Alterations in cord blood leukocyte subsets of patients with severe hemolytic disease after intrauterine transfusion therapy

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Objectives: The aim of this study was to compare, at delivery, the cord blood mononuclear cells of infants with severe hemolytic disease who received intrauterine transfusion (IUT) therapy with the cord blood mononuclear cells of healthy nonimmunized control neonates.

Study design: The expression of leukocyte markers on CBMNC of 14 IUT-treated and 18 control neonates was analyzed by means of a panel of well-defined monoclonal antibodies and flow cytometry.

Results: Patients with severe hemolytic disease requiring IUT treatment displayed significant altered expression of some leukocyte markers when compared with control subjects. The circulating CD34+ progenitor cells were significantly increased in comparison with cord blood of nonimmunized neonates. IUT-treated patients also showed a statistically significant decrease in natural killer (NK) cell associated markers (CD16, CD57, and CD69), which correlated with a lower expression of CD56. In these patients an increased expression of CD3/CD45RO and CD3/CD5 was also noted. Although these latter alterations were statistically significant in a single-parameter analysis, the significance disappeared after multi-parameter analysis because of a loss of statistical power.

Conclusions: Compared with nonimmunized healthy newborn infants, patients who underwent IUT also exhibited a down-regulation of NK cells and NK cell associated markers, as well as increased numbers of CD34+ progenitor cells. (J Pediatr 1997; 130:718-24)
METHODS

Patients and control subjects. Fourteen patients received unrelated-donor erythrocytes to treat severe hemolytic disease of the fetus. On average, the first IUT was performed at 27 weeks of gestation (range, 19 to 34 weeks). Before the first IUT the mean hemoglobin level of the fetuses was 3.5 mmol/L (range, 1.2 to 6.6 mmol/L). The mean number of IUTs was 3 (range, 1 to 6), and deliveries took place at 37 weeks of gestation (range, 32 to 39 weeks). In one case the infant was born by cesarean delivery; the other 13 infants were born by vaginal delivery.

Eighteen untreated neonates were used as control subjects. All these infants were born by vaginal delivery at an average of 40 weeks of gestation (range, 38 to 41 weeks).

Preparation of IUT. The IUT was prepared from fresh (≤24 hours old) donor erythrocytes that were compatible with maternal erythrocyte antibodies. Donor blood was collected in citrate-phosphate-dextrose solution. Erythrocytes were filtered after buffy-coat removal and contained fewer than 5 x 10^6 leukocytes per unit. The hematocrit was adjusted to approximately 85% (0.85) by using 0.9% saline solution. Erythrocytes were irradiated with 25 Gy and administered within 3 hours after preparation.

Collection of cord blood samples. Cord blood (30 to 50 ml) was obtained from the umbilical vein immediately after delivery and collected in heparinized tubes. CBMNC were isolated by Ficoll-Hypaque density gradient sedimentation within 12 hours after collection. To exclude contamination of cord blood with donor leukocytes, we analyzed only the CBMNC of those infants whose time between the last IUT and delivery was at least 2 weeks.

Monoclonal antibodies. To determine the expression of leukocyte cell markers, we used either fluorescein isothiocyanate-, phycoerythrin-, or peridinin chlorophyll protein conjugated monoclonal antibodies. When none of these conjugated monoclonal antibodies was available, unconjugated antimouse monoclonal antibodies were used and fluorescein isothiocyanate or phycoerythrin goat antimouse immunoglobulin was added after incubation with the unconjugated monoclonal antibodies. All monoclonal antigens (CD1, CD3, CD4, CD8, CD16, CD19, CD20, CD25, CD34, CD45, CD45RA, CD45RO, CD56, CD57, CD69, anti-HLA-DR) were purchased from Becton Dickinson UK Ltd. (Oxford, England).

Staining procedures. For each test, 5 x 10^5 CBMNC were incubated with 20 μl (12 mg/ml) pooled human immunoglobulin for 10 minutes at room temperature, to prevent non-specific labeling, and then washed and incubated with 50 μl monoclonal antibodies for 30 minutes at 4°C. After incubation, the cells were washed twice with 0.9% saline solution containing 1% bovine serum albumin. If unconjugated monoclonal antibodies were used, 50 μl goat antimouse immunoglobulin labeled with phycoerythrin or fluorescein isothiocyanate was subsequently added and incubated for 30 minutes at 4°C. After the second incubation the cells were washed twice. When a combination of conjugated monoclonal antibody and unconjugated monoclonal antibodies were used, incubation with the unconjugated monoclonal antibody was first performed, followed by blocking with 2% normal mouse serum before the addition of the conjugated antibody. After the labeling procedure, 3 ml Becton Dickinson lysis solution was then added and left for 10 minutes at room temperature to reduce the number of erythroblasts and erythrocytes. In some newborn infants a high number of erythroblasts is present in cord blood, especially in patients undergoing hemolysis. Because erythrocyte lysing solution does not completely remove erythroblasts from cord blood, it remains contaminated with cells of the erythroid lineage. To establish reliable values for different CBMNC subsets, we corrected the expression of the different cell markers for the percentage of glycophorin A expressing (erythroid lineage) and CD45^+ mononuclear cells present in the gated cell population.

Flow cytometry. Either one-, two-, or three-color flow cytometry was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) and gated on a forward angle versus 90-degree light scatter and the use of back-gating with cluster designation 45 (CD45) and CD14.
**Table.** Hematologic parameters and lymphocytes subsets on CBMNC at delivery

<table>
<thead>
<tr>
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<th>IUT-treated neonates</th>
<th>Control subjects</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
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<tr>
<td><strong>Hematologic parameters</strong></td>
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<td>Hemoglobin/(mmol/L)</td>
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<td>3.6-8.9</td>
<td>9.8</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>18-42</td>
<td>49.0</td>
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<td>Leukocytes (×10⁹/L)</td>
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<td>0.00-1.18</td>
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<td>Platelets (×10⁹/L)</td>
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<td>Lymphocytes (%)</td>
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<tr>
<td>HLA-DR</td>
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<td>14-63</td>
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Results expressed as mean percentage and range percentage of expression of CD45* cells after subtraction of glycophorin A-expressing cells. The numbers displayed in bold typeface resemble statistical significance in comparison with those of control subjects ($p < 0.05$).

NS, Not significant.

*IUT-treated neonates; n = 12; control subjects: n = 18.

†IUT-treated neonates; n = 14; control subjects: n = 18.

‡Corrected $p$ value after multiparameter analysis = 0.0016.

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staining. A minimum of 10,000 gated events were analyzed for each sample.

**Leukocyte and differential cell counts.** Leukocyte and differential cell counts were performed with a Hemalog one-cell counter (Technicon Instruments Corp., Tarrytown, N.Y.). When the analysis revealed extremely low numbers of a particular parameter, a differential manual count was performed.

**Statistical analysis.** The data on the IUT-treated patients and on the untreated neonates were compared with the two-tailed nonparametric unpaired Mann-Whitney test. Results were also corrected for the number of parameters analyzed by using the method of Edwards. A $p$ value less than 0.05 was considered statistically significant.

**RESULTS**

**Hematologic values.** The hematologic values of cord blood from 12 IUT-treated patients and 18 healthy control subjects are summarized in the Table. As expected, the hematocrit and hemoglobin levels in patients with hemolytic disease were significantly lower than in untreated control subjects because of the continuing breakdown of fetal red cells by maternal antibodies and the limited lifespan of transfused donor erythrocytes. Consequently, cord blood of the IUT-treated neonates contained more erythroblasts and reticulocytes because of the compensatory production of red cells. Both the absolute number of leukocytes in both groups and the leukocyte differentiation were similar. Five IUT-treated neonates had thrombocytopenia (platelet count, <150 × 10⁹/L), which resulted in a significantly lower mean platelet count in the IUT-treated patients.

**Mononuclear cells and immunophenotyping.** The expression of cell surface markers on CBMNC of 14 IUT-treated and 18 non-IUT-treated neonates is summarized in the Table.

**Hematopoietic progenitor cells.** The percentage of progenitor cells analyzed with anti-CD34 monoclonal antibody was significantly higher in the IUT group (3.1%) than in the control group (1%) (Figure, A).

**NK cells.** In comparison with cord blood of untreated ne-
onates, the cord blood of IUT-treated fetuses expressed statistically significant lower numbers of CD56<sup>+</sup> cells before multiparameter correction (Figure, B). There was a significant decrease in cells expressing Fcγ-receptor III (CD16) in the IUT group (Figure, C). CD57 was expressed in low numbers on CBMNC of the control subjects (1.4%) and was almost undetectable in IUT-treated patients (Table).

**T cells.** The expression of CD4 and CD8 on T cells was similar in both groups (Table). Coexpression of CD3 and CD5 (Figure, D) was 34% in the IUT-treated group and significantly higher than in the control subjects (22%). The two isoforms of CD45 that distinguish between “naïve” and “memory” T cells were analyzed by using anti-CD45RA and anti-CD45RO. CD3<sup>+</sup> CBMNC coexpressing CD45RO (Figure, E) were significantly increased in the IUT group (8%) in comparison with untreated neonates (2%). It should be noted that these apparently significant differences were lost after correction for the number of parameters analyzed because of a loss in statistical power. The expression characteristics of both CD45RO and CD5 on CD3<sup>+</sup> cells in control subjects and in IUT-treated patients revealed a wide distribution.

**B cells.** The percentage of B cells (Table), as defined by the expression of CD19 and CD20, was slightly elevated in

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**Fig. 1.** Individual (●) and mean (horizontal bars) percentage of expression of CD34 (A), CD56 (B), CD16 (C), CD3/CD5 (D), CD3/CD45RO (E), and CD69 (F) on CBMNC of IUT-treated patients (n = 14) and untreated control subjects (n = 18). p-corr, Corrected p value for the number of parameters analyzed (17). In 11 IUT-treated patients (n = 11), the expression of CD69 (F) was 0%.
the IUT group (19% and 20%) in comparison with the control subjects (16% and 15%). The percentages of B cells expressing CD5 were evenly distributed in IUT-treated patients and control subjects (7% vs 6%). HLA-DR was expressed on nearly all B cells in both groups.

Activated cells. We did not observe any difference in expression of CD25, the low-affinity interleukin-2 α-chain receptor, in both groups (Table). However, the time between the last transfusion and birth was longer than 2 weeks, which was probably too long to measure an effect on these transiently expressed molecules. Moreover, the percentage of HLA-DR+ CBMNC was similar in both groups (Table). Expression of CD69 was significantly lower after IUT treatment and in most cases absent in the IUT group (Figure, F).

DISCUSSION

The normal distribution of lymphocyte subsets in cord blood of healthy fetuses, neonates, and young children has been extensively studied.15-28 In general, distribution, expression, and range of expression of cell surface markers in fetuses and neonates differs from those in adults. Neonatal mononuclear cells express more naïve CD4+ T cells (>90% CD45RA+) than do those of adults (40% to 60%) and more immature CD5+ B cells (30% vs 5%). Neonates are generally physiologically immunodeficient concerning immunoglobulin production, class switch, and magnitude of the alloimmune response.29-31 The observed maturational and developmental changes occurring in lymphocyte populations during life can be largely attributed to antigenic challenges.26

Severe anemia in fetuses with hemolytic disease requiring IUT therapy results in nonselective lymphopenia proportional to the degree of anemia.32 To determine whether IUT treatment resulted in reversal of these changes or imposed additional effects on lymphocyte subsets after exposure to donor alloantigens, we compared CBMNC of IUT-treated patients with those of control neonates. For this purpose we used a broad selection of monoclonal antibodies, which defines various subsets of mononuclear cells. This limited the conclusions that could be drawn from this relatively small study population because we had to correct the statistical significance for the number of monoclonal antibodies tested.

The significant increase of CD34+ progenitor cells in IUT-treated patients may reflect the compensatory hematopoietic stimulation in favor of the erythroid cell lineage in the fetuses with hemolytic anemia.33-35

Although the statistical significance of the reduction in CD56-expressing cells in the IUT-treated group disappeared after correction for the number of parameters analyzed, an overall decrease in expression of this marker was observed. The statistically significant lower expression of CD16 in this group of patients was probably the result of down-regulation in addition to diminished numbers of NK cells. The published observations on the expression of CD56 and CD16 on CBMNC of healthy newborn infants show a wide range, and both increased and decreased values have been found when compared with those for adults.18-20 The expression of CD57 (subset of NK cells with low natural killer activity) is low in neonates, and expression increases slowly throughout life.23-28 A large percentage of healthy neonates (63%) have severely reduced NK function in vitro, corresponding to a low expression of CD56 on CBMNC, whereas the expression of CD16 and that of CD57 were found to be within normal ranges.28 We found the expression of CD57 to be low in control subjects and almost absent in the IUT group. In addition, the diminished expression of the early activation marker CD69 in the IUT group could be due to reduced numbers of NK cells as well as active down-regulation of this marker, because this marker was expressed only on NK cells and was virtually absent on T and B cells in both groups (data not shown). In general, a clear tendency down-regulation of NK cells and a statistically significant decrease in NK cell associated markers was observed in the IUT-treated patients. This might reflect reduced NK cell activity and a more immature profile of NK cells in these patients.

In patients with hemolytic disease who received IUT treatment, the number of leukocytes, leukocyte differentiation, and relative percentages of T and B cells are comparable to those of control patients. Within the T-cell lineage, the percentage of CD4+ and CD8+ T cells is similar in both groups. CD5-expressing T cells represent 95% of the circulating adult T cells. Both in IUT-treated patients and in untreated control subjects, the expression of CD5 on T cells was reduced and represents 68% and 43% of circulating T cells in the two groups, respectively. This difference may reflect a more mature pattern of expression on T cells in the IUT-treated group (Figure, D). Another reflection of premature T-cell maturation is the increased coexpression of CD45RO on CD3+ cells in these patients, suggesting a higher number of "memory" T cells induced in utero (Figure, E). An increased percentage of more than 17% of CD45RO+ T cells in neonatal blood was proposed as a marker for the presence of a congenital infection.36 None of the neonates in this study had any signs or symptoms of a congenital infection. The method of delivery and the length of gestation have profound effects on leukocyte subpopulations.37,38 Considering these factors, which can influence lymphocyte subpopulations in neonates, it is difficult to distinguish the observed effects from specific transfusion effects. However,
the increased expression of CD3/CD45RO+ cells is likely to be the result of an antigenic challenge in utero. The decreased numbers of NK cells and associated cell markers are in agreement with the earlier study of Thilaganathan et al. They also observed diminished numbers of T cells (CD4+ and CD8+) and B cells before the start of IUT therapy in patients with hemolytic disease. These differences in T and B cells were even more apparent earlier in gestation, probably correlating with the severity of the disease. We found that in IUT-treated patients, after birth, the percentages of CD4+ and CD8+ T cells as well as B cells were comparable to those in the control subjects.

In conclusion, evidence is presented that patients with immune hemolytic disease who received IUT therapy displayed a significant decrease in NK cell associated markers in addition to alterations in the activation status of T cells. Further analyses of material derived from additional IUT-treated patients and control subjects are required to allow confirmation of the trends that we detected in this study. The observed differences with regard to the development of memory T cells and the decrease in NK cells must be investigated in more detail to unravel the relationship to transfusion therapy and hemolytic disease.

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