td>
</tr>
<tr>
<td>Anti-CD11a</td>
<td>9 ± 4</td>
<td>10 ± 3</td>
<td></td>
</tr>
<tr>
<td>Anti-CD11a, anti-CD11b</td>
<td>9 ± 5</td>
<td>8 ± 1</td>
<td></td>
</tr>
<tr>
<td>fMLP</td>
<td>52 ± 8</td>
<td>96 ± 3</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD11b</td>
<td>46 ± 7</td>
<td>56 ± 8</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD11a</td>
<td>15 ± 7</td>
<td>52 ± 4</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD11a + anti-CD11b</td>
<td>13 ± 35</td>
<td>14 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Isolated neutrophils were preincubated in PBS or PBS containing fMLP (10 nmol/L) and/or 5 μg/mL of MoAb directed at CD11b (904) and/or CD11a (R3.1) for 300 seconds at 37°C before assay. Values shown represent the mean ± 1 SD percent of adherent neutrophils contacting the protein substrate.

**Table 2. Neutrophil Adhesion to KLH-Coated Glass Substrates**

<table>
<thead>
<tr>
<th>Neutrophil Incubation Conditions</th>
<th>Level of Neutrophil Adhesion</th>
<th>Neutrophil Adhesion</th>
<th>Neutrophil Adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>13 ± 5</td>
<td>11 ± 7</td>
<td></td>
</tr>
<tr>
<td>fMLP</td>
<td>27 ± 10</td>
<td>52 ± 11*</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD11b</td>
<td>24 ± 9</td>
<td>10 ± 8*</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD11a</td>
<td>30 ± 8</td>
<td>49 ± 11†</td>
<td></td>
</tr>
<tr>
<td>fMLP + anti-CD18</td>
<td>27 ± 4</td>
<td>11 ± 7†</td>
<td></td>
</tr>
</tbody>
</table>

Isolated neutrophils were preincubated in PBS or PBS containing fMLP (10 nmol/L) and/or 5 μg/mL of MoAb directed at CD11b (904), CD11a (R3.1), or CD18 (R15.7) for 300 seconds at 300°C before assay. Values shown represent the mean ± 1 SD percent of adherent neutrophils contacting the protein substrate.

**DISCUSSION**

The results in this report show that neonatal neutrophils exhibit a significantly reduced capacity to migrate through IL-1-stimulated HUVEC monolayers. In an effort to identify cellular abnormalities that might account for this deficient migration, experiments designed to assess the functional integrity of CD18-dependent adhesion were used. MoAbs previously shown to block the adhesive functions of Mac-1 and LFA-1 were used, as well as conditions known to promote CD18-dependent adhesion, ie, stimulation of the endothelial cell monolayer with IL-1 for 4 and 8 hours, and IL-1β-stimulated HUVEC monolayers.

**Table 3. Comparison of Transendothelial Migration by Neonatal, LAD, and LAD Heterozygote Neutrophils**

<table>
<thead>
<tr>
<th>Cell Donor (n)</th>
<th>Migration Score</th>
<th>Neutrophil Surface Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD homozygote (2)</td>
<td>0.5 ± 1.1*</td>
<td>0.5 ± 0.1†</td>
</tr>
<tr>
<td>Adult LAD heterozygote (6)</td>
<td>48 ± 7.0</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Adult LAD heterozygote (6)</td>
<td>50 ± 6.5</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>Neonate (11)</td>
<td>29 ± 3.14†</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>Healthy adult (2)</td>
<td>53 ± 5.4</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviation: n, the number of separate experiments with one LAD patient, two LAD heterozygotes, 11 neonates, and two adult controls.

*Mean ± 1 SD percent of neutrophils migrating through HUVEC monolayers preincubated with IL-1 (10 U/mL, 4 hours, 37°C).

†Mean ± 1 SD percent of normal levels of Mac-1 (CD11b) expressed on surfaces of healthy adult neutrophils prestimulated with fMLP (10 nmol/L, 15 minutes, 37°C). Values shown were computed from determinations of the specific binding of the anti-CD11b MoAb 904 assessed by immunofluorescence flow cytometry (see Materials and Methods and reference 30).
chemotactic stimulation of the neutrophils. The adhesion of unstimulated adult neutrophils to IL-1-stimulated HUVEC is dependent on transiently expressed CD18-independent determinants,2,13 and LFA-1 interacting with endothelial zICAM-1.13 In addition to LFA-1-ICAM-1 interactions, the adhesion of stimulated adult neutrophils depends on Mac-1 interacting with several possible determinants, including ICAM-1.11,14

The results of these studies clearly indicate that the LFA-1-ICAM-1 interactions of neonatal neutrophils, whether occurring at the endothelial surface or on artificial membranes containing purified ICAM-1, were not different from adult neutrophils. Supporting this conclusion were findings that the levels of LFA-1-dependent adhesion for neonatal neutrophils were the same as adult neutrophils, the extent of inhibition of adhesion and migration caused by the anti-CD11a MoAb was not different for adult and neonatal cells, and the amount of LFA-1 on the surface of neonatal cells (as previously shown by flow cytometry30) was not different from adult cells.

In contrast, none of the assessments of Mac-1-dependent adhesion by neonatal neutrophils reported here yielded results comparable with adult cells. In previous studies, chemotactic stimulation of neonatal neutrophils did not significantly increase cell adhesion to protein-coated glass or plastic3 and failed to normally mobilize Mac-1 to the cell surface.19 In the present studies, chemotactic stimulation failed to induce Mac-1-dependent adhesion to endothelial cell monolayers, purified ICAM-1, or KLH-coated glass, and the anti-CD11b MoAb failed to inhibit neonatal cell adhesion under conditions where adult neutrophil adhesion was significantly inhibited. These results, along with the failure of anti-CD11b MoAb to influence neonatal cell migration, indicate that abnormal function of Mac-1 contributes to the observed deficiency in transendothelial migration in vitro.

Current evidence indicates that the abnormal functions of Mac-1 on neonatal cells are both quantitative and qualitative. There is diminished translocation from specific-granule associated pools to the plasma membrane on exposure to chemotactic factors.32,39 This finding, in turn, is related to abnormalities of specific granules (as originally reported by Amburso et al.),49 which represent a major intracellular pool from which Mac-1 is normally mobilized.32,39,41,42 In addition to these quantitative changes, chemotactic stimulation functionally alters Mac-1,41,42 in a manner that facilitates its binding to ligands such as ICAM-1.14 The finding here that neonatal neutrophils adhere normally to purified ICAM-1 under baseline conditions (an LFA-1-dependent event), but fail to increase binding in response to fMLP, suggests that this functional alteration does not proceed normally in neonatal cells. Additional evidence for functional aberrations was apparent in comparative assays of migration incorporating LAD neutrophils and neutrophils of individuals heterozygous for CD11/CD18 deficiency. fMLP actually elicited less surface Mac-1 on cells from heterozygotes than on neonatal suspensions tested. However, neutrophils from heterozygotes migrated normally. These findings indicate that diminished levels of qualitatively normal Mac-1 on heterozygote cells are sufficient to promote normal migration in vitro, and normal host defense.15,16,46 These findings imply functional (ie, qualitative) abnormalities of Mac-1 when expressed on stimulated neonatal neutrophils, and that diminished levels of this protein are not independently sufficient to account for diminished adhesion to or migration through vascular endothelium in vitro. Although exogenous chemotactic factors were not used in these assays, chemotactic factors of endothelial origin24 would presumably fail to functionally activate Mac-1 on neonatal neutrophils on contact with HUVEC.

The molecular events underlying these observations are uncertain. The Mac-1 heterodimer in neonatal cells does not appear to be intrinsically abnormal. In previous studies, Jones et al48 used immunoblot procedures to show normal immunoochemical properties and normal levels of the heterodimer when assayed in whole cell lysates or subcellular fractions of neonatal neutrophils. Because the functions of Mac-1 evaluated thus far appear to depend on stimulation of the neutrophil, abnormalities along the pathway of this activation may result in failure of a stimulus to evoke Mac-1-dependent adhesion. Global abnormalities in signal transduction appear not to occur. We have previously shown that chemotactic concentrations of fMLP and C5a elicited normal increases in cell surface CR1, though the same neonatal neutrophils failed to normally upregulate Mac-1.30 We have also found that chemotactic stimulation of neonatal neutrophils over a wide concentration range mediates normal bipolar shape change and chemiluminescence, and in unpublished experiments, we found that fMLP stimulated normal levels of superoxide release by neonatal cells. Hilmo and Howard47 also showed that the rate of actin polymerization following fMLP stimulation was generally similar in neonatal and adult neutrophils. However, previous reports have described abnormalities of membrane fluidity and cytoskeletal properties of neonatal neutrophils that are thought to account for abnormal incorporation of other receptors in the plasma membrane of neonatal cells.46,49,50

Our findings clearly do not exclude the possibility that deficits of other neutrophil adhesion molecules contribute to diminished migration in vitro. In fact, the findings of diminished adhesion to HUVEC stimulated with IL-1 for 4 hours (to elicit CD18-independent ligands as well as ICAM-1)12,13 but not for 8 hours (to selectively elicit ICAM-1) support such a possibility (Fig 3). Recent studies have identified a homologue of the murine MEL-14 antigen on human neutrophils that contributes to CD18-independent adhesion to HUVEC. This determinant reacts with unidentified ligand(s) on endothelial cells stimulated with IL-1 for 4 hours but not longer time intervals.51 Functional and qualitative assessments of this neutrophil determinant on neonatal neutrophils deserve attention. Finally, our findings do not exclude the possibility that Mac-1 recognizes endothelial ligands other than ICAM-1 as recently suggested by Lo et al.,52 nor that defective ligation of Mac-1 with these determinants may underlie diminished migration of neonatal neutrophils. Mac-1 is known to recognize several proteins,53-57 These studies support the hypothesis that diminished or delayed localization and/or emigration of blood neutrophils may be causally related to infectious susceptibility in human neonates. Definitive studies of the kinetics and extent of
neutrophil egress in tissues of human neonates cannot be performed. However, experimental models of acute inflammation in neonatal animals will allow such evaluations. Furthermore, they should facilitate studies of the influence of cytokines, including TNFα, IL-1, and IL-6, and the role of neutrophils in the pathogenesis of inflammatory diseases in neonates.

REFERENCES


52. Lo SK, Van Seventer GA, Levin SM, Wright SD: Two leukocyte receptors (CD11a/CD18) mediate transient adhesion to endothelium by binding to different ligands. J Immunol 143:3325, 1989


57. Lo SK, Wright SD: CR3 mediates binding or PMN to endothelial cells (EC) via its RGD binding, not the LPS binding site. FASEB J 2:A1236, 1988 (abstr)

Impaired transendothelial migration by neonatal neutrophils: abnormalities of Mac-1 (CD11b/CD18)-dependent adherence reactions

DC Anderson, R Rothlein, SD Marlin, SS Krater and CW Smith