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Formula Feeding Skews Immune Cell Composition toward Adaptive Immunity Compared to Breastfeeding

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The ontogeny of the immune system and the effect thereon by type of infant feeding is incompletely understood. We analyzed frequencies and composition of immune cells in blood of breastfed (BF) and formula-fed (FF) infants at 1.5, 4, and 6 mo of age. Three formulas with the same protein concentration but with varying levels of α-lactalbumin and caseinoglycomacropeptide were compared. Twenty-nine exclusively BF infants served as reference, and 17 infants in each formula group completed the study. Whole blood and PBMCs were analyzed by flow cytometry and immunoflow cytometry, respectively. Leukocyte count of BF infants increased with time due to increased frequency of neutrophils. Lymphocyte count was high at 1.5 mo and was unchanged over time, as were the relative proportions of CD4+ T cells and CD8+ T cells. The composition of BF infants changed with time, as were the relative proportions of CD4+ T cells and CD8+ T cells. Whole blood and PBMCs were analyzed by flow cytometry and immunoflow cytometry, respectively. Leukocyte count of BF infants increased with time due to increased frequency of neutrophils. Lymphocyte count was high at 1.5 mo and was unchanged over time, as were the relative proportions of CD4+ T cells and CD8+ T cells. B cells, NK cells, and γδ T cells. Most CD45R0+CD3+ cells were HLA-DR+ and hence memory cells. Compared with breastfeeding, formula feeding resulted in a significant decrease in proportion of NK cells, but a significant increase in naive CD4+ T cells and an elevated CD4-to-CD8 ratio, that is, 3.3 in the combined FF groups compared with 2.6 in the BF group. No significant differences were found between the three groups of FF infants. In conclusion, blood cells of lymphoid lineage did not change significantly in frequencies or composition from 1.5 to 6 mo of age in BF infants. In contrast, FF infants displayed an ongoing maturation of adaptive immunity cells and a delayed recruitment of innate immunity cells as compared with BF infants. The Journal of Immunology, 2009, 183: 0000–0000.

B reastfed (BF) infants have fewer and milder infections compared with formula-fed (FF) infants (1), partly because bioactive components in human milk not only contribute to high bioavailability of nutrients but also affect the maturation of innate and adaptive immunity (2). However, little is known about the immunity of the newborn human infant and the development of the immune system. In particular, there are no prospective studies addressing the effect of different types of infant feeding during the first half of infancy using the exclusively BF infant as reference, although an increase in T cells, particularly in Th cells (3, 4), and a decrease in NK cells (4) have been reported for FF infants compared with BF infants at 6 mo of age.

The composition of infant formulas is continuously modified, aiming at a composition more similar to that of human milk. When using cow’s milk as the protein source for infant formulas, the whey-to-casein ratio is adjusted to resemble the whey-predominant human milk. To ensure sufficient intake of all essential amino acids, a higher protein content in formula as compared with human milk is required, which may have adverse effects (5, 6). By increasing α-lactalbumin, a major human milk whey protein, and decreasing the content of β-lactoglobulin, a major bovine whey protein, which is absent from human milk, it is possible to reduce the total protein content in a formula and achieve a plasma amino acid profile in FF infants more similar to that of BF infants (7). Besides having a well-balanced amino acid composition, α-lactalbumin has antimicrobial and also other biological effects (8, 9). Caseinoglycomacropeptide (CGMP) is cleaved off from κ-casein by pepsin digestion in the stomach and has also been shown to have antimicrobial, immunomodulatory, and prebiotic effects (10–13), including promoting growth of bifidobacteria (14).

We hypothesized that the immune cell composition of FF infants would be more similar to that of BF infants if the protein composition of the formula was made more similar to that of human milk, that is, by decreasing the β-lactoglobulin and increasing the α-lactalbumin proportion compared with a standard formula. The aim of this study was therefore to determine the composition of immune cells in blood in the newly newborn (1.5 mo of age) and prospectively follow age-related changes up to 6 mo of age, and to evaluate how formula feeding, depending on the α-lactalbumin and CGMP contents, affect this composition compared with breastfeeding.

Materials and Methods

Study design

Due to the very high initial breastfeeding rate (90% are exclusively BF at 2 mo), healthy term infants were recruited from well-baby clinics at 6 ± 2 wk of age in Umeå, a university town in northern Sweden with 114,000 inhabitants, most of whom are middle class and well educated. After parents had given written consent for their infant to participate, 96 infants fulfilled the following inclusion criteria: gestational age 36–42 wk, and birth weight 2500–5000 g. Thirty-four of them were recruited among mothers who intended to exclusively breast-feed until 6 mo and served as a BF reference group. Sixty-two of them, initially FF or weaned from the breast at 6 ± 2 wk, were randomized to either control formula (CON; 11%...
Faculty of Medicine, Umeå University, approved the study.

Room temperature before analysis (Fig. 1).

Vegetables purees (1–2 tablespoons per day) until 6 mo when the study was stopped.

Both BF and FF infants were allowed taste portions of fruit and vegetables purees (1–2 tablespoons per day) until 6 mo when the study was stopped. Formulas were provided by Arla Foods Ingredients and were produced as described earlier (15). After 4 mo of age both BF and FF infants were allowed taste portions of fruit and vegetables purees (1–2 tablespoons per day) until 6 mo when the study was discontinued. The purees were supplied by the investigators.

Blood samples (3 ml) were taken by venepuncture into heparinized tubes at entry (6 ± 2 wk), at 4 mo, and at 6 mo, and stored for 2–4 h in room temperature before analysis (Fig. 1B).

The Ethics Committee on Research Involving Human Subjects of the Faculty of Medicine, Umeå University, approved the study.

Flow cytometry analysis of whole blood leukocyte composition

Routine hematology was performed at the Department of Clinical Chemistry, Umeå University Hospital. Concentrations of total white blood cells (WBCs), lymphocytes, monocytes, as well as neutrophil, eosinophil, and basophil granulocytes were assessed by forward and side scatter characteristics in flow cytometry (XE-2100; Sysmex).

Phenotyping by immunoflow cytometry

PBMCs were enriched by density gradient centrifugation on Ficoll-Paque (GE Healthcare Biosciences) within 4 h of storage at room temperature. PBMCs (100,000 cells) were stained with mouse mAbs directly conjugated with PE or FITC. Mabs used were: anti-CD3 (clone SK7, IgG1), anti-CD4 (clone SK3, IgG1), anti-CD8 (clone SK1, IgG1), anti-CD16 (clone B73.1, IgG1), anti-CD56 (clones MY11, IgG1), anti-TCR-ϕ (clone WT31, IgG1), anti-TCR-γδ (clone 11F2, IgG1), anti-HLA-DR (clone L243, IgG2a), anti-CD45R0 (clone 1H7, IgG2b), and anti-CD45 (clone 2D1, IgG1), all from BD Biosciences. Fluorochrome, IgG subclass, and concentration-matched irrelevant mAbs served as negative controls (BD Biosciences). Fifteen thousand ungated events were collected in two-color immunofluorescence in a FACScan (BD Biosciences) and analyzed using the CellQuest software program. The instrument was calibrated with calibrate beads. PBMCs were distinguished by forward and side scatter plots and manually gated to include all lymphocytes but exclude possible erythrocytes and granulocytes in the analysis. The proportion of marker-positive cells is given as percentage of CD45 cells in the same sample.

Table I. Contents of α-lactalbumin, CGMP, and β-lactoglobulin in the formulas studied

<table>
<thead>
<tr>
<th>Protein</th>
<th>CON</th>
<th>α-LAC</th>
<th>RCGMP</th>
<th>CON</th>
<th>α-LAC</th>
<th>RCGMP</th>
</tr>
</thead>
<tbody>
<tr>
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<td>25</td>
<td>25</td>
<td>1.4</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>CGMP</td>
<td>14</td>
<td>15</td>
<td>10</td>
<td>1.9</td>
<td>1.9</td>
<td>1.3</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>27</td>
<td>15</td>
<td>19</td>
<td>3.5</td>
<td>1.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Statistical analysis

Comparisons between study groups were performed using linear regression and analysis of covariance. Value at entry was used as a covariate to circumvent that differences at study entry-influenced outcome at 4 and 6 mo of age. Comparisons between time points within a certain study group were performed using general linear model with repeated measures and paired sample t test. The software SPSS v13.0 was used for all calculations. A p value of ≤0.05 was considered statistically significant. Descriptive values are expressed as means ± 1 SD.

Results

Five of the infants in the BF group were excluded because their mothers could not meet the criterion of exclusive breastfeeding for 6 mo. Four infants were excluded from the CON group: two because they developed cow’s milk protein allergy, and the parents withdrew the other two from the study. In the α-LAC group the corresponding numbers were two and one infant, respectively, and in the RCGMP group one and three infants. Thus, the final numbers of infants completing the study were 29, 17, 17, and 17 in the BF, CON, α-LAC, and RCGMP groups, respectively (Fig. 1A).

Time points for collection of blood samples are shown in Fig. 1B. The mean percentages of infants from which sufficient blood samples were obtained to allow phenotyping by immunoflow cytometry were 97, 94, and 96% at 1.5, 4, and 6 mo, respectively.

We found that gradient centrifugation was an adequate way to isolate PBMCs also from blood of very young infants. CD45 was used as a pan-leukocyte marker in the immunophenotyping experiments, and CD45+ cells comprised >95% of the PBMC prepartions. Furthermore, the sum of the major immune cell populations in PBMCs, that is, monocytes (CD14+ cells), T cells (CD3+ cells), B cells (CD19+ cells), and NK cells (CD16+ and/or CD56+CD3– cells), was 97 ± 3.2% (n = 76), 96 ± 3.6% (n = 73), and 96 ± 3.1% (n = 75) at 1.5, 4, and 6 mo, respectively.

The total WBC count in BF infants increased significantly during the first 6 mo of life, mainly explained by an increase in circulating neutrophil granulocytes

In BF infants the total count of WBCs was 8.5 ± 2.3 × 109/L at 1.5 mo of age, increased significantly with time (p = 0.007), and reached 9.9 ± 2.6 × 109/L at 6 mo (Fig. 2A). Lymphocytes constituted the major fraction of the WBCs already at 1.5 mo, and increased marginally during the study period (Fig. 2B). Neutrophil granulocytes constituted the second largest immune cell population at 1.5 mo and continued to be so with a steady increase to 2.9 ± 1.6 × 109/L at 6 mo (p = 0.002) (Fig. 2B). Monocytes constituted the third largest population at 1.5 mo, decreased significantly to 4 mo (p = 0.01), but returned to the level at entry at 6 mo of age (Fig. 2B). Eosinophil granulocytes constituted a minor population at 1.5 mo (0.3 ± 0.1 × 109/L) and basophils were scarce (0.03 ± 0.03 × 109/L). The frequencies of the latter two cell types remained stable throughout the study period (Fig. 2B).
The proportions of different lymphocyte subsets remained constant throughout the first 6 mo of life in BF infants

A panel of mAbs was used in two-color immunoflow cytometry to characterize the lymphocytes with regard to subtypes. Fig. 3 shows an example of analysis of PBMCs from a 1-mo-old BF infant. B cells were defined as CD19+ cells (Fig. 3B), NK cells as non-T cells (CD3- cells) stained by a mixture of mAbs against the NK cell marker CD56 and the Fcy-receptor CD16 (Fig. 3F), and T cells as CD3+ cells. For each child and time point the percentage of CD3+ cells was calculated as the mean of three determinations, that is, anti-CD3 vs anti-CD19 (Fig. 3B), anti-CD3 vs anti-CD56 plus anti-CD16 (Fig. 3F), and anti-CD3 vs anti-HLA-DR (Fig. 3E). T cells were further characterized by TCR usage, that is, αβT cells (TCR-αβ+ cells) and γδT cells (TCR-γδ+ cells; Fig. 3C), helper T cell phenotype (CD4+ cells; Fig. 3D), cytotoxic T cell phenotype (CD8+ cells; Fig. 3D), activated phenotype (HLA-DR+ CD3+ cells; Fig. 3E), memory/activated phenotype (CD45RO+ CD3+ cells; Fig. 3H), and naive phenotype (CD45RA+ CD3+ cells; Fig. 3G). The experimental setup also allowed for detection of possible circulating immature T cells as CD4/CD8 double-positive cells (Fig. 3D) and CD3/CD56 double-positive cells (Fig. 3F).

In the BF infants T cells constituted by far the largest lymphocyte population at 1.5 mo (68.5 ± 6.8%) followed by B cells (16.6 ± 4.7%) and NK cells (6.9 ± 2.8%). None of the lymphocyte types changed significantly in proportion during the study period. The vast majority of T cells expressed TCR-αβ, while γδT cells constituted a minor population (4.4 ± 2.0%) of the CD3+ cells. Furthermore, CD4+ cells (51.9 ± 6.3%) dominated over CD8+ cells (21.9 ± 5.6%) with a CD4-to-CD8 ratio of 2.6 ± 0.9. All of these parameters remained unchanged over time.

Interestingly, although the proportion of naïve T cells (CD45RA+ CD3+ cells) was high (60.9 ± 8.4%), it was less than the total population of T cells, and the proportion of CD45RO+ CD3+ cells was as high as 22.9 ± 10.6% already at 1.5 mo, suggesting that circulating memory and/or activated T cells are present in significant numbers already during the first months of life. The frequency of HLA-DR+ CD3+ cells, however, was low, indicating that most of the CD45RO+ CD3+ cells are memory T cells rather than activated T cells. The proportions of CD45RA+ CD3+ cells, CD45RO+ CD3+ cells, and HLA-DR+ CD3+ were approximately the same at 1.5, 4, and 6 mo (Fig. 4).

The proportion of CD4/CD8 double-positive cells was initially low (<2.5%) with a tendency to decrease over time. The proportion of CD3/CD56 double-positive cells was even lower (<1.5%).
The proportion of CD8^+ cells was the same or slightly lower than in BF infants (Fig. 6F). On average the CD4-to-CD8 ratio was higher in the FF groups compared with the BF group, and the difference reached statistical significance at both 4 and 6 mo for the RCQPM group (Fig. 6B).

The proportion of naive T cells (CD45RA^-CD3^- cells) was significantly higher in all three FF groups compared with the BF group at both 4 and 6 mo of age (Fig. 4A). The increase from 1.5 to 6 mo was statistically significant in the α-LAC group (Fig. 4A). In contrast, there were no significant differences between the groups with regard to the proportion of memory T cells (CD45RO^-CD3^- cells). There was, however, a tendency to a decrease over time in all groups, and the decrease from 1.5 to 6 mo was significant in the α-LAC group (p = 0.04; Fig. 4B). The proportion of activated T cells (HLA-DR^+CD3^- cells) was low in the BF group and even lower in all three FF groups (Fig. 4C). In the RCGMP group this decrease reached statistical significance from 1.5 to 6 mo (p = 0.005) and the proportion was significantly lower than in the BF group at both 4 and 6 mo (Fig. 4C).

Double-positive CD4/CD8 cells were low in all FF groups (<2.1%) and showed a tendency to decrease over time, while the proportion of CD3/CD56 double-positive cells was even lower (<1.2% at 6 mo). All FF groups showed a tendency to lower values of double-positive CD4/CD8 cells at 4 and 6 mo compared with the BF group. No statistically significant differences were found between the FF groups for any of the lymphocyte subsets analyzed.

**Discussion**

An interesting difference between BF and FF infants was that BF infants showed a steady increase in total WBC counts, essentially explained by an increase in circulating neutrophil granulocytes, while FF infants did not. This potential enforcement of innate immunity by breastfeeding in the young infant with a not fully developed immune system has not been observed previously, as the role of early feeding mode has not been studied. Since there is individual variation in both cell counts and composition of immune cell types, changes over time may easily be missed in cross-sectional studies. A stable eosinophil granulocyte count in the BF group but a decline in the FF groups is in agreement with Cordle et al. (16), who compared BF and FF infants at 6 mo of age, and further indicates beneficial effects on innate immunity by breastfeeding.

Lymphocyte counts were stable over time in all study groups, which is in accord with previous studies on healthy, term infants during the first half of infancy (17–19). These studies reported age-dependent changes in proportions of lymphocyte subsets. However, we found that lymphocyte subset composition is strongly influenced by feeding mode, that is, stable from 1.5 to 6 mo of age in BF infants but an increase of αβTh cells on the expense of NK cells, CTLs, and B cells in FF infants. As the impact of feeding mode was not considered in the earlier studies, the reported changes may reflect increasing proportions of FF infants in their material. In line with this notion, neonate cord blood had higher levels of NK cells and lower levels of T cells than did infant peripheral blood (18, 19). Furthermore, Hawkes et al. (4, 20) found a lower proportion of NK cells in FF compared with BF infants at 6 mo of age, which is in agreement with our results. Our study suggests that in some respects the immune system develops slower in FF infants compared with BF infants because there was a more pronounced dominance of naive Th cells over memory T cells as well as of Th cells over CTLs and a slower recruitment of cells with effector functions in innate immunity. The high frequency of Th cells in FF infants could reflect that their immune...
system is more skewed toward adaptive immunity compared with BF infants.

The increase in CD4+ αβT cells resulting from formula feeding confirms and extends the results of previous studies on infants at 6 mo of age (3, 4, 16). In contrast, one study reported that the frequency of T cells is the same in 6-mo-old BF and FF infants (16). This could, however, be explained by the fact that the infants in the latter BF group were exclusive BF only during the first 2 of the 6 mo.

The proportion of circulating naive T cells, CD45RA+CD3+ cells, in BF infants was similar to that in adults, that is, ~60% (19), while the levels of these cells were significantly higher in FF infants. Similarly, the proportion of CD45RA+CD3+ cells was higher in preterm infants fed formula compared with those fed human milk (21), suggesting that exposure to new food Ags in absence of breast milk causes recruitment of naive T cells to the blood. This might reflect propensity for an adaptive immune response rather than for induction of tolerance induction to dietary Ags.

The proportion of CD45RO-CD3+ cells was on average 20% at 1.5 mo of age. No increase narrowing the gap to adult levels, that is, ~55% (19), was noted in either BF or FF infants at 4 or 6 mo of age. Both activated T cells and memory T cells express CD45RO, while only activated T cells express the MHC class II molecule HLA-DR. Since the frequency of CD3+HLA-DR+ cells was <3% we conclude that there is a very significant number of memory T cells already at 1.5 mo of age.

The list of breast milk components with potential to influence immune cell development and function is long and includes bioactive peptides and proteins, for example, cytokines, chemokines, and growth factors, and components that promote colonization of the infant’s gut (22). Several of these may act directly on the infant’s gastrointestinal mucosa, but may also reach the circulation. Decreased mortality in TGF-β-null mice fed milk from heterozygote dams as compared with milk from null dams (23) and enhanced immune tolerance to dietary Ags in mice after oral administration of TGF-β supports this scenario (24). When considering

**FIGURE 4.** Mean proportions of naive T cells, memory/activated T cells, and activated T cells estimated as double-positive cells in two-color immunoflow cytometry with anti-CD3 vs anti-CD45RA (A), anti-CD3 vs anti-CD45RO (B), and anti-CD3 vs HLA-DR (C) stained samples, respectively, in infants of the BF group (●, hatched line), the CON group (■, solid line), the α-LAC group (▲, solid line), and the RCGMP group (▼, solid line) at 1.5 (6 ± 2 wk, i.e., study entry), 4, and 6 mo of age. Bars represent means ± 1 SD and statistically significant differences between the BF group and a FF group are indicated: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

**FIGURE 5.** Frequencies of WBCs (A), monocytes (B), neutrophil granulocytes (C), and eosinophil granulocytes (D) in whole blood of infants in the BF group (●, hatched line), the CON group (■, solid line), the α-LAC group (▲, solid line), and the RCGMP (▼, solid line) group at 1.5 (6 ± 2 wk, i.e., study entry), 4, and 6 mo of age. Cell counts were done by flow cytometry. Bars represent means ± 1 SD, and statistically significant differences between the BF group and a FF group are indicated: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
the observed differences in blood immune cell composition in BF compared with FF infants some of these factors seem to be of particular relevance. Lactoferrin is known to enhance NK and T cell proliferation and function (25, 26). Immune-modulating factors such as IL-2 may provide the BF infant with important signals during significant stages of T cell development (27) and even contribute to the larger thymus in BF compared with FF infants at 4 mo (28) and 10 mo of continued breastfeeding (29). The earlier exposure to new dietary food Ags in FF infants may enhance T cell release from the thymus, causing the significantly higher levels of circulating naive helper T cells in these children. Moreover, granulocyte CSF is suggested to promote eosinophil production and could contribute to the higher levels of eosinophils in BF infants (30, 31).

Since there were no statistically significant differences between the three groups of FF infants but all three FF groups differed significantly from BF infants in several respects, it appears that the differences in α-lactalbumin and CGMP concentration in the formulas studied had no or only minor effects on the distribution of immune cells in peripheral blood during the first 6 mo of life. It is possible that larger differences in proportions of whey proteins in formula are needed to observe significant changes in immune cell distribution. Alternatively, factors in human milk that affect development and distribution of leukocytes were not present in the formulas studied.

Whether the differences found between the FF groups and the BF group are of clinical significance cannot be concluded from this study. However, as previously reported, there were no differences between BF and FF infants with regard to fever episodes, number of days with fever, and episodes of airway infections (7).

In conclusion, the most striking and consistent differences between FF and BF infants were a higher proportion of T cells

FIGURE 6. Mean proportions of T cells (CD3⁺ cells; A), αβ T cells (TCR-αβ⁺ cells; C), γδ T cells (TCR-γδ⁺ cells; D), T cells with helper cell phenotype (CD4⁺ cells; E), T cells with cytotoxic phenotype (CD8⁺ cells; F), B cells (CD19⁺ cells; G), NK cells (CD16⁺/CD56⁺/CD3⁻ cells; H), and the ratio between CD4⁺ cells and CD8⁺ cells (B) as estimated by immunoflow cytometry in PBMCs of infants in the BF group (●, hatched line), the CON group (●, solid line), the α-LAC group (▲, solid line), and the RCGMP group (▼, solid line) at 1.5 (6 ± 2 wk, i.e., study entry), 4, and 6 mo of age. Bars represent means ± 1 SD, and statistically significant differences between the BF group and a FF group are indicated: *, p < 0.05; **, p < 0.01; and *** , p < 0.001.
caused by a selective increase in naïve Th cells, a lower proportion of NK cells, and lower eosinophil granulocyte cell counts occurring already after 3 mo and persisting after 5 mo of formula feeding. Thus, it seems that the development of the immune system in FF infants is preferentially forced toward cells in adaptive immunity, while the development of innate immunity is slower than in BF infants.

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Disclosures

Co-author Gitte Graverholt is an employee of Arla Foods Ingredients. She contributed to the design of the study and product composition but had no influence on the data compilation and analysis or on the interpretation of the results. None of the other authors declare any conflicts of interest.

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