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Expression of the Complement Receptors CR1 and CR3 and the Type III Fc_γ Receptor on Neutrophils from Newborn Infants and from Fetuses with Rh Disease

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ABSTRACT. Developmental defects in neutrophil function, including diminished expression of plasma membrane receptors, may play an important role in the susceptibility of the newborn infant to infection. We used monoclonal antibodies and flow cytometry to study the expression of complement receptor type one (CR1), complement receptor type three (CR3), and Fc_γ receptor type three (FcR_{III}) on neutrophils from six fetuses with Rh disease, 10 preterm infants, nine term infants, and nine adults. Expression of the complement receptors on unstimulated cells was similar for all groups, but significant differences in complement receptor expression were observed after stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Fetal, preterm, and term infant neutrophils expressed less CR3 than FMLP-stimulated neutrophils of adults [61 ± 2, 48 ± 4, and 66 ± 4% (mean ± SEM) of the mean for adults, $p < 0.05$]. FMLP-stimulated CR1 expression for these groups was 61 ± 6, 73 ± 6, and 91 ± 9% of the adult mean ($p < 0.05$, fetal *versus* term infant and adult). Expression of both CR3 and CR1 increased with postconceptional age in the infants ($r^2 = 0.49$, $p < 0.001$ for CR3; $r^2 = 0.23$, $p < 0.05$ for CR1). Neutrophils of the preterm and term infants expressed less FcR_{III} than adult neutrophils (68 ± 10 and 77 ± 7% of the adult mean, $p < 0.05$, for FMLP-stimulated cells), whereas fetal neutrophil FcR_{III} expression did not differ from that of the adult. The fluorescence distributions showed a peak for eosinophils that was distinct from the FMLP-stimulated neutrophil peak, allowing separate analysis for the two cell types. Eosinophils constituted an unexpectedly large proportion of granulocytes in fetuses with Rh disease, averaging 61% of granulocytes in seven specimens at 20 to 26 wks gestation and 37% in 11 specimens at 28 to 34 wks. (*Pediatr Res* 28: 120–126, 1990)

Abbreviations

CR1, complement receptor type one (CD35)
CR3, complement receptor type three (CD11b/CD18)
FcR_{III}, Fc_γ receptor type three (CD16)
FMLP, *N*-formyl-methionyl-leucyl-phenylalanine

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Human neonates, especially those born prematurely, are highly susceptible to serious infections with bacterial, viral, and fungal pathogens, including some that do not ordinarily cause systemic disease in immunocompetent older children or adults (1). In concert with developmental deficiencies in other parts of the immune system, abnormalities of neutrophil function play an important role in the enhanced susceptibility of newborn infants to infection (2). Study of fetal neutrophils, made possible by *in utero* sampling of umbilical cord blood (3), may provide new insight into the interplay of developmental and environmental effects on neonatal neutrophil function.

Neutrophils from newborn infants have well-recognized defects in adherence-related functions, including an impaired ability to increase adherence after stimulation with chemotactic factors (4). Although the mechanisms responsible for this defect are incompletely understood, abnormal upregulation of plasma membrane expression of CR3 may be an important factor (5). Neutrophils from term infants stimulated with chemotactic factors such as FMLP express significantly less CR3 than neutrophils from adults (5, 6), and this impairment in stimulated expression of CR3 is correlated with the degree of impairment in stimulated adherence (5).

In addition to its role in adherence, CR3 is an important opsonic receptor, with specificity for the C3bi component of complement. Other neutrophil opsonic receptors include CR1, with primary affinity for C3b (7), and receptors for the Fc portion of IgG (8–10). In contrast to CR3, CR1 expression of stimulated neutrophils from term infants is not diminished compared with adult neutrophils (5–6). To date, there have been no studies of these receptors on neutrophils from preterm infants.

The purpose of our study was to measure the resting and FMLP-stimulated expression of CR1, CR3, and FcR_{III} on neutrophils from preterm infants and from a group of human fetuses with suspected Rh disease in comparison with neutrophils from term infants and adults. We found significant differences between adult and fetal or preterm infant neutrophils for all three receptors. We also found that eosinophils constituted an unexpectedly large proportion of the cord blood granulocytes in fetuses with Rh disease at 20 to 30 wk of gestation.

MATERIALS AND METHODS

Patient populations. Human fetal blood samples were obtained during clinically-indicated percutaneous umbilical cord sampling under ultrasound guidance. Fetal wt and gestational age were

estimated by ultrasound examination before each sampling. In fetuses receiving intrauterine transfusions, samples for analysis were obtained before the transfusion was given, and at least two weeks after any prior transfusion. Premature infants in the neonatal intensive care unit were studied within the first few weeks of life during recovery from respiratory distress syndrome. Blood samples were obtained via indwelling catheter or by venipuncture. Blood from infants at term was obtained from the placental side of the umbilical cord immediately after delivering the infant and clamping the cord. Gestational ages of preterm and term infants were obtained from the obstetrical history and the Dubowitz examination. Postconceptional age was defined as the gestational age (with menstrual dating) plus the postnatal age. Samples from the adult volunteers were obtained by venipuncture. Informed consent for blood samples was obtained in accordance with approved institutional protocols.

Analysis of receptor expression was performed on specimens from six fetuses with Rh disease at estimated wt of 312 to 2212 g (mean 1060 g). Sequential specimens were studied for two fetuses, with four specimens obtained from one and three from the other, at intervals of 3 or more wk. CR3 expression was studied on 10 specimens from five fetuses (three males) at 22 to 34 wk (mean 28.7 wk), CR1 expression was studied on eight specimens from five fetuses (three males) at 21 to 34 wk (mean 28.8 wk), and FcR_{III} expression was studied on seven specimens from three fetuses (two males) at 25 to 34 wk of gestation (mean 29.5 wk). Three of the fetuses had only mild anemia and never required transfusions. Two received repeated intrauterine transfusions and were healthy at delivery. One did not receive the recommended transfusions and developed severe anemia. Ten preterm infants (nine males) with birth wt ranging from 700 to 2200 g (mean 1380 g) were studied at 1 to 17 d postnatal age (mean 7.8 d) and at postconceptional age of 26 to 35 wk (mean 30.5 wk). All of the infants were recovering from respiratory distress syndrome, and had no acute illness at the time of sampling. Three had had only mild respiratory distress and were never intubated. Five were still intubated at the time of the study. All but one had received antibiotics, but none had positive blood cultures, and none had any sign of active infection at the time of study. Cord blood was obtained from nine healthy infants born at term (three males), with birth wt ranging from 2990 to 4180 g. Six were delivered operatively (five because of prior cesarean section, one for breech presentation). Blood was also obtained from nine healthy adult donors (seven males) as experimental controls.

The cell-sorting and granulocyte differential experiments used specimens from 13 additional fetuses who underwent umbilical blood sampling at 18 to 38 wk for a variety of clinical indications: Rh disease (seven fetuses, 20 to 31 wk), congenital anomalies (two fetuses, 18 and 20 wk), nonimmune hydrops fetalis (one fetus, 28 wk), hemolysis due to anti-Kell antibodies (one fetus, 31 wk), and isoimmune thrombocytopenia (two fetuses, 34 and 38 wk).

Materials. All buffer solutions used Hank's Balanced Salt Solution without bicarbonate or phenol red and contained 5% by volume heat-inactivated FCS (GIBCO Laboratories, Grand Island, NY). Buffers were free of Ca and Mg except as specified. Stock solutions were adjusted to pH 7.35–7.40, passed through a 0.22-μm filter, and maintained sterile at 4°C. Aliquots of FMLP (Sigma Chemical, St. Louis, MO) were stored at −30°C at a concentration of 2×10^{-3} M in dimethylsulfoxide and diluted to the working concentration in buffer with Ca and Mg just before use.

Monoclonal mouse antihuman CR3 (anti-Leu-15) and antihuman CR1 antibodies were purchased from Becton Dickinson (Mountain View, CA). Anti-Fc receptor antibody (clone 3G8) was the generous gift of Dr. Jay C. Unkeless (8). Aliquots from a single lot of fluorescein-conjugated goat $F(ab')_2$ antimouse IgG (Tago, Inc., Burlingame, CA) were stored at −30°C. Monoclonal mouse isotype-specific control antibodies (IgG_1 for anti-CR1 and 3G8, and IgG_{2a} for anti-CR3) were purchased from Coulter Corp. (Hialeah, FL). Fluorescent beads measuring 10 μm (2% bright) from Coulter were used as a fluorescent reference standard for flow cytometry.

Specimen collection and processing. Blood samples were transferred into EDTA-anticoagulated tubes and immediately placed on ice. Samples were maintained at 0 to 4°C throughout the procedure, except for the stimulation step as specified below. Red blood cells were removed by two cycles of hypotonic lysis at 0°C. After centrifugation at $250 \times g$ for 10 min, the supernatant was discarded and the cell pellet resuspended in 1 mL of buffer and counted. Aliquots of the cell suspension containing 10^5 polymorphonuclear cells were then placed in replicate pairs of 12×75 mm polypropylene tubes. One tube of each pair was an unstimulated control, which contained 1 mL of buffer without Ca or Mg and was kept at 0°C. The duplicate tube was incubated with 10^{-6} M FMLP for 30 min at 37°C in 1 mL of buffer containing Ca and Mg.

After the incubation, the specimens were placed back on ice and 1 mL of cold buffer with 0.1% sodium azide (Sigma) was added to each tube. After centrifugation at $200 \times g$ for 5 min at 4°C, the supernatants were removed and the cells resuspended in 0.1 mL of the azide buffer. Aliquots of 100 μL of solutions in azide buffer of the monoclonal antibodies and isotype controls were then added to replicate tubes of control and stimulated cells. The concentrations used, 5.0 μg/mL for 3G8 and 2.5 μg/mL for the others, were determined in preliminary experiments to provide saturating amounts of antibody for 10^5 FMLP-stimulated adult neutrophils. The cell suspensions were thoroughly mixed, and after reaction for 30 min at 0°C with intermittent agitation, the cells were washed, centrifuged, and resuspended in 0.1 mL of azide buffer. A 50-μL aliquot of a 1:20 dilution of the fluorescein-conjugated goat antimouse IgG antibody was then added and the suspension incubated for 30 min at 0°C, with intermittent agitation. After a final wash, the cells were resuspended in 0.3 mL of azide buffer for flow cytometric analysis.

Flow cytometry. Cell analysis was performed using an Epics C Cell Sorter (Coulter) with a 5000 mW Argon laser operating at 315 mW at a wavelength of 488 nm. Each measurement was based on an accumulation of signals from 2500 granulocytes, determined by gating the characteristic region of the forward *versus* 90° light scattering distribution. Histograms for green fluorescence intensity were recorded simultaneously on both linear and logarithmic scales, with the gain for the fluorescence signal amplifier adjusted for each sample to the maximum setting that produced no accumulation in channel 256 of the linear distribution. The photomultiplier voltage was maintained constant at 1400 mV. The mean linear channel fluorescence (MLF) of aliquots from a single lot of fluorescent beads was used as a reference standard. The mean relative fluorescence (RF) of a sample was expressed as a percentage of the reference bead fluorescence according to the formula

$$RF = 100 \times (\text{sample MLF}/\text{sample gain}) \times (\text{bead MLF}/\text{bead gain})^{-1} - RF_O$$

where RF_O was the background relative fluorescence of the monoclonal isotype control. The cutoff for positive fluorescence was defined as the 99th percentile of the distribution for the isotype control-labeled cells. For all specimens, the mean fluorescence for neutrophils was determined by limiting the analysis to the region of the distribution containing the neutrophil peak, to exclude consistently the region containing the eosinophil peak in the fetal specimens.

Cell sorting and granulocyte differentials. For the cell-sorting experiments, fetal cell suspensions were stimulated with FMLP, labeled with anti-CR3 and the fluorescein-conjugated second antibody, and then fixed in PBS containing 1% paraformaldehyde and 2% FCS. An Epics V Cell Sorter (Coulter) was used to isolate cells from the two peaks of the log-fluorescence distri-

bution. Because the vibrations used to induce droplet formation cause significant distortion of the 90° light scatter signal, gating was performed with the forward light scatter and log-fluorescence distributions only. The sorting windows were adjusted to exclude the small region of overlap between the two peaks of the fluorescence distribution. For granulocyte differentials, aliquots of cell suspensions were cytocentrifuged onto microscope slides and stained with a water-soluble modification of Wright's stain (Leukostat, Fisher Scientific, Pittsburgh, PA). The relative proportions of eosinophils and neutrophils in each specimen were determined by counting at least 200 granulocytes.

Statistics. Analysis of variance was used to test for differences among the four groups of subject specimens. When significant intergroup variation was found, differences among the groups were assessed with the Newman-Keuls multiple comparison test (11). Except where indicated otherwise, the reported p values are those of the Newman-Keuls test. Results are expressed as the mean ± SEM. The StatView 512+ (Brainpower Inc., Calabasas, CA) and CLR ANOVA (Clear Lake Research, Houston, TX) programs for the Macintosh computer (Apple Computer, Cupertino, CA) were used.

RESULTS

Neutrophil CR3 expression. Figure 1 shows representative log-fluorescence histograms for CR3-labeled granulocytes from adult and fetal blood. The histogram for unstimulated adult cells is shown in Figure 1*A*. After FMLP stimulation (Fig. 1*B*), the peak of the distribution was shifted to the right with a 16-fold increase in mean linear fluorescence. Distributions for the preterm and term infant granulocytes (not shown) were qualitatively similar to the adult distributions, with a single major peak both before and after stimulation with FMLP. The unstimulated fetal distribution (Fig. 1*C*) was similar to the unstimulated adult distribution, but the fluorescence distribution for the FMLP-stimulated fetal cells (Fig. 1*D*) had two well-separated peaks at fluorescence values distinct from the single peak of the adult distribution. As detailed below, the bright peak of the fetal CR3 distribution was shown to contain neutrophils, and the dim peak to contain eosinophils. This enabled us to analyze CR3 expression of FMLP-stimulated neutrophils separately from that of eosinophils.

The results for mean CR3 fluorescence for FMLP-stimulated neutrophils are shown in Figure 2. Mean CR3 expression of

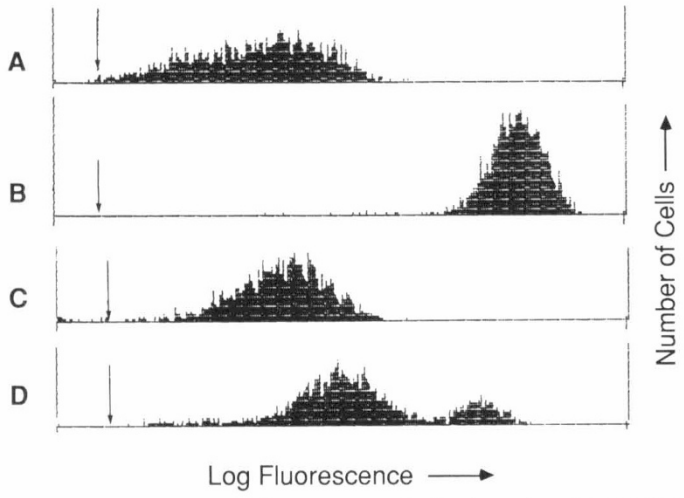

Fig. 1. Representative fluorescence distributions for CR3-labeled granulocytes from an adult and a 25-wk fetus. The *arrow* indicates the cutoff for the negative antibody control. *A*, adult, unstimulated. *B*, adult, FMLP-stimulated. *C*, fetal, unstimulated. *D*, fetal, FMLP-stimulated. The distribution for the FMLP-stimulated fetal cells shows distinct peaks for neutrophils (*right*) and eosinophils (*left*).

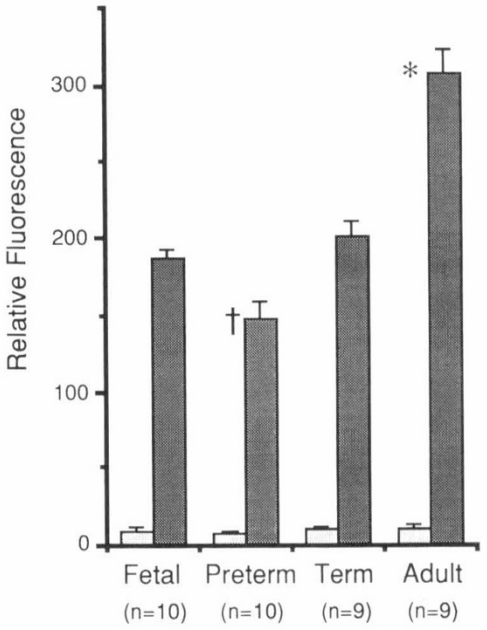

Fig. 2. CR3 expression (mean ± SEM) of FMLP-stimulated neutrophils (*shaded bars*) for the four study groups. n is the number of specimens for each group. * $p < 0.05$, adult *versus* term infant and $p < 0.01$ adult *versus* fetal and preterm infant. † $p < 0.01$, preterm *versus* term infant and $p < 0.05$, preterm infant *versus* fetal. CR3 expression of unstimulated granulocytes (*lightly stippled bars*) did not differ among the groups.

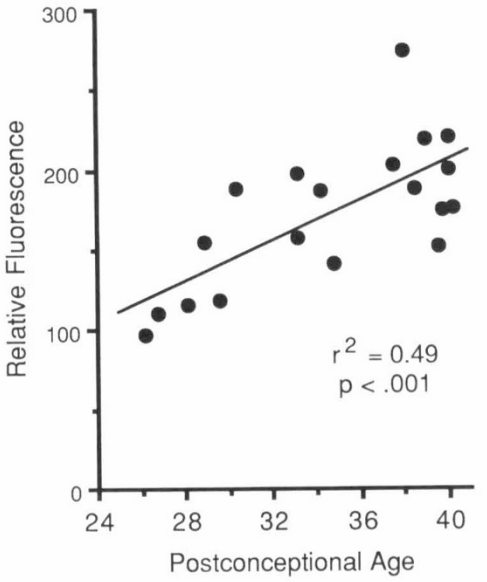

Fig. 3. Variation with postconceptional age of CR3 expression on FMLP-stimulated neutrophils from preterm and term infants.

FMLP-stimulated neutrophils increased by a factor of 10 or more in all groups compared with the values for unstimulated granulocytes, which did not differ among the groups. CR3 expression of FMLP-stimulated neutrophils for the fetal, preterm, and term infant groups was 61 ± 2, 48 ± 4, and 66 ± 4%, respectively, of the mean for adult neutrophils (each $p < 0.05$ *versus* adult). CR3 expression of the preterm infant neutrophils was significantly less than that of the term infant and fetal neutrophils ($p < 0.01$ and $p < 0.05$, respectively). As shown in Figure 3, there was a significant increase in CR3 expression with postconceptional age in the infants ($p < 0.001$, $r^2 = 0.49$). In contrast, there was no significant correlation with gestational age in the fetal group.

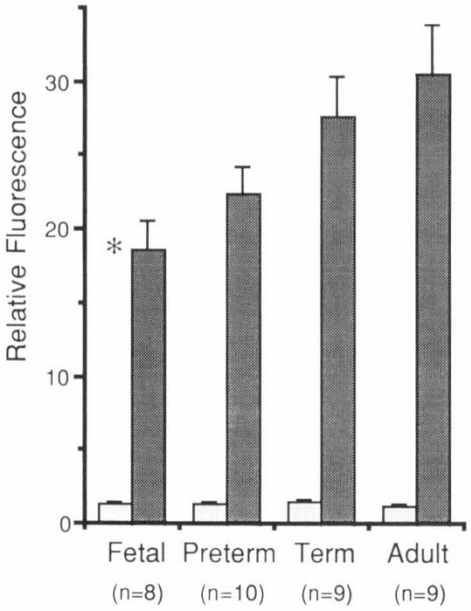

Fig. 4. CR1 expression (mean ± SEM) of FMLP-stimulated neutrophils (*shaded bars*) for the four study groups. *n* is the number of specimens for each group. * $p < 0.05$, fetal *versus* term infant and adult. CR1 expression of the unstimulated granulocytes (*lightly stippled bars*) did not differ among the groups.

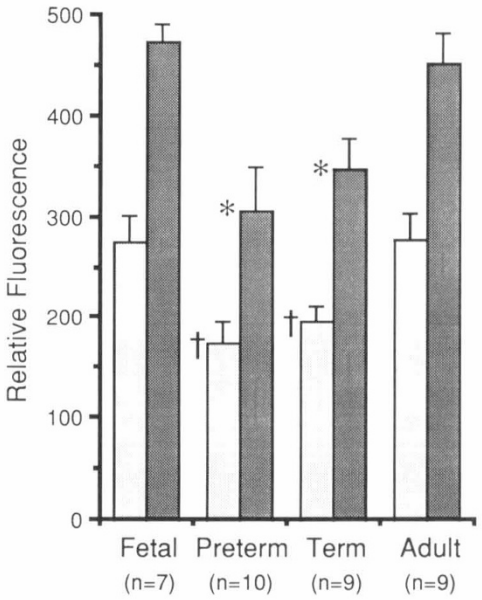

Fig. 6. FcR_{III} receptor expression (mean ± SEM) of unstimulated neutrophils (*lightly stippled bars*) and of FMLP-stimulated neutrophils (*shaded bars*) for the four study groups. *n* is the number of specimens for each group. * $p < 0.05$, preterm and term infant *versus* fetal and adult FMLP-stimulated cells. † $p < 0.05$, preterm and term infant *versus* fetal and adult unstimulated cells.

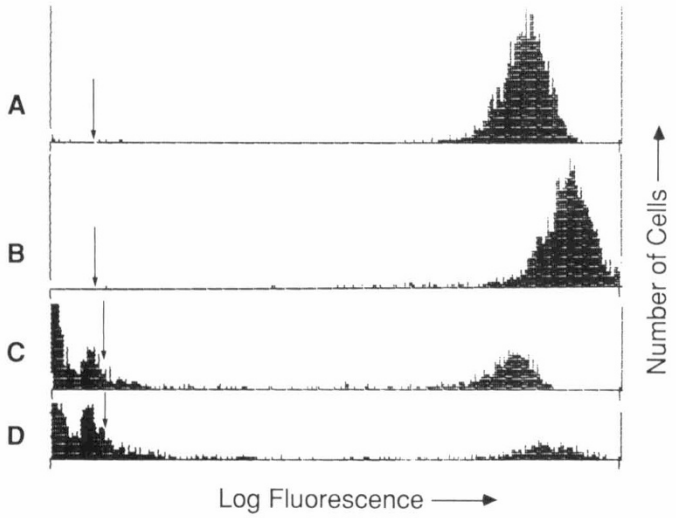

Fig. 5. Fluorescence distributions of FcR_{III}-labeled granulocytes from the same adult and 25-wk fetus as in Figure 1. The *arrow* indicates the cutoff for the negative antibody control. *A*, adult, unstimulated. *B*, adult, FMLP-stimulated. *C*, fetal, unstimulated. *D*, fetal, FMLP-stimulated. Distinct peaks for neutrophils (*right*) and eosinophils (*left*) are seen in the fetal distributions both before and after stimulation with FMLP.

Neutrophil CR1 expression. The fluorescence distributions for CR1 were similar to those for CR3: the FMLP-stimulated fetal granulocyte distributions, but not those of the other groups, had distinct peaks corresponding to neutrophils and eosinophils. CR1 expression of FMLP-stimulated neutrophils increased by a factor of eight or more in all groups compared with the values for unstimulated granulocytes, which did not differ among the groups (Fig. 4). FMLP-stimulated CR1 expression of the fetal, preterm infant, and term infant neutrophils was 61 ± 6, 73 ± 6, and 91 ± 9%, respectively, of the adult mean ($p < 0.05$, fetal *versus* term infant and adult). Although the difference between the preterm and term infant group means was not significant, there was a significant increase in stimulated CR1 expression with postconceptional age in the infant groups ($p < 0.04$, $r^2 =$ 0.23). There was no significant change in CR1 expression of the fetal neutrophils with gestational age.

Neutrophil FcR_{III} expression. A typical distribution for unstimulated adult granulocytes labeled with anti-FcR_{III} is shown in Figure 5*A*. The unstimulated neutrophils were brightly labeled, and the increase that occurred after stimulation with FMLP (Fig. 5*B*) was much less dramatic than for the complement receptors. The unstimulated fetal granulocyte distribution, shown in Figure 5*C*, had a large population with negative or very low fluorescence (subsequently identified as eosinophils), and a small neutrophil peak located at nearly the same high fluorescence values as the unstimulated adult peak in Figure 5*A*. After FMLP stimulation (Fig. 5*D*), the fetal neutrophil peak shifted to increased fluorescence values, but there was no change in the low fluorescence population.

The mean FcR_{III} fluorescence values for neutrophils are shown in Figure 6. In contrast to the complement receptors, FcR_{III} expression of neutrophils could be distinguished from that of eosinophils in unstimulated as well as FMLP-stimulated specimens. Neutrophils of all groups increased FcR_{III} expression significantly after FMLP stimulation ($p < 0.001$). The mean increase was 72 ± 5% and did not differ significantly among the groups. For both unstimulated and FMLP-stimulated cells, preterm and term infant neutrophils expressed significantly less FcR_{III} than the adult neutrophils ($p < 0.05$). Unstimulated neutrophils of preterm and term infants expressed 63 ± 8 and 70 ± 5%, respectively, of the mean for unstimulated adult neutrophils. After stimulation, the corresponding values were 68 ± 10 and 77 ± 7%. Fetal neutrophil expression of FcR_{III} was significantly greater than that of the preterm and term infant neutrophils ($p < 0.05$), and did not differ from adult neutrophils (99 ± 9 and 105 ± 4% of the adult mean, for unstimulated and stimulated cells). There was no significant change in neutrophil FcR_{III} expression with postconceptional age in the infant or fetal groups.

Fetal neutrophils and eosinophils. As illustrated in Figures 1 and 5, distinct subpopulations of fetal granulocytes were evident in the complement receptor distributions after stimulation with FMLP, and in both the control and stimulated FcR_{III} distributions. For a given specimen, nearly the same proportion of cells

was found in the bright peak of the distribution for each receptor. Thus, among all specimens, the percentage of cells in the bright peak of the CR1 distribution was highly correlated with the percentage of CR3-bright cells, with a slope of 0.91 and $r^2 = 0.88$. For the proportion of bright cells in the FcR_{III} distribution compared with those in the CR3 distribution, the slope was 0.87 with $r^2 = 0.87$. This implied that the brightly labeled subpopulation of cells was the same for all three antibodies, and, similarly, that the cells with low or absent expression of FcR_{III} were the cells more dimly labeled with anti-CR1 and anti-CR3. No changes in the distributions were seen when granulocytes isolated by centrifugation through Ficoll-Hypaque were compared with the whole white blood cell preparation from the same fetus, which ruled out the possibility that one of these peaks represented monocytes.

We initially thought that the dim peak in the receptor distributions represented a subpopulation of neutrophils, inasmuch as the number of eosinophils on whole blood smears appeared to be too small to account for this peak. However, we subsequently realized that the differential counts performed on the whole blood smear could be inaccurate because of the very small number of granulocytes present in fetal blood and the susceptibility of the eosinophil to mechanical damage during the preparation of the smear (12). If present in sufficient numbers, eosinophils could account for the dimly-labeled FcR_{III} subpopulation, inasmuch as eosinophils lack FcR_{III}, or express it at very low levels compared with neutrophils (9, 13). In addition, eosinophils would account for the minimal increase in CR1 and CR3 expression by the cells in the dim peak, inasmuch as eosinophils lack a specific receptor for FMLP, and FMLP exposure fails to stimulate eosinophils, as determined by chemotaxis, membrane potential change, or nitro blue tetrazolium reduction (14).

To determine whether the dimly-labeled subpopulation did indeed consist of eosinophils, we performed cell-sorting studies on blood specimens from two additional fetuses with Rh disease, at 28 and 31 wks gestation. The cells were stimulated with FMLP, labeled with anti-CR3, and analyzed by flow cytometry as before. The proportions of cells in the dim peaks of the fluorescence distributions of the two specimens were 49 and 35%. Counts of cytocentrifuge preparations of the same specimens showed that eosinophils constituted 50 and 34% of the granulocytes, respectively. Thus, the proportion in the dim peak was nearly identical to the percentage of eosinophils counted directly. Next, cells from each peak of the fluorescence distributions were isolated using a cell sorter, and cytocentrifuge preparations of the sorted cells were made. Eosinophils constituted 87 and 93% of the granulocytes from the dim peaks, whereas 97 and 99% of granulocytes sorted from the bright peaks were neutrophils. Thus, the bright peaks contained neutrophils almost exclusively and the dim peaks contained eosinophils with a small admixture of neutrophils.

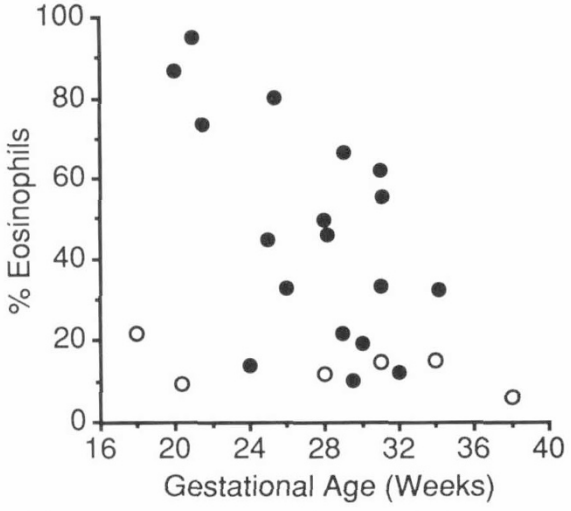

Fig. 7. The percentage of eosinophils among fetal granulocytes as a function of gestational age for fetuses with Rh disease (*closed circles*) and with other conditions (*open circles*).

Additional data on the proportion of eosinophils in fetal blood were obtained from cytocentrifuge granulocyte differentials of specimens from five more fetuses with Rh disease and six with other conditions. Figure 7 shows the percentage of eosinophils in these specimens and the two studied by cell-sorting, along with the data derived from the complement receptor distributions for the 11 specimens of the original group of fetuses with Rh disease. In fetuses with Rh disease, the proportion of eosinophils was markedly elevated in the specimens studied at 20 to 22 wk, and decreased with advancing gestation, ($p < 0.02$, $r^2 = 0.34$). The proportion also declined in successive specimens from each of the fetuses with Rh disease studied sequentially, one of whom was never transfused. Among fetuses with Rh disease, eosinophils constituted an average of 61% of the granulocytes in single specimens from seven fetuses at 20 to 26 wk of gestation, and an average of 37% in 11 specimens from eight fetuses at 28 to 34 wk. In contrast, eosinophils averaged 13% of granulocytes in the six specimens from fetuses with other conditions.

To determine whether the high proportion of eosinophils observed in fetuses with Rh incompatibility was related to the severity of disease, we compared fetuses who required one or more intrauterine transfusions with fetuses with mild Rh disease who were not transfused. The proportion of eosinophils was significantly greater in the fetuses who required transfusion (57 *versus* 30%, $p = 0.04$). This was not attributable to a difference in the time of sampling, as the gestational ages of the specimens from transfused and nontransfused fetuses were not significantly different (mean of 27.9 *versus* 28.0 wk, respectively). Transfusion per se did not appear to be responsible for the difference, since high eosinophil percentages were observed in several fetuses before any transfusion.

DISCUSSION

In these experiments, neutrophils from preterm infants expressed significantly less CR3 on the plasma membrane in response to stimulation with FMLP than did neutrophils from term infants, which expressed less CR3 than neutrophils from adults. Neutrophils from both preterm and term infants expressed less FcR_{III} than neutrophils from adults and neutrophils from fetuses with Rh disease. The fetal neutrophils expressed CR3 at levels comparable to neutrophils from term infants, but expressed less CR1 than neutrophils from term infants and adults. We observed that the fluorescence distributions for the FMLP-stimulated fetal granulocytes had two distinct peaks, and showed that these represented neutrophils and eosinophils. Eosinophils constituted a surprisingly large proprotion of granulocytes from the fetuses with Rh disease.

Previous studies of neutrophils from term infants by Bruce *et al.* (6) and Anderson *et al.* (5) reported diminished FMLP-stimulated expression of CR3 compared with neutrophils from adults, but no difference for CR1. We also found no difference in CR1 expression between term infant and adult neutrophils, and our result for CR3 expression of term infant neutrophils was in close agreement with these studies. Contrary results were reported by Adinolfi *et al.* (15), who found greater expression of CR1 and CR3 on cord blood neutrophils than on the corresponding adult controls, both before and after FMLP stimulation. The reason for this discrepancy is unclear.

Our study is the first to investigate the expression of CR1 and CR3 on neutrophils from preterm infants. We found a strong correlation of FMLP-stimulated CR3 expression with postconceptional age, and found that the mean CR3 expression of the preterm infant neutrophils was significantly less than that of the term infant group. The importance of CR3 in normal neutrophil function is exemplified by the profound defects in neutrophil

adherence and exudation seen in patients with congenital CR3 deficiency (16). An association between diminished CR3 upregulation and diminished adherence in response to stimulation has been demonstrated in term infant neutrophils (5). It appears possible, therefore, that the further impairment of CR3 upregulation we observed in neutrophils of preterm infants may have a role in their enhanced susceptibility to serious bacterial infection. In contrast, CR1 expression on neutrophils of preterm infants did not differ significantly from that of term infants or adults. Because CR1 expression increased with postconceptional age, a study with a larger sample size might demonstrate a difference for infants less than 30 wk postconceptional age.

We also report diminished expression of FcR_{III} on neonatal neutrophils compared with neutrophils from adults. This is consistent with the recent study of Masuda *et al.* (17) showing diminished Fc-mediated rosetting by cord blood neutrophils, although differences in rosetting could also be mediated by the type II Fc_γ receptor (9, 13), which we did not measure. Diminished expression of Fc as well as complement receptors may contribute to the deficiencies of binding and phagocytosis shown when neonatal neutrophils are tested with low concentrations of opsonins (2). An earlier study did not show a difference in Fc rosetting (10). This may have been due to selection of an unrepresentative subpopulation by the glass-adherence procedure used (17, 18).

We observed that neutrophils of all four study groups increased their expression of FcR_{III} after stimulation with FMLP. In contrast to CR1 and CR3, whose plasma membrane expression increased by a factor of more than 10, the increase in FcR_{III} expression was of the same magnitude as the increase in plasma membrane surface area that occurs with stimulation (19). It is possible, therefore, that the increase in FcR_{III} expression simply reflects the change in exposed membrane surface area, and does not involve a specific translocation of the receptor from an intracellular pool as is thought to occur with CR1, CR3, and other neutrophil receptors (20–22).

Adinolfi *et al.* (15) previously reported that FMLP increased the expression of CR1 and CR3 on fetal granulocytes from autopsy specimens, but did not distinguish neutrophils from eosinophils or provide quantitative data. In our study, CR1 expression on neutrophils of the fetuses with Rh disease was significantly diminished compared with that of term infants and adults, and slightly less than that of the preterm infants. In contrast, fetal neutrophil expression of both CR3 and FcR_{III} was significantly greater than that of the preterm infant neutrophils, and FcR_{III} expression was equal to that of adult neutrophils. These differences may have been due in part to Rh disease. Alternatively, the relatively high fetal expression of CR3 and FcR_{III} may reflect depressed function of the neonatal neutrophils, as observed recently for bovine neutrophils by Clifford *et al.* (23). Whatever the mechanism, the differences in the relative expression of CR1 and CR3 between the fetal and infant groups are consistent with data indicating that the intracellular locations and mechanisms of upregulation of these receptors are distinct (24, 25).

Our finding that eosinophils constituted a large proportion of granulocytes in many of the fetuses with Rh disease was unexpected. Although we did not perform cytocentrifuge differentials on the initial specimens, the subsequent experiments indicated that the proportion of cells in the dim peak of the receptor distributions gave an accurate estimate of the eosinophil population, and confirmed the finding of an elevated proportion of eosinophils in fetuses with Rh disease. In contrast, the granulocyte differentials of specimens from fetuses with other conditions were consistent with reported blood smear differentials for normal fetuses at these gestational ages (26, 27). We suggest that future studies of fetal granulocytes include a differential based on a cytocentrifuged preparation or on other methods that allow a more accurate quantitation of cell types present in small numbers.

As noted above, fetal neutrophils expressed FcR_{III} at levels comparable to adult neutrophils. This finding cannot be attributed to contamination with maternal neutrophils, inasmuch as aliquots of the same cell suspensions were used for all three receptors and the expression of CR1 and CR3 on the fetal neutrophils was significantly lower than the adult levels. The same considerations apply to the unlikely possibility that neutrophils from previous therapeutic transfusions could have remained in the fetal circulation. For preterm infants, the number of neutrophils received in previous erythrocyte transfusions would be insignificant in relation to the endogenous circulating pool, and the data show no evidence of contamination with adult neutrophils. However, it is possible that blood loss and transfusions may indirectly affect neutrophil kinetics.

CONCLUSION

Neutrophils from preterm infants recovering from respiratory distress syndrome expressed significantly less CR3 after stimulation with FMLP than neutrophils from term infants or adults. In addition, CR3 expression of the FMLP-stimulated neonatal neutrophils was strongly correlated with gestational age. In view of its association with an impaired adherence response in term infant neutrophils, we speculate that diminished CR3 upregulation may play a role in the enhanced susceptibility of preterm infants to bacterial infection. Neutrophils from fetuses with Rh disease expressed more CR3 and FcR_{III} than neutrophils from the preterm infants, and eosinophils constituted a markedly increased proportion of granulocytes in these fetuses. This suggests that receptor expression is not determined solely by developmental age, and that future studies should address the influence of illness on both neutrophils and eosinophils.

REFERENCES

1. Wilson CB 1986 Immunological basis for increased susceptibility of the neonate to infection. J Pediatr 108:1–12
2. Hill HR 1987 Biochemical, structural, and functional abnormalities of polymorphonuclear leukocytes in the neonate. Pediatr Res 22:375–382
3. Ludomirski A, Weiner S 1988 Percutaneous fetal umbilical blood sampling. Clin Obstet Gynecol 31:19–26
4. Anderson DC, Hughes BJ, Smith CW 1981 Abnormal mobility of neonatal polymorphonuclear leukocytes. J Clin Invest 68:863–874
5. Anderson DC, Freeman KLB, Heerdt B, Hughes BJ, Jack RM, Smith CW 1987 Abnormal stimulated adherence of neonatal granulocytes: impaired induction of surface MAC-1 by chemotactic factors or secretagogues. Blood 70:740–750
6. Bruce MC, Baley JE, Medvik KA, Berger M 1987 Impaired surface membrane expression of C3bi but not C3b receptors on neonatal neutrophils. Pediatr Res 21:306–311
7. Fearon DT, Collins LA 1983 Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. J Immunol 130:370–375
8. Fleit HB, Wright SD, Unkeless JC 1982 Human neutrophil Fc receptor distribution and structure. Proc Natl Acad Sci USA 79:3275–3279
9. Looney RJ, Ryan DH, Takahashi K, Fleit HB, Cohen HJ, Abraham GN, Anderson CL 1986 Identification of a second class of IgG receptors on human neutrophils: a 40 kilodalton molecule also found on eosinophils. J Exp Med 163:826–836
10. Pross SH, Hallock JA, Armstrong R, Fishel CW 1977 Complement and Fc receptors on cord blood and adult neutrophils. Pediatr Res 11:135–137
11. Godfrey K 1985 Comparing the means of several groups. N Engl J Med 313:1450–1456
12. Archer RK 1963 The Eosinophil Leukocytes. Blackwell Scientific Publications, Oxford, p 4
13. Kulczycki Jr A 1984 Human neutrophils and eosinophils have structurally distinct Fc gamma receptors. J Immunol 133:849–854
14. Roberts RL, Gallin JI 1985 Rapid method for isolation of normal human peripheral blood eosinophils on discontinuous Percoll gradients and comparison with neutrophils. Blood 65:433–440
15. Adinolfi M, Cheetham M, Lee T, Rodin A 1988 Ontogeny of human complement receptors CR1 and CR3: expression of these molecules on monocytes and neutrophils from maternal, newborn, and fetal samples. Eur J Immunol 18:565–569
16. Anderson DC, Springer TA 1987 Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Ann Rev Med 38:175–194
17. Masuda K, Kinoshita Y, Kobayashi Y 1989 Heterogeneity of Fc receptor expression in chemotaxis and adherence of neonatal neutrophils. Pediatr Res 25:6–10

18. Krause PJ, Malech HL, Kristie J, Kosciol CM, Herson VC, Eisenfeld L, Pastuszak WT, Kraus A, Seligmann B 1986 Polymorphonuclear leukocyte heterogeneity in neonates and adults. Blood 68:200–204
19. Hoffstein ST, Friedman RS, Weissmann G 1982 Degranulation, membrane addition, and shape change during chemotactic factor-induced aggregation of human neutrophils. J Cell Biol 95:234–241
20. Borregaard N, Heiple JM, Simons ER, Clark RA 1983 Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J Cell Biol 97:52–61
21. Fletcher MP, Seligmann BE, Gallin JI 1982 Correlation of human neutrophil secretion, chemoattractant receptor mobilization, and enhanced functional capacity. J Immunol 128:941–948
22. Yoon PS, Boxer LA, Mayo LA, Yang AY, Wicha MS 1987 Human neutrophil laminin receptors: activation-dependent receptor expression. J Immunol 138:259–265
23. Clifford CB, Slauson DO, Neilsen NR, Suyemoto MM, Zwahlen RD, Schlafer DH 1989 Ontogeny of inflammatory cell responsiveness: superoxide anion generation by phorbol ester-stimulated fetal, neonatal, and adult bovine neutrophils. Inflammation 13:221–231
24. O'Shea JJ, Brown EJ, Seligmann BE, Metcalf JA, Frank MM, Gallin JI 1985 Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. J Immunol 134:2580–2587
25. Berger M, Birx DL, Wetzler EM, O'Shea J, Brown EJ, Corss AS 1985 Calcium requirements for increased complement receptor expression during neutrophil activation. J Immunol 135:1342–1348
26. Millar DS, Davis LR, Rodeck CH, Nicolaides KH, Mibashan RS 1985 Normal blood cell values in the early mid-trimester fetus. Prenat Diagn 5:367–373
27. Forestier F, Daffos F, Galactéros F, Bardakjian J, Rainaut M, Beuzard Y 1986 Hematological values of 163 normal fetuses between 18 and 30 weeks of gestation. Pediatr Res 20:342–346