Abnormal Stimulated Adherence of Neonatal Granulocytes: Impaired Induction of Surface MAC-1 by Chemotactic Factors or Secretagogues

By Donald C. Anderson, Katherine L. Becker Freeman, Barbara Heerdt, Bonnie J. Hughes, Richard M. Jack, and C. Wayne Smith

To identify possible secretory determinants of impaired hyperadherence and stimulated migration of neonatal granulocytes (NGs), we performed correlative studies of: (a) specific granule content and exocytosis, (b) secretagogue-mediated upregulation of f-met-leu-phe (fMLP) receptors, (c) the chemotactic induction of the adhesive glycoproteins Mac-1α (complement receptor 3) and β, and (d) morphometric assessments of specific (peroxidase-negative) granule depletion following chemotactic stimulation. Lactoferrin (LF) content of NG suspensions (cord blood or peripheral blood cells) was profoundly diminished (mean ± SD 51% ± 18% of normal) as compared with healthy adult granulocytes (AGs). Despite diminished cellular content, LF release by NG suspensions in response to fMLP was comparable to that of AGs. In contrast, LF release by NG suspensions was significantly diminished in response to phorbol myristate acetate (PMA) or calcium ionophore A23187 and/or during stimulated cell spreading, experimental conditions promoting overall greater LF depletion than chemotactic stimuli. In addition, NGs demonstrated an impaired capacity to upregulate fMLP receptors in response to PMA or A23187 when tested under the same experimental conditions. Baseline expression of the adhesive glycoproteins Mac-1α and β on NG surfaces was normal, but induction or upregulation of these proteins by chemotactic concentrations of fMLP, C5a as well as secretory (high) concentrations of PMA and A23187, was significantly diminished as compared with AGs. In contrast, chemotactic induction of the surface expression of the complement receptor-1 (CR-1) on NGs was normal. An impaired induction of Mac-1α or β was directly related to an impaired enhancement of adherence of NG in response to fMLP over a chemotactically relevant concentration range (10⁻¹⁰ to 10⁻⁹ mol/L). Moreover, in blocking-incubation experiments using anti–Mac-1α/β monoclonal antibodies (MAbs), significantly less inhibition of adherence by these MAbs was evident with fMLP-stimulated NG as compared with AG suspensions. Under selected chemotactic conditions, ultrastructural assessments of NGs demonstrated diminished peroxidase-negative granule loss in association with diminished granule-membrane fusion and the “addition” of plasma membrane. These studies suggest that abnormal expression of multiple surface determinants derived from peroxidase-negative granules or other intracellular pools may contribute to deficient chemotaxis or other inflammatory functions of NGs. Specifically, they indicate that diminished stimulated adherence of NGs is functionally linked to an insufficient chemotactic induction of surface Mac-1 protein subunits.

©1987 by Grune & Stratton, Inc.
Mac-1, LFA-1 glycoprotein family.\textsuperscript{12-14,24} Infectious susceptibility and impaired inflammatory functions recognized in both pathologic entities have been partially attributed to abnormalities of neutrophil and/or monocyte adherence. Of considerable relevance to our studies reported herein, Ambruso and co-workers recently reported diminished LF content as well as quantitative and qualitative abnormalities of specific granules in NGs.\textsuperscript{26} The relationship(s) of their findings to impaired chemotaxis or other adherence-dependent functions of NGs have not been previously evaluated.

To identify possible secretory determinants of impaired hyperadherence and stimulated migration of NGs, we performed correlative studies of: (a) specific granule content and exocytosis; (b) secretagogue-mediated up-regulation of fMLP receptors; (c) the induction of the “adhesive” glycoproteins Mac-1 α, β, and p150,95α by chemotactic or secretory stimuli; and (d) ultrastructural and morphometric assessments of peroxidase-negative granule depletion or “membrane addition” effected by chemotactic stimuli. Our studies suggest that abnormal expression of multiple surface determinants derived from specific granules or other intracellular pools may contribute to deficient chemotaxis or other inflammatory functions of NGs. In addition, our findings indicate that impaired chemotactic induction of Mac-1 subunits represents a major pathogenic mechanism accounting for their diminished stimulated adherence properties. Finally, these studies provide further evidence for diverse secretory contributions to neutrophil inflammatory functions.

MATERIALS AND METHODS

Blood samples. Heparinized venous blood was drawn from the placenta or peripheral veins of normal full-term or premature infants within 5 minutes of birth. Peripheral blood samples were also drawn from healthy neonates (age 1 to 30 days), healthy maternal pairs, Mac-1, LFA-1–deficient individuals, and healthy adult laboratory donors. All blood samples were processed within 10 minutes after phlebotomy. Informed consent was obtained from patients or their legal guardians prior to obtaining blood samples.

Isolation of granulocytes. Granulocytes were purified from heparinized, dextran-sedimented venous blood samples over Ficoll-Hypaque gradients and suspended in Dulbecco’s phosphate-buffered saline (DPBS) (GIBCO Laboratories, Grand Island, NY), pH 7.4, containing 0.2% dextrose as described.\textsuperscript{1} For use in N-formyl-Met-Leu-Phe (fMLP) (Sigma Chemical, St Louis, MO) and fMLP\textsuperscript{[3H]} binding studies, erythrocytes were eliminated by hypotonic lysis.\textsuperscript{1} To minimize degranulation or “spontaneous up-regulation” of C3b receptors (CR-1) or Mac-1 glycoprotein (iC3b receptor or CR-3) surface expression during cell purification, acellularized blood samples were processed immediately at 4°C, and all purification procedures were performed at 0°C to 4°C with ice-cold buffers as described by Fearon and Collins.\textsuperscript{27}

Preparation of reagents. Stock solutions of f-Met-Leu-Phe (fMLP) (Sigma Chemical, St Louis, MO) and fMLP\textsuperscript{[3H]} (New England Nuclear, Boston) were prepared in DPBS. A low-mol-wt chemotactic factor (CF) referred to as C5a in this article, was prepared from activated human sera.\textsuperscript{28} Zymosan, calcium ionophore A23187, phorbal myristate acetate (PMA), phenylmethyl sulfonyl fluoride (PMSF), and both supernatants and sonicates were stored at -70°C prior to enzyme analysis.

A radiometric technique previously described was used to assay for LF.\textsuperscript{29} In brief, purified anti-LF antibody was radioiodinated using lactoperoxidase immobilized on Sepharose beads. Polystyrene beads (6-mm diameter; Clifton Plastic, Clifton Heights, PA) were coated with anti-LF IgG (200 μg/mL) in freshly prepared carbonate buffer (0.2 mol/L, pH 9.4) by overnight incubation at 4°C. Unlabeled fMLP binding assay

Immuno-fluorescence flow cytometry. Indirect immuno-fluorescence assessment of intact granulocytes were performed by using saturating concentrations of MABs and FITC-conjugated goat anti-mouse IgG or goat anti-rabbit IgG F(ab')\textsuperscript{2}, Surface-sminated cells were fixed in 1% paraformaldehyde and analyzed in a flow cytometer (Coulter Epics V; Coulter Electronics, Hialeah, FL) as previously described.\textsuperscript{30}

Assay of granule-associated enzymes. Unstimulated or stimulated granulocyte suspensions (5 x 10\textsuperscript{6} cells/mL DPBS) were immersed in an ice bath to terminate extracellular release conditions and centrifuged at 800 g for 5 minutes. Supernatants were carefully aspirated, and cell pellets were suspended in 0.5 mL DPBS containing 1.0 mL of Triton X-100 surfactant/L and then sonicated on ice for 120 seconds. Cell sonicates were diluted 50-fold with 2 mmol/L PMSF, and both supernatants and sonicates were stored at -70°C prior to enzyme analysis.

fMLP\textsuperscript{[3H]} binding assay

Modification of methods previously described were used.\textsuperscript{1,12} Triplicate suspensions of unstimulated or prestimulated granulocytes (5 x 10\textsuperscript{6}) were incubated in 100 μL DPBS containing 40 nmol/L fMLP\textsuperscript{[3H]} or fMLP\textsuperscript{[3H]} plus 40 μmol/L unlabeled fMLP at 0°C with gentle mixing. Reactions were terminated after 12 min. of incubation by rapid dilution with 2 mL ice-cold DPBS followed by rapid filtration and washing (5 mL twice) of mixtures through Whatman GF C glass fiber filters (Whatman, Clifton, NJ).\textsuperscript{31} Washed filters were placed into 10 mL Scinta Verse II scintillation cocktail, and radioactivity was quanti-
tated in a Packard TriCarb liquid scintillation spectrometer. Non-specific binding, defined as the amount of binding occurring in the presence of a 1,000-fold excess concentration of fMLP, represented 20% to 30% of total binding values. Specific binding was defined as the total amount of fMLP<sup>3H</sup> bound minus that proportion nonspecifically bound.

**Ultrastructural evaluations: Granule characterization and quantitation and morphometric assessments.** The experimental conditions for ultrastructural evaluations were based on preliminary experiments to determine optimal stimulation conditions in shape change assays. As shown by Nomarski optics, stimulation with fMLP (1 or 100 nmol/L) for 20 seconds elicited cell activation evidenced by circumferential membrane ruffling but no bipolar shape change. For transmission electronmicroscopy preparation, stimulated cell suspensions were fixed in 1.8% (vol/vol) glutaraldehyde in 0.2 mol/L sodium cacodylate made in 1% sucrose, pH 7.4, cells were resuspended in DAB 0.5 mg/mL and 0.05 mol/L Tris-HCl, pH 7.6, made 0.01% (vol/vol) hydrogen peroxide and 1% (wt/vol) sucrose. Following 30-minute incubation at room temperature, neutrophils were washed three times with 0.05 mol/L acetate-veronal buffer (A-V buffer), pH 7.4, and postfixed with 1% (wt/vol) osmium tetroxide in A-V buffer containing 5% sucrose for 1 hour. Cells were washed twice with A-V buffer and stained en bloc for 1 hour with 0.5% (wt/vol) uranyl acetate dissolved in A-V buffer containing 4% sucrose. Gold-silver sections were stained with saturated aqueous uranyl acetate and counterstained with Reynold's lead citrate.

Sections were examined using a JEOL 100S or 100 CX transmission electron microscope at 60 kV. To standardize the ultrastructural granule quantitations and morphometric measurements, negatives (7,000 x) were taken only if the cell profile contained at least three nuclear lobes and/or the centriole.

Intracellular granule characterizations were made based on cytochemical staining. Primary granules (evidenced by the DAB reaction) were easily distinguishable from the secondary granules. Intracellular granule quantitation assessments were expressed as the average number of peroxidase-positive or peroxidase-negative granules per neutrophil profile. Measurements of cell perimeter and area were made on a Houston graphics tablet interfaced to an Apple computer equipped with software by Optomax. Ratios of surface to volume (S/V) were calculated from area and perimeter measurements of micrographs based on the formula: S/V = 3/2 (P/A), where P = perimeter and A = area. Surface membrane area was calculated from the S/V using 397 μm<sup>2</sup> as the mean cell volume as previously reported. 13

**Adherence assays.** Granulocyte adherence to serum-coated glass substrates was evaluated as previously described using Smith-Hollers chambers. 19 The capacity of granulocytes to anchor (spread) on glass coverslips placed in 35-mm diameter plastic Petri dishes was assayed as described. 18 Supernatants of these preparations were assayed for granule-associated enzymes as described above.

**Statistical analysis.** Student's t test was used to determine statistical significance between the means of control and stimulated values among both neonatal and healthy adult individuals in most assays. Unless otherwise stated, all P values refer to the two-tail test. For comparisons of calculated surface area between neonatal and adult neutrophil profiles, Bonferroni T tests were applied. Linear regression analysis and statistical programs to generate an exact 95% confidence interval around regression lines were used to analyze correlations of adherence increments versus protein expression values.

### RESULTS

**Quantitation of granule-associated proteins.** The granulocyte content of granule-associated proteins of healthy full-term neonates (n = 31), premature neonates (n = 3), healthy adults (n = 32), or Mac-1, LFA-1-deficient patients (n = 3) is shown in Table 1. Mean values for LF and vitamin B-12 transport protein content among neonatal suspensions were significantly diminished (LF; P < .001, vitamin B-12 transport protein; P < .05 as compared with healthy adult or Mac-1 deficiency mean values), whereas mean values for β-glucuronidase and lysozyme determinations were comparable among all test groups. Among neonates tested, the lowest LF values were observed in those of three premature infants. LF content of cord blood granulocytes (n = 20 samples) was comparable to that of cells obtained by phlebotomy from neonates aged 1 to 30 days (n = 14) and was unrelated to the mode of delivery (P = .5, vaginal vs cesarean section delivery).

### Table 1. Primary and Secondary Granule Content of NGs, Healthy AGs or MAC-1–Deficient Granulocytes

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>(n)</th>
<th>β-Glucuronidase (nmol/10&lt;sup&gt;9&lt;/sup&gt; cells)</th>
<th>Lysozyme (μg/10&lt;sup&gt;9&lt;/sup&gt; cells)</th>
<th>LF (μg/10&lt;sup&gt;9&lt;/sup&gt; cells)</th>
<th>Vitamin B12 Transport Protein (ng/10&lt;sup&gt;9&lt;/sup&gt; cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonates</td>
<td>(34)</td>
<td>308 ± 12 *</td>
<td>7.88 ± 1.9</td>
<td>30.6 ± 6.1†</td>
<td>6.7 ± 2.4‡</td>
</tr>
<tr>
<td>Healthy adults</td>
<td>(32)</td>
<td>320 ± 18</td>
<td>8.32 ± 1.8</td>
<td>59.6 ± 5.5</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>Mac-1, LFA-1 deficient</td>
<td>(3)</td>
<td>290 ± 18</td>
<td>8.51 ± 2.1</td>
<td>54.1 ± 3.9</td>
<td>8.1 ± 2.1</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†P < .001 as compared with healthy adult or Mac-1, LFA-1–deficient groups.
‡P < .05 as compared with healthy adult or Mac-1, LFA-1–deficient groups.

### Table 2. LF Release by NGs or Healthy AGs in Response to f-Met-Leu-Phe

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Neonates</th>
<th>Healthy Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPBS</td>
<td>0.72 ± 0.31 (2.4)</td>
<td>0.84 ± 0.1 (1.4)</td>
</tr>
<tr>
<td>f-Met-Leu-Phe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 nmol/L</td>
<td>0.84 ± 0.41 (2.8)</td>
<td>0.91 ± 0.21 (1.5)</td>
</tr>
<tr>
<td>1.0 nmol/L</td>
<td>1.35 ± 0.60 (4.5)</td>
<td>1.22 ± 0.31 (2.1)</td>
</tr>
<tr>
<td>10.0 nmol/L</td>
<td>1.86 ± 0.80 (6.0)</td>
<td>2.17 ± 0.61 (3.5)</td>
</tr>
<tr>
<td>100.0 nmol/L</td>
<td>2.74 ± 1.10 (9.1)</td>
<td>3.21 ± 0.74 (4.7)</td>
</tr>
</tbody>
</table>

*Cell suspensions were prewarmed for 5 minutes, at 37 °C and then incubated at 37 °C for 20 minutes with or without f-met-leu-phe, after which reaction tubes were immersed in an ice bath to terminate release conditions.
†Pooled data from analyses of four cord blood samples and four specimens obtained by phlebotomy from neonates aged 2 to 16 days.
‡Pooled data from analyses of six healthy adult laboratory donors.
§LF release expressed as μg/10<sup>9</sup> granulocytes (mean ± 1 SD) and, in parentheses, mean percentage of release of total cell content (Neonates averaged 30 μg/10<sup>9</sup> cells. Adults averaged 60 μg/10<sup>9</sup> cells.)

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
**LF release.** The release of LF by suspensions of NGs or healthy adult granulocytes (AGs) in response to fMLP stimulation is summarized in Table 2. Following a 20-minute exposure at 37°C to 0.1, 1, 10, or 100 nmol/L fMLP, neonatal suspensions demonstrated extracellular LF release equivalent to that of healthy adult suspensions. As a result of their diminished total LF content, calculated percentage release values were significantly ($P \leq .02$) greater for neonatal suspensions under each test condition. In contrast, abnormalities of LF release by suspensions of NGs were observed in response to the secretagogues A23187 and PMA under conditions eliciting overall greater release than occurred following fMLP stimuli. Stimulating conditions chosen were shown to elicit preferential release of specific granules; essentially no release of $\beta$ glucuronidase was observed under each of the incubation conditions illustrated in Figs 1 and 2. Assessments of fMLP receptor up-regulation were concurrently performed on the same test suspensions in response to these secretory stimuli (as discussed below).

Following a 20-minute exposure to $\geq 5 \times 10^{-8}$ mol/L A23187 at 37°C, significantly ($P < .01$) diminished release of LF by NG as compared with healthy AG suspensions was observed. In kinetics experiments using $10^{-7}$ mol/L A23187, diminished release of LF by NGs was also evident following incubation intervals of $>5$ minutes ($P \leq .015$ as compared with healthy adult controls). In addition, moderately diminished exocytosis of vitamin B-12 transport protein, another secondary granule marker, was also apparent among six neonatal suspensions maximally stimulated with A23187 (2.5 x 10^{-7} mol/L, 20 minutes) ($P < .01$) (data not shown).

Similar but less striking differences between neonatal and control suspensions were evident in dose–response or kinetics experiments using PMA as a stimulant (Fig 2). Comparable LF release was observed under some experimental conditions, but diminished release by NGs was evident when they were exposed to high concentrations (100 or 500 ng/mL) of PMA for 20 minutes ($P < .05$). No significant ($P = .05$) differences were evident with respect to vitamin B-12 transport protein release among neonatal and healthy adult suspensions tested under the same conditions (data not shown). LF and vitamin B-12 transport protein release by Mac-1, LFA-1–deficient granulocyte suspensions was normal in response to both PMA and A23187 stimuli, as previously reported.13

Diminished LF release by NGs was also evident during
cell spreading on glass surfaces either in the presence of chemotactic factors or secretagogues (data not shown). Mac-1, LFA-1-deficient granulocytes were used as experimental controls in these experiments because they fail to anchor but have a normal LF content. Under all test conditions, neonatal and healthy adult suspensions demonstrated comparable anchorage or spreading following a 20-minute incubation interval at 21°C. However, LF release during anchorage by stimulated neonatal cells was significantly diminished as compared with healthy adult cells (P < .05), and even lower values were observed for Mac-1, LFA-1-deficient cells (P < .0001). Although fMLP (10^-6 mol/L), and A23187 (2.5 × 10^-7 mol/L), and PMA (500 μg/mL) enhanced LF release by neonatal cells above baseline values, mean values in each case were significantly diminished as compared with healthy adult cells.

fMLP receptor induction. Induction of fMLP receptor expression was also assessed with respect to the same neonatal or control suspensions assayed for LF release, as shown in Figs 1 and 2. Methods previously described were used to quantitate specific, saturable binding of fMLP[P]P before and following secretory stimulation. Binding studies were carried out at 0°C to prevent downregulation (pinocytosis) of fMLP[P]P-receptor complexes. In dose–response experiments, diminished fMLP[P]P binding by NGs was observed over a range of ionophore concentrations from >2.5 × 10^-10 mol/L to 2.5 × 10^-7, and longer incubation intervals (>3 minutes) were required to up-regulate (enhance above baseline values) fMLP[P]P binding by AGs as compared with healthy adult suspensions under most conditions, but diminished values for neonatal suspensions were evident when a maximal stimulus (500 ng/mL) was used for ≥20 minutes (P < .05). Lower left panel: Significantly (P < .027) diminished f-Met-Leu[P]P binding by NGs as compared with healthy adult suspensions was demonstrated following exposure to PMA (500 ng/mL, 20 minutes). In kinetics assessments (right lower panel) up-regulation of f-Met-Leu[P]P binding by AGs was evident following a 5-minute exposure to PMA (100 ng/mL), whereas a 10-minute exposure was required to enhance NG fML[P]P binding significantly above baseline values. Values shown in all panels represent the mean ± 1 SD of individual determinations (adults, n = 5 to 11; neonates, n = 3 to 15, for each test condition).

Somewhat similar relationships were observed when NGs or AGs were stimulated with PMA (Fig 2). Among both neonatal and healthy adult suspensions, concentrations of PMA ≥ 100 ng/mL elicited a significantly increased number of fMLP[P]P binding sites as compared with baseline values. However, significantly (P < .027) enhanced fMLP[P]P binding by adult cells as compared with neonatal conditions.
ADHERENCE PROPERTIES OF NEONATAL GRANULOCYTES

suspensions was demonstrated using PMA at a concentration of 500 ng/mL for 20 minutes. FMLP receptor up-regulation by adult cells was evident following a 5-minute exposure to PMA (100 ng/mL), whereas at least a 10-minute exposure was required to enhance NG fML[3H]P binding significantly above baseline values. Under conditions of maximal PMA stimulation, healthy AGs demonstrated a mean increase of specific fML[3H]P binding of 305% ± 70%, whereas neonatal suspensions demonstrated a mean increase of only 120% ± 30% above baseline values.

Induction of surface Mac-1 α and β by chemotactic factors A23187 or PMA. The surface expression of Mac-1 α, LFA-1 α, their common β subunit, the complement receptor I (CR-1), and the HLA A,B, antigen of neonatal or healthy AGs was evaluated using subunit-specific MAbs to each antigen in immunofluorescence flow cytometry studies (Fig 3). Cell suspensions were studied prior to or following stimulation with fMLP (10⁻⁴mol/L, 20 minutes), PMA (100 ng/ml, 20 minutes), or A23187 (10⁻⁷ mol/L, 20 minutes). Fluorescent histograms derived from these studies generally demonstrated a single population of cells among both adult and neonatal test suspensions. Under unstimulated conditions (DPBS), the mean surface expression values for each protein subunit were comparable among neonatal and healthy adult test groups (P > .1). Similar values were also determined when flow cytometric assessments were performed on suspensions of leukocyte-rich plasma among neonates as compared with adult controls (data not shown). However, with respect to either FMLP, PMA, or A23187 (data not shown) stimuli, NGs generally demonstrated an impaired capacity to up-regulate the surface expression of Mac-1 α or β subunits. Mean fold increases of Mac-1 α or β expression were significantly diminished among neonatal suspensions as compared with adult suspensions assessed under comparable conditions (neonate—fMLP, 2.4 (Mac-1 α), 2.3 (β); PMA, 1.6 (Mac-1 α), 1.7 (β); healthy adult—fMLP, 5.5 (Mac-1 α), 4.9 (β); PMA, 4.9 (Mac-1 α), 4.8 (β) (P < .04 to .001). Individual values for stimulated Mac-1 α and/or β expression among neonatal suspensions were considerably less than healthy adult suspensions when compared in 17 of 22 experiments. As expected, no induction of LFA-1 α or HLA A,B, by fMLP, PMA, or A23187 was evident among test or control suspensions, and the surface expression of these proteins was comparable among healthy adult and neonatal suspensions.

The capacity of chemotactic factors or PMA to up-regulate the surface expression of the CR-1 on NGs was also evaluated (Fig 4); such assessments were of particular interest since important differences between CR-1 and the CR-3 have been reported with respect to the anatomic location of their intracellular pools and their functional activities. For these studies, Mac-1, LFA-1–deficient

---

**Fig 3.** Expression of Mac-1α, β, LFA-1α, and HLA A,B, on NGs or healthy AGs as assessed with subunit specific monoclonal antibodies and immunofluorescence flow cytometry. NGs (●) or healthy AGs (●) were incubated in DPBS, fMLP (10 nmol/L) or PMA (100 ng/mL) for 20 minutes at 37 °C prior to antibody staining. Individual determinations and mean ± 1 SD values (bars) are shown for each test condition. Studies of neonatal suspensions included six cord blood and five peripheral blood samples.
granulocytes were used as experimental controls because: (a) they express CR-1 normally, and (b) they contain and secrete (in suspension) normal quantities of specific granule constituents.

In contrast to Mac-1 α or β expression, no differences were evident among neonatal, healthy adult, and genetically deficient cell suspensions with respect to fMLP-mediated CR-1 expression (Fig 4). In contrast to their diminished capacity to express Mac-1 α or β, neonatal suspensions exposed to ≥ 10⁻⁴ mol/L fMLP expressed CR-1 normally. Mean fold increases of CR-1 expression (over baseline) following fMLP (10 nmol/L, 20 minutes) exposure were 3.1, 3.8, and 3.9 for neonatal, healthy adult, and Mac-1-deficient suspensions, respectively. Similar relationships were evident in dose-response experiments using C5a (1.5 to 20 μg/mL concentration range), and normal CR-1 expression was also evident on NGs exposed to PMA (100 ng/mL, 20 minutes) (data not shown).

Relationship of Mac-1 glycoprotein expression to stimulated adherence properties. As illustrated in Fig 5, NGs, Mac-1-deficient granulocytes, and healthy AGs were stimulated with a range of concentrations of fMLP extending above and below that promoting optimal chemotaxis or hyperadherence. A sharp enhancement of adherence of healthy AGs was evident following their exposure to fMLP (≥ 10⁻⁸ mol/L, 10 minutes, 21 °C). The correlation between percentage of enhancement of adherence and percentage of increase of Mac-1α surface expression in response to a range of fMLP concentrations (10⁻¹¹ to 10⁻⁴ mol/L) were highly significant (r = .89; P < .001). Although baseline (DPBS) adherence of neonatal granulocyte suspensions was comparable to that of healthy adult cells as previously reported, markedly diminished adherence increments were evident for each concentration of fMLP between 10⁻¹⁰ and 10⁻⁸ mol/L (P < .001). Adherence increments among stimulated neonatal and adult suspensions were directly related to relative enhancement of surface Mac-1α or β expression. Similar relationships were observed for both healthy adult and neonatal suspensions in dose-response experiments using C5a or PMA as a stimulant (data not shown).

As shown in Fig 5, the degree of inhibition of adherence (percentage) by the anti-Mac-1 α MAb LM2.1 F(ab')₂ + the anti-β MAb TS1/18 F(ab')₂ was directly related to the mean level of stimulated adherence as well as the level of surface Mac-1 expression (as previously shown in Figs 3 and 4) on granulocytes among each test group. Significantly less (P ≤ .02) inhibition of stimulated adherence was evident among neonatal suspensions as compared with healthy adult suspensions with respect to each fMLP concentration inclusive of 5 × 10⁻⁹ to 1 × 10⁻⁷ mol/L. As expected, no inhibitory effects were observed using Mac-1-deficient cells. Binding control antibodies including rabbit anti-CR-1 F(ab')₂ and anti-HLA A,B, MAb demonstrated no inhibitory effects among neonatal, Mac-1-deficient or healthy adult suspensions (data not shown).

Ultrastructural evaluations: intracellular granule quantitation and morphometric determinations. No significant differences between unstimulated neonatal and healthy adult cells were evident with respect to the number of peroxidase-positive or peroxidase-negative granules per profile. However, neonatal cells treated with 1 nmol/L fMLP did not exhibit a significant decrease in peroxidase-negative

![Fig 4. Expression of Mac-1α, β, and CR-1 on NGs, Mac-1, LFA-1-deficient granulocytes, or healthy AGs as assessed by immunofluorescence flow cytometry. Neonatal (O), Mac-1, LFA-deficient (Δ), or healthy AGs (□) were exposed to a range (0.01 to 10 nmol/L) of fMLP concentrations for 10 minutes at 21 °C prior to antibody staining. Values shown for each patient category represent the mean of five to nine separate determinations for each experimental condition. Neonatal suspensions assessed included four to six cord blood and two to four peripheral blood samples. Significant (P ≤ .001) differences between healthy AG and NG Mac-1α or β expression were observed using ≥ 1 nmol/L fMLP. Comparable baseline and stimulated expression of CR-1 was observed among each of the test groups under each test condition.](/bloodjournal.org)
ADHERENCE PROPERTIES OF NEONATAL GRANULOCYTES

Fig 5. Adherence properties of NGs, Mac-1, LFA-1-deficient granulocytes, or healthy AGs with or without fMLP or anti-Mac-1α + anti-β monoclonal antibodies. Granulocytes of each test group were exposed to a range of fMLP concentrations similar to that used in immunofluorescence flow cytometry experiments (Fig 4). As shown, the hyperadherence response of healthy AGs is observed over a similar range of stimulating concentrations that significantly enhance their surface expression of Mac-1α and β (Fig 4). Although baseline adherence of neonatal suspensions was normal, adherence increments in response to fMLP were significantly diminished as compared with healthy adult values. Profoundly diminished baseline adherence and essentially no enhancement of adherence was observed using Mac-1-deficient granulocytes exposed to fMLP. Dashed lines demonstrate the effects of anti-Mac-1α plus β preincubation on the adherence and hyperadherence responses of granulocytes of each population. Values shown represent the mean ± 1 SD of individual determinations in 7 healthy adults, 7 neonates, and 4 Mac-1 deficient patients.

Table 3. Quantitation of Granules in Healthy Adult or Neonatal Neutrophils: Effects of f-Met-Leu-Phe Stimulation

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Healthy Adult Neutrophil Granules*</th>
<th>Neonate Neutrophil Granules†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peroxidase +</td>
<td>Peroxidase -</td>
</tr>
<tr>
<td>Baseline‡</td>
<td>40</td>
<td>74.2 ± 13.7§</td>
</tr>
<tr>
<td>f-Met-Leu-Phe 1 nmol/L,</td>
<td>22</td>
<td>72.6 ± 14.5</td>
</tr>
<tr>
<td>f-Met-Leu-Phe 100 nmol/L</td>
<td>25</td>
<td>79.5 ± 11.3</td>
</tr>
</tbody>
</table>

*Data pooled from five individuals.
†Data pooled from five individuals.
‡Cells incubated in DPBS for 5 minutes at 37°C prior to fixation.
§Mean ± SD: number of granules/profile (n = number of profiles examined).
‖Cells incubated in DPBS for 5 minutes at 37°C, f-Met-Leu-Phe added, and incubation continued for an additional 20 seconds prior to fixation.
†P < .01 v baseline determination.

These studies provide additional evidence that impaired chemotaxis of NGs is functionally linked to abnormalities of cellular effector mechanisms contributing to stimulated adherence (hyperadherence) properties.1-5,36,40 We previously observed impaired C5a, fMLP, or bacterial chemotactic factor-mediated hyperadherence of NGs and abnormal redistribution of their surface "adhesions sites" under conditions of sequential chemotactic factor stimulation and in response to colchicine.1,3 Krause and co-workers later reported abnormalities of granulocyte adherence in populations of healthy neonates4 and in "stressed" infants studied during the course of acute respiratory or cardiopulmonary illness.2 The molecular basis of these functional deficits has not been clearly elucidated. Defects of membrane deformability,40 lectin binding and/or capping41 microtubule assembly,2 and expression of membrane receptors for granules, as was observed for healthy adult suspensions studied under these conditions (Table 3). Both neonatal and adult cells exposed to 100 nmol/L fMLP for 20 seconds did exhibit a significant reduction of peroxidase-negative granules, and no loss of peroxidase-positive granules was observed under these experimental conditions.

Morphometric evaluations of membrane perimeter and associated profile areas were also performed. Nonactivated neonatal neutrophils exposed significantly less plasma membrane (mean ± SD, 474 ± 108 μ2; S/V ratio, 1.19 ± 0.27; n = 38) than did nonactivated adult cells (552 ± 128 μ2; S/V ratio, 1.39 ± 0.32; n = 71). Moreover, following activation with an optimum shape change inducing concentration of fMLP (1 nmol/L) for both neonatal and adult neutrophils,1 the mean area determinations of neonatal and adult cells were comparable, but the exposed plasma membrane of newborn cells (450 ± 57 μ2; S/V ratio, 1.13 ± 0.14; n = 39) was significantly (P < .01) less than that of stimulated adult cells (648 ± 194 μ2; S/V ratio, 1.63 ± 0.49; n = 42). The increase in S/V ratio and calculated surface membrane caused by chemotactic stimulation of adult neutrophils were statistically significant (P < .01). There was no significant change in the neonate neutrophil. Thus, under at least selected experimental chemotactic conditions, neonatal neutrophils failed to demonstrate the peroxidase-negative granule loss and surface membrane increase seen in adult neutrophils.

DISCUSSION

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
fMLP	extsuperscript{42} have been reported. Heterogeneity, including increased proportions of immature granulocyte forms	extsuperscript{6} or subpopulations of cells expressing less surface antigen identified by the 31D8 monoclonal antibody	extsuperscript{23} in neonatal or cord blood samples may contribute to the observed abnormalities. Our studies were designed to evaluate in NGs the secretory processes that contribute to normal stimulated cellular adherence and/or migration.

The positively charged glycoprotein, LF, a known constituent of specific granules, appears to influence granulocyte–endothelial cell adherence interactions, homotypic granulocyte aggregation, and cell surface charge.	extsuperscript{22} Moreover, deficits of adherence and directed migration have been well documented in studies of patients with genetic deficiency of neutrophil-specific granules.	extsuperscript{20,21} Of considerable relevance to this report, Ambruso et al.	extsuperscript{20} previously documented profoundly diminished LF content in cord blood granulocytes, as well as diminished numbers and abnormal morphology of electron-dense (specific) granules. Our findings confirm diminished LF content of cord blood as well as peripheral blood granulocytes of healthy neonates. However, unlike Ambruso et al., we were unable to demonstrate quantitative morphologic abnormalities of peroxidase-negative granules in profiles of unstimulated cord blood or neonatal cells. Differences observed among these two studies may reflect the application of different ultrastructural techniques. Despite its diminished cellular content, LF released by neonatal cells under conditions of chemotactic stimulation was comparable to that of healthy adult cells in both studies. Indeed, in response to a chemotactically relevant range of fMLP concentrations, we observed that the percentage of LF released by neonatal samples was actually enhanced as compared with healthy adult controls.

Diminished LF release by NGs was clearly documented with secretagogue stimulation and during stimulated cell spreading, conditions that promoted overall greater granule depletion than chemotactic stimuli (Figs 1 and 2). Based on the previously determined number of LF molecules required to saturate granulocyte surfaces, it can be estimated that chemotactic conditions applied in these studies would promote sufficient LF release to saturate cell surfaces of both NGs and healthy AGs. Thus, the relevance of diminished secretagogue-mediated LF release to diminished chemotactic function in neonates is uncertain. Assessments of LF release by NGs undergoing directed migration in Boyden chambers also demonstrated a comparable extent of release as compared with healthy adult cells (data not shown). Thus, although direct assessments of cell surface charge were not performed in these studies, it is unlikely that diminished LF cell surface deposition and consequent abnormalities of surface charge could account for abnormal stimulated adherence properties of NGs.

Previous reports have clearly documented that degranulating stimuli modulate the ability of the chemoattractant fMLP to bind to human neutrophils. Increased fMLP receptor expression and enhanced oxidative responsiveness to fMLP have been functionally related to preferential specific granule release.	extsuperscript{17,19} Such data support the concept that specific granules may represent a source of additional membrane and chemoattractant receptors that can be integrated into the cell membrane at its leading edge during granule–membrane fusion associated with directed locomotion in vitro or exudation in vivo.	extsuperscript{17} Our findings suggest a limited capacity of NGs to up-regulate fMLP receptors in response to PMA or A23187 under the same experimental conditions demonstrating their diminished LF release. However, baseline fMLP receptor expression when studied at 0°C was comparable among neonatal (cord blood) and healthy adult suspensions tested.	extsuperscript{1} If LF and fMLP receptors are packaged in the same intracellular compartments and mobilized to cell surfaces in a similar manner, these latter findings would suggest that diminished LF content of NGs did not result from in vivo or artificial in vitro specific granule release prior to our in vitro assessments. Further studies using fractionated granulocytes will be required to determine if abnormal surface expression results from diminished intracellular granular pools or as a result of abnormal translocation of fMLP receptors or other granule-associated constituents to cell surfaces. Our findings of diminished peroxidase-negative granule depletion under selected chemotactic conditions supports the latter possibility. These studies do not address the alternative possibility that NGs express only low-affinity as opposed to high-affinity fMLP receptors.	extsuperscript{45,46}

Our studies provide the first reported evidence that abnormal stimulated adherence and/or directed migration of NGs involves deficits of mobilization and/or surface expression of the adhesive protein, Mac-1 (iC3b receptor or CR-3). Mac-1, LFA-1, and p150,95 molecules constitute a family of structurally and functionally related human leukocyte surface glycoproteins that are critical determinants of cellular adhesive properties. Inherited deficiency of these glycoproteins has been recognized in a number of patients with recurrent bacterial infection, persistent granulocytosis, and profoundly diminished leukocyte mobilization into extravascular inflammatory sites (reviewed in ref 53).	extsuperscript{13,14} Adherence-dependent functional defects of patient leukocytes have been reproduced by incubating normal cells with MAbs directed at Mac-1 subunits.	extsuperscript{51-55} In unstimulated peripheral blood granulocytes, Mac-1 and p150,95 are present in an intracellular compartment as well as on the cell surface. Inflammatory mediators, including C5a and fMLP stimulate a three-to-seven-fold increase in Mac-1 and p150,95 on the cell surface. This up-regulation appears to be of great importance in regulating granulocyte and monocyte adhesiveness. Mac-1 has been shown to cosediment with secondary granules in fractionated granulocytes on sucrose gradients.	extsuperscript{23} Impaired granulocyte hyperadherence and motility associated with genetic deficiency of Mac-1/LFA-1/p150,95 appears to be related directly to impaired induction of surface Mac-1 and p150,95 glycoproteins.	extsuperscript{13,14}

Our findings demonstrate significantly diminished surface expression of Mac-1, β, and p150,95s (data not shown) on neonatal granulocytes exposed to chemotactic concentrations of fMLP. C5a (data not shown), as well as PMA and A23187 (data not shown). As also recently described by Bruce et al.,	extsuperscript{45} these deficits appear to be relatively specific for Mac-1 subunits since chemotactic induction of the CR-1
ADHERENCE PROPERTIES OF NEONATAL GRANULOCYTES

(C3b receptor) on NGs is normal. Such findings provide increasing evidence of important biologic differences between CR-1 and CR-3 with respect to function, intracellular anatomical location, and mechanisms regulating intracellular translocation and/or cell surface expression. On the basis of our MAb-inhibition studies, our findings suggest that impaired hyperadherence of NGs is directly related to impaired Mac-1α/β up-regulation. Findings of normal baseline expression and up-regulation of CR-1, CR-3, and CR-5 on NGs suggest a role for these mechanisms in NGs. Direct assessments of intracellular Mac-1 pools using fractionated cells will be required to evaluate these possibilities further.

The implications of our findings with respect to deficits of inflammation in human neonates are uncertain, but at least two distinct pathogenic mechanisms may be operative. Impaired chemotactic induction of the surface expression of Mac-1 or other adhesive molecules on NGs may contribute preferentially to disturbed cellular interactions with microvascular endothelium which, in turn, may diminish cellular localization at inflammatory sites. Alternatively, secretory abnormalities (lysosomal granule release, fMLP receptor up-regulation) recognized in our in vitro studies using relatively high stimulus conditions might be expected to contribute to deficits of motile, microbicidal, and/or cytotoxic functions of neonatal leukocytes infiltrating inflamed tissues. Confirmation and/or dissection of these other in vivo inflammatory aberrations should be possible with applications of molecular probes (ie, anti-Mac-1 MAB) in studies of neonatal animal models.

REFERENCES

26. Ambruso DR, Bentwood B, Henson PM, Johnston RB Jr: Oxidative metabolism of cord blood neutrophils: Relationship to...
Abnormal stimulated adherence of neonatal granulocytes: impaired induction of surface Mac-1 by chemotactic factors or secretagogues

DC Anderson, KL Freeman, B Heerdt, BJ Hughes, RM Jack and CW Smith