Allergy development and the intestinal microflora during the first year of life

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Background: The intestinal microflora is a likely source for the induction of immune deviation in infancy.
Objective: The purpose of this study was to prospectively relate the intestinal microflora to allergy development in 2 countries differing with respect to the prevalence of atopic diseases.
Methods: Newborn infants were followed prospectively through the first 2 years of life in Estonia (n = 24) and Sweden (n = 20). By that age, 9 Estonian and 9 Swedish infants had developed atopic dermatitis and/or positive skin prick test results. Stool samples were obtained at 5 to 6 days and at 1, 3, 6, and 12 months, and 13 groups of aerobic and anaerobic microorganisms were cultivated through use of standard methods.
Results: In comparison with healthy infants, babies who developed allergy were less often colonized with enterococci during the first month of life (72% vs 96%; $P < .05$) and with bifidobacteria during the first year of life (17% to 39% vs 42% to 69%; $P < .05$). Furthermore, allergic infants had higher counts of clostridia at 3 months (median value, 10.3 vs 7.2 $\log_{10}$; $P < .05$). The prevalence of colonization with *Staphylococcus aureus* was also higher at 6 months (61% vs 23%; $P < .05$), whereas the counts of *Bacteroides* were lower at 12 months (9.9 vs 10.6 $\log_{10}$; $P < .05$).
Conclusion: Differences in the composition of the gut flora between infants who will and infants who will not develop allergy are demonstrable before the development of any clinical manifestations of atopy. Because the observations were made in 2 countries with different standards of living, we believe that our findings could indicate a role for the intestinal microflora in the development of and protection from allergy. (J Allergy Clin Immunol 2001;108:516-20.)

Key words: *Allergy, atopic dermatitis, infants, microflora, bifidobacteria, enterococci, clostridia, Staphylococcus aureus, Bacteroides, prospective study*

Abbreviations used
CCFA: Cefoxitin-cycloserine-fructose agar
CNA: Columbia agar with colistin and nalidixic acid
COA: Columbia agar with colistin sulfate and oxolinic acid
CONS: Coagulase-negative staphylococci
FAA: Fastidious anaerobes agar
LAM: Leeds acinetobacter medium

Numerous studies over the past 8 years have demonstrated that the prevalence of atopic diseases is lower in the formerly socialist countries of Central and Eastern Europe than in Western European countries.[1-3] The reasons for the difference are unknown. They are clearly environmental, however, and cannot be explained by genetic differences between populations.

It has been suggested that environmental factors associated with a Western lifestyle might decrease the overall exposure to microbial stimulation in infancy and early childhood and, as a consequence, lessen the stimulation of T_H1-type immunity.[4] If this is indeed the case, then the intestinal microflora would be of particular importance, inasmuch as it comprises approximately 10^{14} microorganisms—10 times more than the number of cells that the body consists of.[5]

We have previously shown infants in Estonia and infants in Sweden to be different with respect to microflora, with a low prevalence of allergies in the former and a high prevalence of allergies in the latter.[6,7] For example, the counts of aerobic bacteria (coagulase-negative staphylococci [CONS], enterococci, enterobacteria, and lactobacilli) were 10- to 1000-fold higher in Estonian than in Swedish newborn babies during the first week of life.[7] Furthermore, lactobacilli were more commonly found in Estonian children at 1 month[7] and 1 year.[6]

There are also differences in the composition of the intestinal microflora between allergic and nonallergic 2-year-old children.[8] Thus the prevalence of bifidobacteria was low in the allergic infants, whereas the counts of *Staphylococcus aureus* and enterobacteria were higher. Very recently, differences in the composition of the intestinal microflora between allergic infants and nonallergic infants were also confirmed by other methods.[9] Thus, isocaproic acid, which might be an indicator of colonization with *Clostridium difficile*, was found in 6 of 25 allergic 1-year-old infants but only 1 of 47 healthy babies. These studies were all cross-sectional, however, and did not address the issue of whether the differences were primary or secondary to disease. Yet in a recent

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prospective study, more clostridia were detected in the feces of 3-week-old infants who developed allergy during their first year of life.[10] They also tended to have less bifidobacteria at 3 weeks.

Because differences in microbial stimulation affecting the development of the immune system would occur very early in life, we have tested the hypothesis that early childhood allergy could be related to differences in the intestinal microflora. To exclude the influence of unknown local factors, this study was done prospectively in 2 countries, one with a low and the other with a high prevalence of atopic diseases.[1,2]

MATERIAL AND METHODS

Study groups and design

The study group consisted of 18 allergic infants (9 Estonian and 9 Swedish) and 26 nonallergic infants (15 Estonian and 11 Swedish). The 24 Estonian babies (12 of whom were boys) were born at the Women's Clinic of Tartu University Clinics between February 1997 and June 1998. The 20 Swedish babies (12 of whom were boys) were born at the Linköping University hospital between March 1996 and August 1998. The 2 groups were selected from participants in a prospective study of the development of immune responses to allergens and the development of allergy in relation to environmental factors. In each case, a family history of allergy was obtained, though this was not a criterion for inclusion. A positive family history was defined as a clear history of allergic rhinitis, asthma, or flexural, itching dermatitis in the parents. Selection criteria were vaginal delivery at term, an uncomplicated perinatal period, and availability of stool samples collected 5 to 6 days after birth and at 1, 3, 6, and 12 months. All of the children were immunized according to the routine procedures used in their respective countries. In Sweden, diphtheria, tetanus, inactivated polio, and *Haemophilus influenzae* type B vaccination was done at approximately 3, 5, and 12 months, and rubella, mumps, and measles vaccination was done at 18 months. In Estonia, BCG vaccination was done on day 3, 4, or 5 after birth, diphtheria, tetanus, pertussis, and oral poliovirus (Sabin) vaccination at 3, 4.5, and 6 months, and rubella, mumps, and measles vaccination at 12 months.

Clinical follow-up, focusing on allergic symptoms and skin prick testing, was performed at 3, 6, and 12 months and 2 years. The clinical assessment was done by one of us (T.V.) in Estonia and by a trained research nurse in Sweden.

Fecal samples were collected at 5 or 6 days and at 1, 3, 6, and 12 months of age. Approximately 1 to 2 g of voided stool was collected into sterile plastic containers by the ward staff or by the parents. Samples collected at home were kept in a refrigerator at 4°C for no more than 2 hours before transportation to the laboratory, where they were frozen at –70°C until analysis. Samples from Swedish children were transported to Estonia in dry ice for bacterial analyses.

Diagnostic criteria

Atopic dermatitis was defined as flexural, itching dermatitis, as characterized by Hanifin and Rajka[11]; recurrent wheezing was defined as reversible bronchial obstruction occurring at least 3 times and verified at least once by a doctor. Allergic rhinitis/rhinoconjunctivitis was defined as the appearance of symptoms at least twice after exposure to allergen and unrelated to the infection. The allergic children had atopic dermatitis and/or at least 1 positive skin prick test result. Thus symptoms from the respiratory tract were not regarded as allergy in the absence of a positive skin prick test result during the first 2 years of life.

Skin prick testing

Skin prick tests were performed in duplicate on the volar aspects of the forearms with natural egg white and fresh skimmed cow's milk (lipid concentration, 0.5%) at 3 and 6 months. At 12 months and 2 years of age, the children were also tested with standardized extracts of *Dermatophagoides pteronyssinus*, cat and dog dander, birch, timothy (Soluprick, ALK, Hørsholm, Denmark), and cockroach (Bayer, Spokane, Wash). Histamine hydrochloride (10 mg/mL) was used as a positive control and glycerol as a negative control. The test result was regarded as positive if the mean diameter of one of the wheals was ≥3 mm.

Bacteriologic analyses

Weighed samples of feces were serially diluted (10^{-2} to 10^{-9}) in prereduced phosphate buffer (pH 7.2) in an anaerobic glove box (Sheldon Manufacturing, Inc, Cornelius, Ore) with a gas mixture consisting of 5% CO_2, 5% H_2, and 90% N_2; they were then cultivated on 11 freshly prepared media. The order of analysis of samples from the Estonian and Swedish allergic and nonallergic infants was random, except that samples from the same infant were analyzed as closely in time as possible.

Yeast extract agar was used for total aerobes; yeast extract agar with 6.5% sodium chloride for staphylococci; Endo agar for enterobacteria; Leeds acinetobacter medium (LAM) with vancomycin at 10 mg/L, cefsulodin at 15 mg/L, and cephradine at 50 mg/L for acinetobacteria[12]; de Man-Rogosa-Sharpe agar (Oxoid, Hampshire, United Kingdom) for microaerophiles such as lactobacilli and streptococci; Columbia agar with colistin sulfate and oxolinic acid supplement (COA; Oxoid) for β-hemolytic streptococci; fastidious anaerobes agar (FAA; LAB M, Bury, United Kingdom) for total anaerobes; BBL Schaedler agar (Becton, Dickinson and Company, Franklin Lakes, NJ) with vancomycin and nalidixic acid supplement (Oxoid) for gram-negative anaerobes; BBL Columbia agar with colistin and nalidixic acid (CNA; Becton, Dickinson and Company) for gram-positive anaerobes; cefoxitin-cycloserine-fructose agar (CCFA; Oxoid) with egg yolk and sodium taurocholate for *C difficile*; and Sabouraud dextrose agar with penicillin (50,000 U/L) and streptomycin (40,000 U/L) for yeasts and fungi. The counts of clostridia were estimated on FAA after ethanol treatment.[13]

Seeding of anaerobes and incubation of FAA, Schaedler agar, CNA, and CCFA for 5 to 6 days was performed in the anaerobic glove box. The yeast extract agar, salt-yeast-extract agar, Endo agar, LAM, COA, and Sabouraud medium were incubated aerobically by 37°C and inspected after 24 and 48 hours. The de Man-Rogosa-Sharpe agar medium was incubated in a microaerophilic atmosphere (IGO 150 CO_2 incubator, Jouan Inc, Winchester, Va) for 72 hours.

Colonies that differed morphologically and were growing on the plate with the highest dilution of bacteria were Gram-stained and subjected to microscopy. The microorganisms were identified primarily at the genus level (CONS, enterococci, streptococci, acinetobacteria, *Candida*, bifidobacteria, *Bacteroides*, *Eubacterium*, clostridia) and the species level (lactobacilli, β-hemolytic streptococci, enterobacteria, *S aureus*, *C difficile*). The detection level of the various microorganisms was 3 $\log_{10}$ CFU/g.[7]

Standard methods were used for identification of enterobacteria and other gram-negative bacteria.[14] A coagulase test (Oxoid) was used for differentiation of *S aureus* and CONS. Streptococci and enterococci were identified by the absence of catalase production and differentiated by fermentation of esculin. β-hemolytic streptococci were identified by their growth on COA medium and through use of a latex test (Oxoid). Colony and cellular morphology and a negative catalase production identified lactobacilli grown on selective media. The anaerobes were identified up to the family or genus level by growth on selective media, colony and cellular morpholo-

TABLE I. Prevalence of certain species of fecal microorganisms in allergic (n = 18) and healthy (n = 26) children during the first year of life

	Prevalence (%)									
	1 week		1 mo		3 mo		6 mo		12 mo	
Microorganisms	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**
S aureus	62	56	65	56	50¶	50	23*¶	61*	27	39
Enterococci	96*	67*	96†	72†	96	89	96	100	96	89
Lactobacilli	8*‖	39*	46‖	56	34	56	38	39	38	44
Bifidobacteria	50†	17†	69	39	62‡	28‡	42	28	69§	22§
Bacteroides	46	61	62	72	65	83	81	94	96	89
Clostridia	27	50	27	28	46	28	65	50	54	67

Prevalence of microorganisms in allergic infants vs healthy infants at a given age: *P = .02; †P = .03; ‡P = .04; §P = .05.
Prevalence of microorganisms in healthy children at different ages: ‖P = .02; ¶P = .05.

gy, and Gram-stain reaction. We diagnosed anaerobic gram-negative rods as *Bacteroides*, gram-positive rods as eubacteria, bifidobacteria, or clostridia, and gram-positive cocci as anaerobic cocci. *C difficile* was identified by its ability to grow on CCFA, by colony and cellular morphology, by positive Gram staining, and by the typical smell. All anaerobic microorganisms were tested for the absence of growth under aerobic and microaerophilic conditions.

Statistical methods

The total count ($\log_{10}$ CFU/g) of microorganisms and the counts of various genera and species were calculated for each stool sample. In addition, the relative amount of each of the particular microbes was expressed as a percent of the total count in that sample.[6]

The Fisher exact test was used to compare allergic and nonallergic infants with respect to the prevalence of colonization at different ages. The counts (Mann-Whitney rank sum test) and the proportions of different microorganisms (Student *t* test) were compared through use of the computer program Statgraphics (Statistical Graphics Corp, Rockville, Md).

Ethical aspects

Informed consent was obtained from the parents of the babies. The Institutional Review Boards at Tartu and Linköping Universities approved the study.

RESULTS

The prevalence of colonization with enterococci was lower at 1 week and 1 month in infants who developed allergy during the first 2 years of life than in those who did not (67% vs 96% [P = .02] and 72% vs 96% [P = .03]; Table I). Similarly, the prevalence of colonization with bifidobacteria was lower in allergic infants through the first year of life, though significantly so only at 1 week (17% vs 50% [P = .03]) and at 3 (28% vs 62% [P = .04]) and 12 months (22% vs 69% [P = .005]). Bifidobacteria were isolated from 21 of 26 healthy and 8 of 18 allergic infants (P = .02) at 5 days and/or 1 month and in 24 of 26 healthy and 9 of 18 allergic infants at least once during the first 3 months of life (P = .003).

The differences between those infants who developed allergy and those who did not were less pronounced after the first month of life. As shown in Table II, however, higher counts of clostridia were recorded in the allergic infants at 3 months (10.3 vs 7.2 $\log_{10}$ [P = .01]). Furthermore, the prevalence of *S aureus* was higher at 6 months (Table I), and the counts of *Bacteroides* were lower at 12 months (Table II).

The prevalence and counts of lactobacilli increased in healthy children during the first month (Tables I and II). The high proportion of aerobic CONS decreased from 1 to 3 months (mean, 20% of the flora at 1 month vs 2% of the flora at 3 months [P = .02]). At the same time, the proportion of anaerobic bifidobacteria increased from 15% (mean of total bacterial count in the sample) at 1 month to 40% at 3 months (P = .01).

Two children with recurrent wheezing and negative skin prick test results in the group of nonallergic children had a colonization pattern similar to that seen in the other nonallergic children. The exclusion of these infants did not appreciably alter any of the relationships between microflora and allergy.

The duration of breast-feeding was 6 months (median) in both the allergic and the nonallergic infants. The numbers of babies treated with antibiotics during the first year of life were 9 of 18 in the allergic group and 12 of 26 in the nonallergic group. We also analyzed the different subgroups in Estonia and Sweden separately. Although the numbers then became too small for statistical analysis, the differences between allergic and nonallergic infants were similar in the 2 countries (data not shown).

DISCUSSION

This prospective study extends previous reports of differences in the composition of the intestinal flora between allergic infants and nonallergic infants[8,9] and demonstrates that they are already present during the first week of life. Differences in diet and antibiotic treatment cannot explain the findings; the allergic and nonallergic infants were similar with respect to diet and the use of antibiotic drugs in both countries.

In at least 3 recent studies, differences in the composition of the gut flora were observed between allergic and nonallergic infants.[8-10] Thus the counts of *S aureus* were higher and the prevalence of *Bacteroides* and bifidobacteria lower

TABLE II. Counts of certain fecal microorganisms in allergic (n = 18) and healthy (n = 26) children during the first year of life

	Count (CFU log/g)									
	1 week		1 mo		3 mo		6 mo		12 mo	
Microorganisms	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**	**Healthy**	**Allergic**
S aureus	8.2	7.1	8.8	7.2	7.6	5.9	6.1	5.3	5.4	4.6
	4.3-11.7	3.8-11.5	4-11.3	3.3-10.3	4.2-11.7	3.6-9.3	3.6-9.3	3.3-9.3	5.3-11	3.6-9.3
Enterococci	10.3	9.3	10.1	9.8	10.1	10.2	10.1	9.6	10.3	9.5
	3.9-11.8	3.6-11.7	6.9-12.2	6.3-11	5.2-11.8	3.3-11.7	5.9-11.6	4.3-11.8	4.2-11.8	4.3-12
Lactobacilli	5.8‡	8.8	8.6‡	7.5	9.3	7.8	8.4	9.3	10.2	7.8
	4.3-7.3	4.4-10.1	6.4-10.8	3.3-10.4	3.8-10.9	4.3-10.4	4.3-10.3	5.9-10.3	4.2-11.3	3.3-10.6
Bifidobacteria	10.3	10.8	10	10.3	10.8	11.2	10.3	10.8	10.6	10.9
	7.1-11.7	9.6-11.6	7.3-11.7	7.1-11.1	7.3-12.1	8-11.8	8.3-11.7	7.3-11.6	8.3-11.5	9.3-11.7
Bacteroides	11	9.9	9.8	10.1	9.8	10.1	10.1	9	10.6†	9.9†
	3.3-12	7.3-11.3	3.6-11.6	6.6-11.8	6.8-11.7	7.8-11.6	3.3-11.5	6.3-11.8	8.3-11.8	5.3-11.6
Clostridia	5.1	4.3	6.3	7.6	7.2*	10.3*	7.3	6.6	8.5	6.6
	3.3-10.6	3.6-11.7	3.3-7.9	3.3-9.3	3.6-10.8	7.8-12	3.6-9.3	4.3-10.1	3.6-12	3.3-11.8

The median values are given for positive samples.
Counts of microorganisms in allergic infants vs healthy infants at a given age: *P = .01; †P = .03.
Counts of microorganisms in healthy children at different ages: ‡P = .02.

in allergic children at 2 years.[8] In the present prospective study, lower counts of *Bacteroides* were observed at 12 months and the prevalence of bifidobacteria was lower through the first year of life. Furthermore, the allergic infants had higher counts of clostridia at 3 months but not later. Very recently, higher counts were reported in allergic infants at 3 weeks but not at 3 months or later.[10] Support for a higher prevalence of colonization with *C difficile* in allergic infants at 12 months was obtained in a considerably larger study, though an indirect method was used.[9]

The differences between the findings of the present study and those of previous studies might be due to differences in statistical power and the use of different methods. It should also be noted that the composition of the intestinal microflora is largely unknown; the observed differences were therefore possibly due to alterations in other, unknown components of the flora. Colonization with bifidobacteria and low counts of *Bacteroides* and *C difficile* appear to be associated with protection against allergy, however.

An early and more extensive colonization with aerobic bacteria in healthy infants could conceivably induce a strong stimulation of the immune system, including a stimulation of IL-12 by gram-positive bacteria.[15] The higher prevalence of bifidobacteria in healthy children very early in life is also of interest, because these microorganisms are known to elicit a T_H1-type immune response[15,16] According to the "hygiene hypothesis," atopy is associated with less microbial stimulation and as a consequence, with a prolonged propensity towards T_H2-skewed immune responses.[4] Interest has recently been focused on the protective role of early infections on the development of allergy, possibly through T_H2-inhibitory effects of cytokines released during host defense responses to infections.[17] It would seem unlikely, however, that a stimulus that is potentially harmful to the host should be necessary for the postnatal maturation of a balanced immune system. It has therefore been suggested that the primary signal for such maturation during infancy and early childhood is provided not by pathogens but by stimulation from the commensal microbial flora—in particular, the flora of the gastrointestinal tract.[18] The present study could support this hypothesis. Furthermore, a very recent prospective study suggested that lactobacilli given during the first 6 months of life might protect against the development of allergy.[19]

It is tempting to speculate that the differences in gut flora between babies who will and babies who will not develop clinical manifestations of atopy indicate that these flora are primary causes of allergy—either promoting immune deviation in healthy infants or delaying it in allergic babies. Our findings do not exclude the possibility, however, that atopy is associated with mucosal conditions' facilitating a certain microflora. Thus it is possible that the differences in the gut flora and the differences in the development of immunity are both consequences of one or more other, as-yet-unknown factors associated with the atopic genotype. Only a prospective intervention study or studies in gnotobiotic animals could settle this issue.

In conclusion, our results show that differences in the composition of the gut flora are demonstrable very early in life and before the development of any clinical manifestations of atopy. If the microbial flora drives the maturation of the immune system, changes in its composition as a consequence of altered lifestyles and diet in industrialized societies might play a role in the higher prevalence of allergy. Because our observations were made in 2 countries with different standards of living, we believe that these findings could indicate a major role for the intestinal microflora in protection from allergy.

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