Abnormal FcRIII Expression by Neutrophils From Very Preterm Neonates

By Robert Carr and John M. Davies

To further investigate the neutrophil dysfunction of newborn infants, we have measured expression of the neutrophil Fcγ receptors FcRII and FcRIII in extremely immature preterm neonates born at 24 to 32 weeks of gestation. Fc receptor expression was measured by FACS analysis of cells stained with monoclonal antibody Leu1 b for FcRlll and IV-3 for FcRII. "Well" preterm neonates displayed reduced FcRIII, 51.05 ± 2.0 (mean fluorescence channel ± SE) when compared with term neonates, 69.24 ± 5.5 and adult controls, 71.83 ± 3.0. "Stressed" preterm neonates with severe respiratory distress syndrome or septicemia had a further downregulation of FcRIII, 32.67 ± 3.0 and 35.75 ± 1.8, respectively, associated with grossly abnormal cellular

Neonates have increased morbidity and mortality from bacterial infections, with the incidence of severe infection rising sharply in extremely immature preterm neonates born weighing less than 1,500 g.¹ The neutrophil polymorphonuclear leukocytes (PMNs) provide important defense against bacterial infection, and are functionally impaired in newborn infants.²³

In well term neonates the major neutrophil abnormality is impaired chemotaxis associated with an impaired ability to upregulate surface expression of the leukocyte adhesion complex Mac-1 (CR3, CD11/18).³ Phagocytosis and bacterial killing is normal in well term neonates, but is abnormal in stressed and low birth weight infants.²⁶ The study of infants with birth weights less than 1,500 g reported impaired phagocytosis at the time of birth that became normal during the first 2 weeks of life.⁴ Part of the impaired bactericidal capacity of neonatal neutrophils stems from reduced hydroxyl radicle generation by the respiratory burst. However, several studies have demonstrated that initiation of the respiratory burst by opsonized zymosan, as indicated by superoxide generation, is normal even in stressed infants.⁴⁰⁻¹²

Human neutrophils express two classes of Fcγ receptor that together mediate the binding, ingestion, and killing of bacteria. FcRII (CD32) is a 40-Kd transmembrane structure¹¹ that mediates immunoglobulin G (IgG)-induced phagocytosis,¹⁴,¹⁵ and initiates superoxide generation and the respiratory burst.¹⁵ Neutrophil FcRIII is a 50- to 70-Kd phosphoinositol-linked (PI-linked) structure¹⁷ that provides the major binding capacity of neutrophils for immune complexes and IgG-opsonized particles.¹⁴,¹⁵ FcRIII contributes toward antibody-dependent cytotoxicity,¹⁸ and has an additional activation function in that it is able to initiate phagocytosis of nonopsonized bacteria that possess lectin-like substances on their surface.¹⁹

To investigate the molecular basis of the impaired Ig-mediated neutrophil functions observed in preterm and stressed neonates, we have measured Fcγ receptor expression in extremely immature preterm neonates in a variety of clinical settings. We have identified reduced levels of FcRIII expression in clinical situations in which impaired phagocytosis and killing would be expected, while FcRII expression is less variable, corresponding to the reported normality of superoxide production. At the same time we have studied Mac-1 expression and chemotaxis as indicators of PMN activation and functional maturity.

MATERIALS AND METHODS

Study population. Twenty-seven preterm infants were studied soon after birth and at intervals during the first weeks of life. The median gestational age was 27 weeks (range 24 to 32 weeks), and median birth weight was 962 g (693 to 1,800 g). Neonates were defined as "well" if they had no evidence of infection and did not require mechanical ventilation for pulmonary insufficiency. "Stressed" neonates either required ventilation for severe respiratory distress syndrome (RDS) with the typical x-ray changes of hyaline membrane disease, or were clinically septicemic with positive blood cultures. The preterm infants were divided into groups for analysis: Group 1, well preterm neonates studied soon after birth at a median postnatal age of 1.5 days, (range 1 to 5), n = 12. Group 2, well preterm infants studied for a second time on median day 10 (7 to 20), n = 12; seven of these infants had been well from birth and five were previously stressed. Group 3, well preterm infants studied on median day 56 (42 to 70), n = 6. Group 4, stressed preterm neonates with RDS studied at a median postnatal age of 1.5 days (1 to 4), n = 8. Group 5, stressed preterm neonates with sepsis studied on median day 3 (1 to 21), n = 5. For comparison, nine healthy term neonates (group 6) were studied at the time of delivery by elective cesarian section, median gestational age 38 (38 to 40) weeks. All infants were studied in parallel with healthy adult controls (group 7).

The study was approved by and all blood sampling was in accordance with the Liverpool Maternity Hospital Ethical Committee guidelines.

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Isolation of PMNs. Polymorphonuclear leukocytes from preterm neonates were isolated from umbilical arterial catheter or peripheral venous samples; from term neonates using cord blood collected at birth; and from adults by venepuncture. Blood was collected into heparin 10 U/mL and PMNs separated on a sodium metrizoate/dextran gradient (Polyprep, Nycomed Ltd, Buckingham, UK). Residual red blood cells were removed by hypotonic lysis and resuspended in RPMI medium with Ca\(^{2+}\) and Mg\(^{2+}\) (Flow Laboratories, Hertfordshire, UK), the cell suspensions and all reagents being kept on ice. The purity and morphologic maturity of the neutrophil PMN preparations were assessed by visual inspection of cytopsin preparations. Viability, by trypan blue exclusion, was greater than 95% in all cases.

Preparation of reagents. Stock solutions of f-Met-Leu-Phe (fMLP) (Sigma Chemical Company Ltd, Poole, UK) were prepared in phosphate-buffered saline (PBS). Casein (Sigma) was prepared as a 5% stock solution in PBS and before use diluted 1:10 in Hank's Balanced Salt Solution (HBSS) (Sigma). Immune complexes containing human IgG (16 g/L) were prepared by incubating tetanus toxoid (28 Lf/mL) (Wellcome, Beckenham, UK) with human anti-tetanus Ig (200 IU/mL) (Blood Products Laboratory, Elstree, UK) for 1 hour at 37°C. Insoluble complexes were pelleted by centrifugation at 13,000g for 1 minute, washed once in PBS, and resuspended in the original volume of PBS.

Detection of PMN receptors. Fc\(a\)RIII (CD16) was detected using monoclonal antibody (MoAb) Leu11b (Becton Dickinson, Abingdon, Oxford, UK); Fc\(a\)RII (CD32) using MoAb IV-3b (a generous gift from Dr M.W. Fanger, Dartmouth Medical College, Hanover, NH); Mac-1 (CD11b) was detected using MoAb Leu15 (Becton Dickinson) and measured after stimulation with 10\(^{-3}\) mol/L fMLP for 15 minutes at 37°C. Cells were labeled for indirect immunofluorescence using saturating concentrations of MoAbs and FITC-conjugated F(ab')\(^2\) fragments of goat antiserum to human IgG (Wellcome, Beckenham, UK). Neutrophil-associated IgG and immune complexes were detected by FITC-conjugated F(ab')\(^2\), fragments of goat antiserum to human IgG (Kallestad, supplied by Northumbria Biologicals, Cramlington, UK). Surface-stained cells were fixed in 1% paraformaldehyde and analyzed on a Beckton Dickinson FACScan Analyzer I. Forward and 90° light scatter were used to identify and isolate the PMN population for analysis. Fluorescence intensity distribution of 5,000 cells was recorded on a 3-decade logarithmic scale, and the mean fluorescence channel for each cell population was calculated.

Chemotaxis. Chemotaxis through a 3-\(\mu\)m cellulose filter (Millipore Ltd, Harrow, UK) was measured in a modified Boyden chamber. PMNs, resuspended in HBSS with 1% bovine serum albumen, were placed in the upper chamber and migrated toward casein placed in the lower chamber. After a 90-minute incubation the leading front distance was measured on a calibrated microscope.

Statistical analysis. Results are expressed as cell population mean \pm SEM. Statistical significance of difference between groups was sought using Student's two-sample t-test.

RESULTS

Fc\(a\)RIII expression. The number of Fc\(a\)RIII receptors on the surface of PMNs was assessed using the MoAb Leu11b (CD16). The neutrophils from normal term babies at the time of birth (group 6) had Fc\(a\)RIII receptor numbers similar to those found on normal adult cells, and displayed a similar bimodal distribution of fluorescence intensity (Figs 1 and 2A, b). Well preterm neonates studied at or soon after birth (group 1) showed significantly reduced expression of Fc\(a\)RIII compared with term neonates (P = 0.02) and adults (P < 0.001) (Fig 1, Table 1). The fluorescence distribution curve was broader, representing a wider variation in receptor number from cell to cell, but the distribution of brightly fluorescent cells was symmetrical and the normal bimodal pattern was maintained (Fig 2A, c). Sequential postnatal study of well preterm infants (groups 2 and 3) showed a progressive increase in Fc\(a\)RIII expression, with normal adult values being established by the third postnatal week (Fig 1, Table 1).

Clinically stressed infants, either being ventilated for severe RDS (group 4) or ill with bacteriologically proven sepsis (group 5), showed gross downregulation of Fc\(a\)RIII expression compared with healthy infants of similar age and maturity. Not only was the mean fluorescence significantly reduced (P < 0.001) (Fig 1), but the fluorescence distribution curves were broad and skewed to the left with loss of the bimodal pattern, representing a progressive reduction in receptor expression by a proportion of the cell population (Fig 2A, d). This pattern was seen in infants with severe RDS when tested soon after birth and developed again in preterm neonates who became septic during their second or
Fluorescence intensity (log scale)

Fig 2. Representative fluorescence distribution curves of PMNs labeled with MoAb to (A) FcRIII and (B) FcRII from: (a) adult control; (b) healthy term neonate; (c) "well" preterm neonate, soon after birth; (d) "stressed" preterm neonate with severe RDS. Note the large FcRIII "dull" peak in the term neonate (b) due to 15% eosinophils and the loss of a well-defined "bright" FcRIII peak in the "stressed" preterm neonate (d).

subsequent postnatal weeks, after normal FcRIII had been established. Fluorescence intensity and distribution returned to normal as the clinical state of the infant improved.

Chemotaxis and Mac-1 expression. Chemotaxis through a cellulose filter and the ability to upregulate Mac-1 expression in response to fMLP stimulation, as detected by MoAb Leu15 (CD11b), were measured and related to FcRIII on PMNs in the different neonate populations. Well preterm neonates had impaired chemotactic responses and reduced stimulated expression of Mac-1 at birth. During the first 3 postnatal weeks, when FcRIII developed normal levels of expression, chemotaxis and the ability to upregulate Mac-1 improved in parallel, although neither achieved adult levels over this period (Table 2).

In contrast, stressed neonates with severe RDS who displayed downregulation of FcRIII tended to have increased stimulated Mac-1 expression, and had significantly enhanced chemotactic ability when compared with well infants of similar age and maturity ($P < .001$) (Fig 3).

Morphologic maturity and purity of neonatal PMN preparations. Neonates, especially when clinically stressed, have increased numbers of circulating immature myeloid cells. FcRIII, as identified by Leu11b, does not develop full expression on neutrophils until the late metamyelocyte stage. The increase in immature forms might account for some of the observed changes. In groups 1, 4, and 5, with impaired FcRIII expression, the percentage of bands and more immature myeloid cells in the purified PMN preparations was $18.7 \pm 3.5$. In groups 2, 3, and 6, where FcRIII expression was at or near adult levels, the percentage of immature myeloid cells was $16.3 \pm 3.0$. There was no correlation between intensity of FcRIII expression and the number of bands and more immature myeloid cells in the PMN preparations ($r = -0.59$).

FcRIII has only minimal expression on eosinophils. The proportion of eosinophils contaminating the PMN prepar-

<table>
<thead>
<tr>
<th>Study Group (postnatal age)</th>
<th>FcRIII</th>
<th>FcRII</th>
</tr>
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<tbody>
<tr>
<td>1. Well preterm (day 1–5)</td>
<td>51.05</td>
<td>30.95</td>
</tr>
<tr>
<td>2. Well preterm (day 7–20)</td>
<td>68.00</td>
<td>28.99</td>
</tr>
<tr>
<td>3. Well preterm (day 42–70)</td>
<td>74.78</td>
<td>27.92</td>
</tr>
<tr>
<td>4. Stressed: RDS (day 1–4)</td>
<td>32.67</td>
<td>25.94</td>
</tr>
<tr>
<td>5. Stressed: septic (day 1–21)</td>
<td>35.75</td>
<td>28.73</td>
</tr>
<tr>
<td>6. Term neonates (day 1)</td>
<td>69.24</td>
<td>35.69</td>
</tr>
<tr>
<td>7. Adult controls</td>
<td>71.83</td>
<td>32.16</td>
</tr>
</tbody>
</table>

There was no significant difference between any study group for FcRII expression. See text for discussion of FcRII.

*Group mean and (SE) calculated from pooled individual mean fluorescence channel values.

Table 2. Progressive Postnatal Improvement in FcRIII Expression, Ability to Upregulate Mac-1, and Chemotaxis in Well Preterm Neonates

<table>
<thead>
<tr>
<th>Study Group (postnatal age)</th>
<th>Mean Fluorescence*</th>
<th>Chemotaxis (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FcRIII</td>
<td>FcRII</td>
</tr>
<tr>
<td>1. (day 1–5)</td>
<td>51.05</td>
<td>64.00</td>
</tr>
<tr>
<td>2. (day 7–20)</td>
<td>68.00</td>
<td>75.09</td>
</tr>
<tr>
<td>3. (day 42–70)</td>
<td>74.78</td>
<td>110.33</td>
</tr>
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Significance levels are for unpaired data. When the seven neonates, well at birth and for whom day 7 to 20 data are available are subjected to paired analysis, the level of significance is increased.

*Group mean and (SE) calculated from pooled individual mean fluorescence channel values.
Fig 4. Adult neutrophils were incubated with dilutions of immune complex suspension (1:25 to 1:1,600) for 30 minutes at 0°C. The cells were washed and labeled with F(ab') fragments of goat anti-human IgG (A) to quantitate adsorbed IgG. The fluorescence intensity plotted for each study group is shown.

DISCUSSION

The mechanisms underlying the defective bactericidal activity of neutrophils from neonates remain unclear. This study has demonstrated reduced expression of FcRIII on neutrophils from preterm and stressed neonates in clinical situations in which phagocytosis and killing are reported to be abnormal. In contrast, FcRII expression did not vary significantly from adult levels in any study group, which corresponds with the reported observation that the initiation of the respiratory burst remains normal, even in stressed infants.

The molecular basis for this reduced neutrophil FcRIII expression, as detected by Leu11b, is unknown. We have excluded the possibility that our observations were due to increased numbers of eosinophils, morphologically immature neutrophils, or the receptors being occupied by Ig or immune complexes. Neutrophil FcRIII is a PI-linked molecule that is
actively synthesized by mature cells\textsuperscript{22,23} and is shed after cell activation.\textsuperscript{24} The parallel improvements in the expression of FcRIII and Mac-1, and in chemotaxis during the early postnatal life of well preterm infants, would seem to suggest a fundamental process of cell maturation. On the other hand, the downregulation of FcRIII that occurs in stressed infants, who simultaneously display upregulated Mac-1 and enhanced chemotaxis, raises the possibility that partial cell activation in vivo may have led to increased FcRIII shedding.

It is not possible to predict from these preliminary observations to what extent the reduced numbers of FcRIII receptor on the cell surface might contribute to the impaired functional responses of neonatal neutrophils. Prediction of how function might be affected in vivo is further complicated by the hypogammaglobulinemia and reduced opsonic capacity of neonates born at less than 32 weeks gestational age.\textsuperscript{25}

While FcRII is the principle Fc\gamma receptor mediating IgG-induced functional activation, Huizinga et al\textsuperscript{15} have demonstrated the importance of IgG binding first to FcRII to increase its affinity for FcRII. FcRIII has the additional role of mediating opsonin-independent phagocytosis.\textsuperscript{19} Therefore, it may be that in conditions of suboptimal opsonization, the number of FcRIII molecules available becomes critical for normal neutrophil activity.

ACKNOWLEDGMENT

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REFERENCES