

Infants colonized with enterotoxin-producing staphylococci at 3 months display a decreased frequency of interferon- γ -producing CD45RO lymphocytes upon stimulation with staphylococcal enterotoxin A at birth but not at 6 months of age

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The aim of the study was to elucidate the relationship between the cytokine response to staphylococcal enterotoxin A (SEA) at birth and subsequent staphylococcal colonization in the first months of life. In a cohort of 45 newborns, cord blood lymphocytes were stimulated with SEA (10 ng/ml) *in vitro*, re-stimulated with PMA (phorbol myristate acetate) and ionomycin at day 3 and assessed for CD45RO expression and cytokine generation by flow cytometry. The infants were classified into three groups according to nasal staphylococcal colonization and enterotoxin generation at 3 months: There were 16 infants with either no colonization or non-enterotoxin-producing staphylococci, 16 infants with enterotoxins B, C, D and E, and 13 infants colonized with SEA-producing staphylococci. At birth, the group without subsequent colonization displayed a significantly higher frequency of CD45RO-positive interferon- γ -producing cells (1.7%; range 0.0–9.3%) in comparison to the SEA-positive group (0.1%; range 0.0–0.4%) and also to the group positive for other enterotoxins (0.50%; range 0.0–2.5%). Comparable but less pronounced results were found for interleukin-5 but not for interleukins 2 and 4. At 6 months, no differences in cytokine generation were detected between the three groups. The results provide evidence that a non-specific immunologic immaturity at birth is a risk factor for early bacterial colonization. Furthermore, it is remarkable that this immaturity is similar to that seen in infants destined to be atopic with respect to disequilibrium of interferon- γ to interleukin-4 generation. Thus the link between early staphylococcal colonization and subsequent atopy requires further investigation.

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Staphylococcus aureus is a common cause of pyogenic infections of the skin, osteomyelitis and pneumonia. Pathogenicity is known to depend on

cell wall components such as protein A, muramic acid and teichoic acid (1). A further determinant of pathogenicity is the presence of toxins.

Diseases such as food poisoning, Kawasaki syndrome (2), atopic dermatitis (3), toxic shock syndrome and even sudden infant death (4) have been attributed to the effect of staphylococcal enterotoxins. Staphylococci are ubiquitous. About 20% of healthy individuals are colonized with enterotoxin-producing staphylococci (5). T lymphocytes respond to staphylococcal enterotoxins with a massive generation of cytokines (6). Excessive cytokine production may result in potentially fatal toxic shock syndrome. On the other hand, deficient interferon production upon stimulation with staphylococcal enterotoxins is associated with increased susceptibility to infection (7). Thus, it is not surprising that the response to staphylococcal enterotoxins is tightly regulated. Prolonged contact with staphylococcal enterotoxins induces a specific non-responsiveness of T lymphocytes (8). The first months of life represent a period with increased susceptibility to tolerance induction (9) as well as to infections (10). Although it is known that babies are colonized with enterotoxin-producing *Staphylococcus aureus* from early on (11), and that neonatal T-cells display a decreased production of interferon- γ *in vitro* (10,12,13), little is known about the interplay between enterotoxin-producing staphylococci and T-cell reactivity in infants. This question was addressed in the present report.

Materials and methods

Study group

The children studied were consecutively born after uneventful pregnancies and deliveries at two obstetric clinics in Bochum (St Elisabeth Hospital, Augusta Krankenanstalten) between May 1996 and August 1996. Parents had to give informed written consent before their infants were included in the study. The study was approved by the ethics committee of the medical faculty. Of the 97 parents whose babies were eligible for the study, 86 agreed to answer a questionnaire. Information with regard to sex of the baby, birth weight, duration of pregnancy, age of parents, number of siblings, smoking, socio-economic status, age of their home, use of a gas stove, wall-to-wall carpets, pets, washable bed cover and type of mattress was recorded. Furthermore, parents answered a previously evaluated questionnaire (14) concerning the hereditary atopic risk. Fifty-two of these parents were willing to participate in the further follow-up evaluation of their children. Only specimens of infants whose parents agreed to

participate in the study were further evaluated. During the study, seven babies were excluded, as the families did not comply during the entire study period. Results obtained with these seven babies were in no regard different from the 45 fully evaluated babies. The only difference between the families who completed the study successfully and those who either refused to participate or dropped out was the presence of atopic risk ($p = 0.0356$). This bias might be due to the fact that atopic dermatitis was mentioned in the information sheet as one of the diseases potentially caused by staphylococci.

Umbilical cord blood was obtained in all infants after maternal blood had been wiped off. Each infant was seen at birth, 3 and 6 months. At each appointment, the children were examined by a physician with special emphasis on skin, respiratory tract, and ear, nose and throat. Parents filled in a questionnaire and gave a structured interview about their infant's diseases, relevant atopic symptoms and nutrition. To keep the bias low the parents kept a diary in which the infant's diet, diseases and important events were recorded. Nasal swabs were taken at 3 months and were cultured as described below. Venous blood was drawn at the 6 month visit.

Culture and isolation of *Staphylococcus aureus*

Swabs taken from the patient's anterior nares were plated onto a selective Vogel Johnson Agar (BAG, Lich, Germany) and incubated at 37°C overnight. Colonies were replated on conventional blood agar. After 48 h of incubation at 37°C, representative colonies were isolated in pure culture for catalase and coagulase testing. Cultures that proved to be positive in these tests were confirmed in the ID32 Staphsystem (Bio Mérieux) to consist of *S. aureus*, and were selected for analysis of the production of enterotoxins. The selected colonies were transferred to brain heart infusion broth (BAG, Lich, Germany) and grown overnight. The bacteria suspension was centrifuged at 6000×g for 10 min and the supernatant was selected for identification of toxin secretion.

Elisa

The ELISA for detection of staphylococcal toxin was carried out in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Wiesbaden, Germany). The plates were coated with 0.1 ml of staphylococcal toxin-specific polyclonal rabbit IgG (Toxin Technology, Sarasota, Florida, USA), diluted to a 10 µg/ml concentration in

0.5 M H_2CO_3 (pH 9.5). The plates were covered and incubated at 37°C for 12–14 h. Excess antibody was removed by washing the plates with 10 mM phosphate buffer containing 0.9% NaCl + 0.1% Tween-20, pH 7.4). Samples and standards were diluted in staphylococcal enterotoxin-free rabbit serum 1:5 and incubated for 30 min to block Protein A activity. The wells were then filled in duplicate with standard concentrations of the appropriate enterotoxin in PBS–Tween and incubated for 1 h at 37°C. One hundred milliliters of an appropriate dilution of horseradish peroxidase-coupled enterotoxin-specific antibody were added and incubated for 1 h at 37°C. The plates were washed 10 times with Tween–PBS and 100 μl substrate were added (0.03% 2,2'-azino-bis-3ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma Deisenhofen Germany), 0.001% Perhydrol in 0.05 M citrate buffer, pH 4.0) incubated for 20 min and read at 410 nm with an ELISA plate reader (Titertec, Meckenheim, Germany). In these assays, serial dilutions of purified toxin solutions (range 0.6–10 ng/ml) were used as standards. The sensitivity of these assays was approximately 1 ng/ml.

Preparation of cord blood cells

Blood was drawn by puncture of the umbilical vein of the placenta. No contamination with maternal IgA was detected. The blood for further cellular analysis was placed in a 50 ml sterile Falcon tube (Falcon, Heidelberg, Germany) containing 10 ml of HBSS with 100 IU penicillin, 100 $\mu\text{g/ml}$ streptomycin and 2.5 $\mu\text{g/ml}$ amphotericin B (Biochrom, Berlin, Germany). Only samples not older than 6 h were processed. Separation and culture were performed as described for peripheral blood mononuclear cells (PBMC).

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were separated from heparinized blood by standard Ficoll (1.077 g/ml; Biochrom, Berlin, Germany) density gradient centrifugation. Cells were suspended in culture medium (RPMI-1640; Biochrom, Berlin, Germany; supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 1 mM sodium pyruvate) at a density of 1×10^7 cells/ml at 37°C in an atmosphere containing 8% CO_2 in 24-well plates (Falcon, Heidelberg, Germany). The cells were stimulated with 10 ng/ml staphylococcal enterotoxin A (SEA; Toxin Technology, Sarasota, Florida, USA).

Stimulation and staining for intracellular cytokines

After 3 days of incubation, cells were re-stimulated with 10 ng/ml PMA (phorbol myristate acetate, Sigma Deisenhofen, Germany) and 1 μM ionomycin (Sigma Deisenhofen, Germany) for 5 h. Cytokine secretion was inhibited by 2.5 μM monensin (Sigma Deisenhofen, Germany). The cytokines were measured as described before (15). Cells were washed in HBSS (Biochrom, Berlin, Germany) and then fixed in ice-cold HBSS containing 4% paraformaldehyde (Riedel-de Haën, Seelze, Germany) for 10 min. Cells were washed twice. Permeabilization of the cell membrane was achieved by resuspension in 100 μl HBSS containing 0.1% saponin monensin (Sigma Deisenhofen, Germany) and 0.01 M HEPES buffer (saponin buffer). The permeabilized cells were incubated with FITC-labelled antibodies against intracellular proteins [interferon- γ (IFN- γ); interleukins 2, 4 and 5 (IL-2, IL-4 and IL-5), Pharmingen, Hamburg, Germany], phycoerythrin-labelled anti-CD45RO (Becton Dickinson, Heidelberg, Germany) and Tricolor-labelled anti-CD3 (Medac, Hamburg, Germany) against surface antigens for 20 min at 4°C. After washing with saponin buffer the cells were resuspended in 200 μl HBSS for flow cytometric analysis.

Data acquisition and analysis

List mode data was acquired on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using LYSIS II software. Typically, 30 000 events were acquired. List mode files were then analyzed using LYSIS II software.

Statistics

The statistical analysis was performed using the SPSS software package. χ^2 tests of association were used to analyze the categorical variables. Continuous variables were analyzed by means of the Kruskal–Wallis test with subsequent Dunn's test in case of a significant result. A p value of less than 0.05 was considered as significant throughout the study. Results are given as median and range.

Results

At 3 months of age, infants displayed a high nasal colonization with *S. aureus*

At 3 months of age, nasal colonization with staphylococci and enterotoxin generation were analyzed. As shown in Table 1, 31 of the 45

infants (68.9%) were found to harbour *S. aureus*. Twenty-nine of the 31 isolated strains (93.5%) produced enterotoxins. The infants were categorized according to the enterotoxin generation. In 16 infants either no staphylococci or staphylococci without enterotoxin generation were found, in an additional 16 infants enterotoxins B, C, D and E were detected, and finally 13 infants displayed colonization with SEA-producing strains. This classification was used for the subsequent analysis of immunologic and clinical data obtained at birth and at 6 months of age.

Cord blood cells from infants subsequently colonized with enterotoxin-producing *S. aureus* displayed a decreased frequency of functionally mature CD45RO-positive lymphocytes

The three groups were first compared for the expression of the marker CD45RO in cord blood lymphocytes cultured with SEA. In the group without enterotoxins, 2.1% (range 0.5–12.5%) of lymphocytes were CD45RO-positive, whereas both groups subsequently colonized with enterotoxin-producing *S. aureus* displayed significantly lower frequencies of CD45RO-positive cells when compared to the non-colonized groups, i.e. 0.5% (range 0.0–4.3%, $p < 0.01$) and 0.65% (range 0.0–2.1%, $p < 0.05$), respectively (Fig. 1).

CD45RO-positive cord blood lymphocytes of infants subsequently colonized with enterotoxin-producing staphylococci displayed a decreased frequency of IFN- γ -generating cells upon stimulation with SEA

Intracellular expression of IFN- γ was determined simultaneously with surface expression of CD45RO. Compared to the non-colonized controls, cord blood lymphocytes of infants from the subsequently SEA-positive group displayed a diminished production of IFN- γ upon stimulation with SEA *in vitro*. This difference was highly significant in CD45RO memory/effector cells (1.7%, range 0.0–9.3%, for non-colonized controls

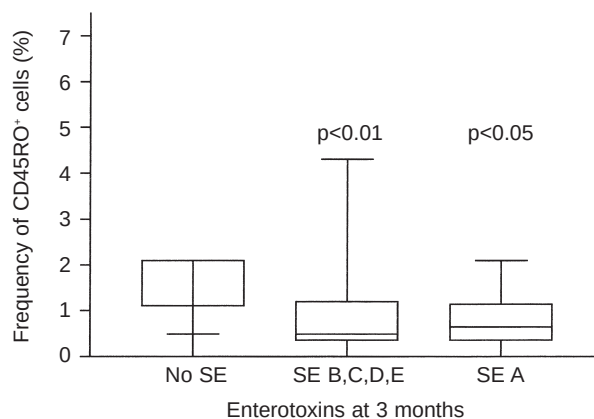


Fig. 1. Expression of CD45RO in cord blood lymphocytes. Cord blood lymphocytes were cultured with SEA for 3 days and re-stimulated with ionomycin and PMA. The expression of CD45RO was measured by flow cytometry as described in Materials and methods. The infants were grouped according to nasal colonization and enterotoxin generation of isolated *S. aureus* at 3 months (16 infants without enterotoxin, 16 infants with enterotoxins B,C,D or E, and 13 infants with SEA). Results are given as box and whiskers plots for percentage of T lymphocytes. Significant differences between the two enterotoxin-positive and the enterotoxin-negative groups are indicated.

versus 0.1%, range 0.0–0.4%, for the SEA-positive group, $p < 0.01$, Fig. 2), whereas there was only a slight but not significant trend in naive CD45RO-negative cells. Although the reduction of IFN- γ -producing cells was more obvious in the SEA-positive group, children colonized with strains producing other enterotoxins also displayed a significantly decreased response to SEA stimulation (1.7%, range 0.0–9.3%, for non-colonized controls versus 0.50%, range 0.0–2.5%, for infants colonized with non-SEA enterotoxin-producing strains, $p < 0.05$, Fig. 2).

Cord blood CD45RO lymphocytes of infants of the SEA-positive group display a significantly reduced frequency of IL-5- but not of IL-4-producing cells

We next asked whether the effect observed with IFN- γ also applied to other cytokines. Again no differences between the three groups were found in naive CD45RO-negative cells. In CD45RO-positive cells, however (Fig. 3), IL-5 generation was reduced in the SEA-positive group (0.25%, range 0.0–2.3%, for non-colonized controls versus 0.0%, range 0.0–0.4%, for the SEA-positive group, $p < 0.05$) as well as in the group positive for other enterotoxins compared to the non-colonized controls (0.0%, range 0.0–0.05%, $p < 0.01$). There was also a trend for a lower IL-2 production, which did not reach statistical significance. For IL-4, however, no differences were seen between the three groups.

Table 1. Microbiological characteristics of isolated staphylococci

	No enterotoxin	Enterotoxin B, C, D, E	Enterotoxin A
n	16	16	13
Catalase-positive	16	16	13
Coagulase	9	16	13
ID32 Staphsystem	2	16	13
Enterotoxin A	—	—	13
Enterotoxin B	—	3	—
Enterotoxin C	—	5	—
Enterotoxin D	—	3	—
Enterotoxin E	—	5	—

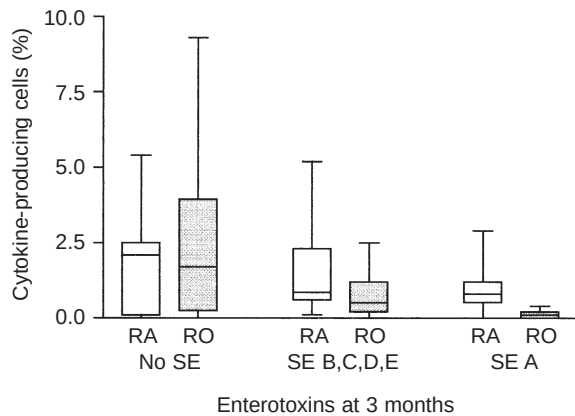


Fig. 2. Simultaneous expression of interferon- γ (IFN- γ) and CD45RO in cord blood lymphocytes. Cord blood lymphocytes were cultured with SEA for 3 days and re-stimulated with ionomycin and PMA. The expression of intracellular IFN- γ and CD45RO was measured by flow cytometry as described in Materials and methods. The infants were grouped according to nasal colonization and enterotoxin generation of isolated *S. aureus* at 3 months (16 infants without enterotoxin, 16 infants with enterotoxins B,C,D or E, and 13 infants with SEA). The proportion of IFN- γ -bearing T lymphocytes is given for CD45RO-positive and CD45RO-negative cells as box and whiskers plots. Significant differences between the two enterotoxin-positive and the enterotoxin-negative group are indicated. RA, CD45RA; RO, CD45RO.

At 6 months of age, CD45RO lymphocytes responded to SEA equally well, independent of former colonization with enterotoxin-secreting staphylococci

The infants were re-examined at 6 months of age. Although IFN- γ generation in CD45RO-positive lymphocytes seemed to be slightly depressed (Fig. 4), there was no significant difference between the three groups. Likewise, there was no difference concerning the frequency of IL-2-, IL-4- or IL-5-producing cells between the three groups.

Colonization with enterotoxin-producing staphylococci is independent of confounding environmental or medical factors

In order to identify possible confounding influences of environmental and medical factors, the children were examined by a physician with special emphasis on skin, respiratory tract, and ear, nose and throat factors at each appointment. Parents answered a questionnaire concerning their infant's diseases, atopic symptoms, nutrition and frequency of diseases. Neither demographic or pregnancy-related factors (gender, atopic risk, age of parents, duration of pregnancy, birth weight), nor environmental (socio-economic status, age of home, wall to wall carpet, indoor plants, pets, smoking, washable bedcover) and nutritional factors (feeding with cows milk or vegetables), nor the frequency of diseases (Table 2) were different between the three groups.

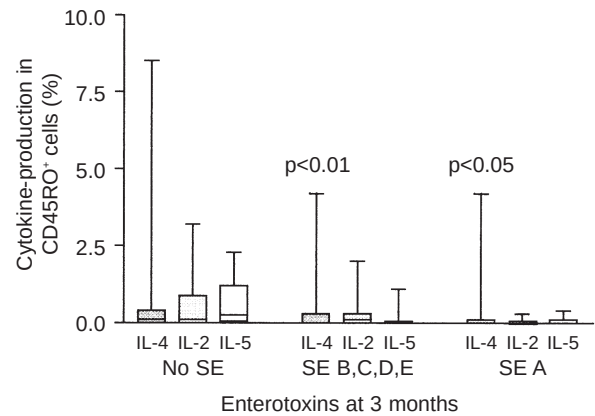


Fig. 3. Expression of interleukin (IL)-4, IL-2 and IL-5 in cord blood lymphocytes. Cord blood lymphocytes were cultured with SEA for 3 days and re-stimulated with ionomycin and PMA. The expression of CD45RO and a particular cytokine was measured by flow cytometry as described in Materials and methods. The infants were grouped according to nasal colonization and enterotoxin generation of isolated *S. aureus* at 3 months (16 infants without enterotoxin, 16 infants with enterotoxins B,C,D or E, and 13 infants with SEA). Results are given as box and whiskers plots for percentage of T lymphocytes. Significant differences between the two enterotoxin-positive and the enterotoxin-negative group are indicated.

Discussion

The present study was performed to elucidate the relationship between staphylococcal colonization and the cellular response to staphylococcal enterotoxins in the first months of life. In a cohort of infants, lymphocytes were stimulated with SEA at birth, nasal colonization with

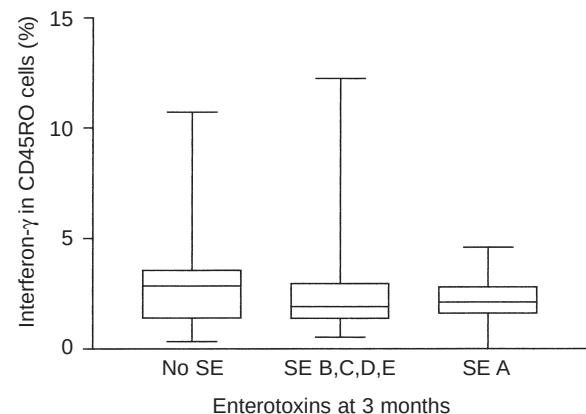


Fig. 4. Expression of interferon (IFN)- γ in lymphocytes isolated at 6 months of age. Lymphocytes isolated from the peripheral blood were cultured with SEA for 3 days and re-stimulated with ionomycin and PMA. The expression of CD45RO IFN- γ was measured by flow cytometry as described in Materials and methods. The infants were grouped according to nasal colonization and enterotoxin generation of isolated *S. aureus* at 3 months (16 infants without enterotoxin, 16 infants with enterotoxins B,C,D or E, and 13 infants with SEA). Results are given as box and whiskers plots for percentage of T lymphocytes. Significant differences between the two enterotoxin-positive and the enterotoxin-negative group are indicated.

enterotoxin-producing staphylococci was assessed at 3 months of age, and the reaction to SEA was re-examined at 6 months of age. The most important result was that the infants colonized with enterotoxin-producing staphylococci displayed decreased IFN- γ generation at birth but not at 6 months. The decrease was confined to the CD45RO lymphocyte sub-population, which is crucial to the effective response in viral and bacterial infection (16,17). It is conceivable that there is a causal relationship between low IFN- γ generation and staphylococcal colonization. The high rate of 68.9% colonized infants found in our study is consistent with previous studies (18).

SEA and other enterotoxins act as super-antigens. These proteins share an ability to bind to major histocompatibility complex proteins. The complex ligand so formed has specificity for a particular part of T-cell receptors, V-beta, and by engaging V-beta can stimulate many T cells (6). Animal studies (19) as well as *in vitro* studies with cord blood cells (20) have shown that contact with super-antigens early in life may result in tolerance and in clonal deletion of the super-antigen-responding cells. In contrast, the data presented here provide evidence for an increased response to SEA after colonization with SEA-producing staphylococci. Since the infants developed no symptoms related to the effect of enterotoxin, the concentrations of toxin were presumably low. Recently, Miethke et al. (21) have shown in mice that only high doses of enterotoxin induce clonal deletion whereas low doses induced a transient anergy that is overcome by clonal growth.

A possible explanation for the decreased frequency of IFN- γ -producing cells at birth is a lack of targets for SEA. Known targets of SEA are V-beta 9, V-beta 16 and V-beta 7.1 (22,23). It is conceivable that the frequency of T cells bearing these V-beta chains might be low in cord blood and increased upon subsequent contact with SEA *in vivo*. However, two observations argue against this hypothesis. First, the most prominent reduction of IFN- γ generation

was seen in cord blood CD45RO cells but not in naive CD45RO-negative cells. Second, reduced IFN- γ generation is also observed in the group of infants colonized with *S. aureus* secreting other enterotoxins but not SEA. Both findings are consistent with a lack of co-stimulatory signals in cord blood from infants subsequently colonized with enterotoxin-producing staphylococci. Co-stimulatory signals are essential for maturation of CD45RO memory cells (24) and are non-specific.

It is well known that the response to enterotoxins is enhanced by IL-12 (25) and by ligation of CD80 or CD86 with CD28 on T cells (26,27), whereas IL-10 has a suppressive effect (28). The cell that primarily provides these co-stimulatory signals is the dendritic cell. There is evidence that neonatal dendritic cells are functionally immature in cord blood (29) as well as in respiratory epithelium (30). Contact with bacterial products results in maturation of dendritic cell (31). It is thus conceivable that colonization with *S. aureus* will enhance maturation of dendritic cells *in vivo* and that most of the colonized infants will display normalized production of IFN- γ in response to SEA when re-tested at 6 months.

Lack of co-stimulatory factors in cord blood may also be responsible for the decrease of IL-5 and IL-2 generation. Interestingly, there were no differences concerning IL-4 generation at birth and at 6 months between the three groups analyzed. Ohshima and Delespesse (32) recently found that IL-4 production can be primed in naive T cells by a low density of dendritic cells, whereas induction of IFN- γ requires a high density of dendritic cells. Although there were no differences in the family history of atopy between the groups characterized in the present study, it is interesting that infants subsequently colonized with enterotoxin-producing staphylococci resemble infants who were shown to developed atopic disease in a study by Warner et al. (33). As with SEA in the present study, the babies described by Warner et al. displayed a reduced generation of IFN- γ *in vitro* upon stimulation with allergens or phytohaemagglutinin (PHA). Moreover, as for the subsequently colonized infants in the present study, the subsequently atopic infants displayed significantly lower numbers of CD45RO-positive cells at birth but not at 6 months of age (33).

The factors responsible for this pattern of immune response are largely unknown. No differences concerning exposure to nutritional or environmental allergens or atopy-related symptoms between the three groups were found in this study. In this regard it is interesting that in

Table 2. Diseases of the infants during the first 6 months

	No enterotoxin	Enterotoxin B, C, D, E	SEA
n	16	16	13
Vaccination (no/yes)	6/10	5/11	4/9
Diarrhea (no/yes)	12/4	14/2	7/6
Atopic dermatitis (no/yes)	14/2	13/3	12/1
Wheezing (no/yes)	13/3	13/3	12/1
IgE (kU/l) at 6 months	2.5 (1–74)	3.2 (1–21)	7.4 (1–45)

a recent study infants who were at risk of atopy could be identified by a reduced response not to enterotoxin alone but to a combination of enterotoxin and lipopolysaccharide, a product of Gram-negative *Escherichia coli* (34). Further work will be needed to define exactly the requirements of maturation and disease susceptibility.

In conclusion, in a cohort of newborns, lymphocytes were stimulated with SEA at birth, nasal colonization with enterotoxin-producing staphylococci was assessed at 3 months of age, and the reaction to SEA was re-examined at 6 months of age. The most significant result of the study is that the infants colonized with enterotoxin-producing staphylococci showed a decreased IFN- γ generation at birth but not at 6 months. This may indicate a predisposition at birth to be colonized with enterotoxin-producing staphylococci at 3 months of age.

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