Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing

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Background: Improved hygiene has altered early microbial exposure by reducing childhood infections, which has been suggested as a cause for the continuously rising prevalence of atopic diseases. On the basis of both intensity and timing of stimulus, it has been hypothesized that exposure to commensal microflora may represent another key protective modulator of immunity against atopy and subsequent atopic diseases.
Objective: We sought to investigate whether differences in early gut microflora precede the later development of atopic sensitization.
Methods: Intestinal microflora from 76 infants at high risk of atopic diseases were analyzed at 3 weeks and 3 months of age by using conventional bacterial cultivation and 2 culture-independent methods, gas-liquid chromatography of bacterial cellular fatty acids and quantitative fluorescence in situ hybridization of bacterial cells. Infants evincing at least one positive skin prick reaction at 12 months were grouped as atopic subjects, and those without positive reactions were grouped as nonatopic subjects.
Results: Atopic sensitization was observed in 22 (29%) of 76 children. At 3 weeks, the bacterial cellular fatty acid profile in fecal samples differed significantly between infants in whom atopy was and was not developing ($P = .005$). By using fluorescence in situ hydridization, atopic subjects had more clostridia (geometric mean [95% confidence interval]: 9.3×10^7 [3.8-22.9 $\times 10^7$] vs 3.3×10^7 [1.8-6.1 $\times 10^7$], $P = .04$) and tended to have fewer bifidobacteria (1.8×10^9 [0.4-7.6 $\times 10^9$] vs 6.1×10^9 [2.5-14.6 $\times 10^9$], $P = .11$) in their stools than nonatopic subjects, resulting in a reduced ratio of bifidobacteria to clostridia ($P = .03$). The differences were not detected by bacterial cultivation.
Conclusion: Differences in the neonatal gut microflora precede the development of atopy, suggesting a crucial role of the balance of indigenous intestinal bacteria for the maturation of human immunity to a nonatopic mode. (J Allergy Clin Immunol 2001;107:129-34.)

Key words: *Atopic sensitization, atopy, gut-associated lymphatic tissue, gut microflora, infant, intestine*

Abbreviations used
DAPI: 4´,6-Diamidino-2-phenylindole
FISH: Fluorescence in situ hybridization
GALT: Gut-associated lymphatic tissue
GLC: Gas-liquid chromatography

The prevalence of atopic diseases has been continuously on the increase in Western societies.[1] Epidemiologic studies have demonstrated an inverted association between atopy and sibling number.[2] On this basis, the hygiene hypothesis proposes the rapid increase in atopy to be related to reduced exposure to infections early in life,[3] when the immune responder phenotype is consolidated.[4] The hypothesis is supported by data showing the immune response to microbial antigens to be accompanied by preferential expression of T_H1 cytokines,[5,6] counterbalancing the T_H2-polarized cytokine production of neonates,[7] the continuity of which might lead to enhanced IgE production, atopy, and atopic disease.[5,6] Could atopy be ascribed to the lack or insufficiency of counterbalancing microbial exposure? Thus far, no prospective clinical evidence is on record to confirm such a conception.

Recent retrospective epidemiologic studies have shown that in particular food-borne and orofecal infections might be essential in prevention of atopy.[8,9] Because the host's major and primary microbial stimulation occurs along with the establishment of the gut microflora,[10] it has been suggested that exposure to commensal microflora or specific strains thereof may represent a key modulator of the immune system against atopy and atopic diseases.[11-13] Because the hypothesis is so far based only on retrospective cross-sectional data, we addressed the question thoroughly by prospectively following 76 high-risk infants during the first year of life. Intestinal microflora were analyzed at 3 weeks and 3 months of age, and development of atopic sensitization was determined at 12 months by using skin prick testing.

Research on the gastrointestinal microflora by conventional bacterial culture has been hampered by the lack of sensitivity in methods of assessment because almost half of the bacteria in the gut are unculturable,[10] if nonetheless viable.[14] The characteristics of the media often cause difficulties in bacterial cultivation irrespective of whether media are not truly specific and growth of unwanted bacterial species disturb reliable counts or

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media are so specific that only certain strains of a bacterial group grow on the plates.[15] To verify the hypothesis, we therefore used, in addition to bacterial cultivation, 2 culture-independent methods, gas-liquid chromatography (GLC) of bacterial cellular fatty acids[16] and quantitative fluorescence in situ hybridization (FISH) of bacterial cells,[14,17] to characterize the gut microflora in infants in whom atopy later did or did not develop.

METHODS

Subjects and study design

One inclusion criterion for the study was a family history of atopic disease (ie, one or more family members [mother, father, and/or older sibling] with atopic eczema, allergic rhinitis, or asthma). Families were recruited in antenatal clinics in the city of Turku (population, 170,000). The study cohort comprised 76 volunteer families with 76 newborn infants. In this study population 30 (39%) of 76 infants had one and 46 (61%) of 76 had several first-degree family members with atopic disease. The infants were born between the thirty-sixth and forty-second weeks of gestation (mean, 40 weeks). They were clinically examined at the ages of 2 days, 3 weeks, and 3, 6, and 12 months. Any signs and symptoms of atopic disease were recorded. Atopic sensitization at the age of 12 months, evaluated by skin prick testing, was taken as the primary endpoint. Infants were considered to be atopic if they evinced at least one positive reaction to the antigens tested, whereas those without positive reaction were considered to be nonatopic. Double-blind, placebo-controlled, cow's milk challenges were applied when symptoms, clinical signs, or skin prick test responses were suggestive of cow's milk allergy.

The study was approved by the Committees on Ethical Practice in Turku University Central Hospital and the Health Office of the city of Turku. Written informed consent was obtained from the children's parents.

Diagnosis of atopy

Atopic sensitization was evaluated by skin prick testing, as previously described.[18] Reactions were read at 10 minutes, and half of the histamine dihydrochloride (10 mg/mL; ALK Abelló, Horsholm, Denmark) reaction sizes (2+) or more was recorded as positive on the condition that the mean diameter of the wheal was at least 3 mm, and the negative control (ALK) at the same time was 0 mm. Antigens tested included milk, wheat, and rye flours diluted both 1:10 (wt/vol) with 0.9% (wt/vol) sodium chloride; gliadin diluted 1:1000 (wt/vol) with 0.9% (wt/vol) sodium chloride; banana, potato, carrot (3 last mentioned by prick-prick technique), egg white, cod, soybean, birch, a mixture of 6 local grasses, cat, dog, and *Dermatophagoides pteronyssinus* allergen Der p 1 (all 13 from ALK); and latex (Stallergens, France). The diagnosis of atopic dermatitis was based on criteria previously described.[19] Briefly, atopic dermatitis was confirmed if the following 3 major features were detected: pruritus, typical morphology and distribution, and chronic dermatitis (duration of 1 month or longer). The diagnosis of cow's milk allergy was based on an unambiguous relationship between ingestion of cow's milk and clinical symptoms (ie, the symptoms disappeared after elimination of cow's milk from the diet), and an unequivocal relapse occurred in a double-blind, placebo-controlled, cow's milk challenge. This challenge was carried out as described elsewhere.[18]

Cultivation of stool samples

A fecal sample from the infant was taken either by nursing staff at a scheduled visit or immediately before it by parents. In the latter case, the sample was stored at 4°C and delivered to the hospital within 24 hours for immediate cultivation. A stool sample was obtained from 71 infants at the age of 20 days (18-21 days) and from 69 infants at the age of 14 weeks (13-14 weeks). The rest of the sample was immediately frozen and stored at –20°C until analyzed with GLC and FISH. No quantitative culture methods were used. The bacteria were cultured on 6 different freshly prepared media: blood agar (Pronadisa, Madrid, Spain) for gram-negative rods; agar (Leiras, Turku, Finland) supplemented with mycological peptone (Oxoid, Basingstoke, United Kingdom) and glucose for yeasts and fungi; bile eskulin azide agar (Difco, Detroit, Mich) for enterococci; blood agar (Pronadisa) supplemented with glucose, yeast extract (LAB M, Bury, United Kingdom), L-cysteine HCl (Merck, Darmstadt, Germany), metadion (Merck), and neomycin sulfate (Sigma, St Louis, Mo) for anaerobes; *Clostridium difficile* Agar (Oxoid) supplemented with hemin (Sigma), neutralred (Merck), D-cycloserine (Sigma), egg, and cefoxitin (MSD, Haarlem, the Netherlands) for *Clostridium difficile*; and Rogosa SL agar (Difco) for *Lactobacillus*-like bacteria. The first 3 media were incubated aerobically and the last 3 were incubated anaerobically at 35°C for 48 hours. Subsequently, identification of different species was made according to their growth on selective media, colonies, color, and cell morphology.

GLC of bacterial fatty acids

The method has previously been described in more detail.[16] After separation of bacterial material from fecal vegetable fibers and free fatty acids, the sample was remixed and allowed to sediment for 15 minutes. Thereafter, the bacterial component was isolated by centrifuging at 1000*g* for 15 minutes at room temperature and removing the supernatant. The collected bacterial mass was saponified and methylated. The methylated fatty acids were then extracted with ethyl ether and hexane. GLC analysis was performed with an HP5890A gas chromatograph (Hewlett-Packard) and an Ultra 2004-11-09B fused silica capillary column (0.2 mm by 25 m; cross-linked 5% phenylmethyl silicone; Hewlett-Packard). A recently developed computerized bacterial identification program was used to analyze the GLC profiles of the fecal samples.[16] The analysis was based on the correlation and cluster analysis of the fatty acid spectra of individual samples. All peaks of individual fatty acids in the chromatograms were used in comparisons.

FISH of bacterial cells

Fecal samples were suspended in 0.1 mol/L PBS (pH 7.0) to give a final concentration of 10% (wt/vol). The slurries were homogenized and centrifuged at low power (250*g* for 2 minutes) to remove particulate matter. Bacterial cells were fixed, and FISH was performed as previously described.[17] In brief, cells were fixed overnight in 4% (vol/vol) paraformaldehyde at 4°C, washed twice in PBS, and stored at –20°C in a PBS/ethanol (1:1) solution. Subsamples of the fixed cells were hybridized overnight in hybridization buffer with 5 $ng \cdot \mu L^{-1}$ Cy3 indocarbocyanin-labeled oligonucleotide probe. Probes included were Bac303 (CCAATGTGGGGGACCTT)[20] specific for bacteroides, Bif164 (CATCCGGCATTACCACCC)[17] for bifidobacteria, His150 (TTATGCGGTATTAA TCT(C/T)CCTTT)[21] for clostridia, and Lab158 (GGTATTAGCA(T/C)CTGTTTCCA)[22] for lactobacilli and enterococci (sequence 5′→3′). Total cell numbers were counted by using a nucleic acid stain 4′,6-diamidino-2-phenylindole. Cells were washed with the hybridization buffer, filtered through a 0.2-μm polycarbonate filter (Millipore Corporation, Bedford, Mass) and mounted on a slide with SlowFade (Molecular Probes Inc, Eugene, Ore). They were counted visually by using a Leica Laborlux D epifluorescence microscope mounted with Cy3 and 4′,6-diamidino-2-phenylindole specific filters. Fifteen microscopic fields were counted per assay.

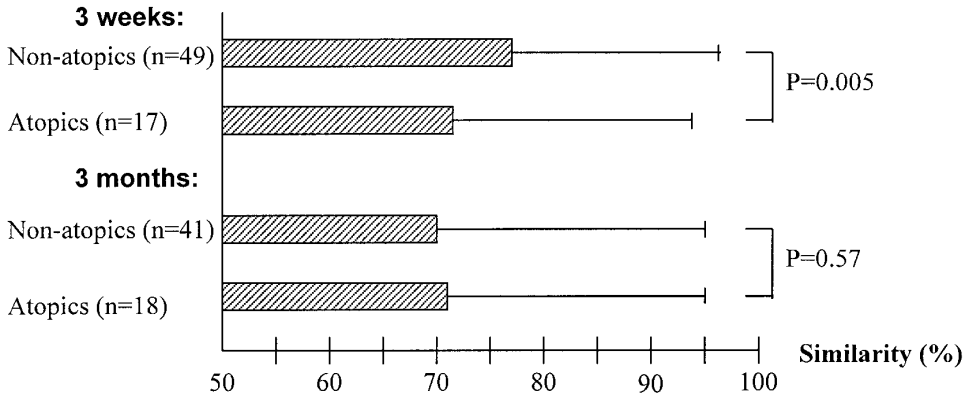

FIG 1. Relative similarities of bacterial fatty acid profiles in fecal samples analyzed by using GLC between samples from atopic and nonatopic subjects at 3 weeks and 3 months. *Column* represents means ± SD. The analysis of the GLC data was based on paired comparisons between individual samples and calculation of similarity indices between them. Similarity indices were presented as correlation matrices and further analyzed by using weighted paired-group cluster analysis of arithmetic means. See the "Methods" section for detailed description of the analysis.

Statistics

Because of skewed distribution, data of FISH of bacterial cells are expressed as geometric means with 95% confidence intervals after logarithmic (log) transformation. Unpaired *t* tests were applied to compare values between the groups. The χ^2 test was used to compare proportions between the groups. The entire analysis of the GLC data was based on paired comparisons between individual samples and calculation of similarity indices between them. Similarity indices were presented as correlation matrices and further analyzed by a weighted paired-group cluster analysis of arithmetic means.[23] The fecal samples were divided into 4 different groups for analysis of the results of GLC of bacterial cellular fatty acids according to the atopic status of the infant (atopic vs nonatopic) and sampling time (age, 3 weeks vs 3 months). Statistical significance between atopic and nonatopic subjects at different time points was calculated by comparing the variation in fatty acid profiles within the groups to that between the groups. The within-group variation was determined by calculating the mean ± SD of the similarity indices of all paired comparisons within the group. That between the different groups was obtained by calculating the mean ± SD of all paired similarity indices between samples in both groups. Subsequently, the intergroup variation was compared with that within the groups by calculating a *z* value, as previously described.[23] The *z* value was used to determine the *P* value by means of a *z* table. A *P* value of less than .05 was considered statistically significant.

RESULTS

Development of atopy

At the age of 12 months, atopic sensitization was observed in 22 (29%) of 76 children. Skin prick test reactivity to egg, cow's milk, wheat, cat, and latex was seen in 19 (86%) of 22, 5 (23%) of 22, 3 (14%) of 22, 2 (9%) of 22, and 1 (5%) of 22 atopic subjects, respectively. No skin prick reactivity to any other antigen tested was seen. Five atopic subjects were polysensitized; that is, they evinced positive skin prick test reactivity to at least 2 different antigens. Half of the atopic subjects, 11 (50%) of 22, manifested atopic dermatitis, and 9 (41%) of 22 were allergic to cow's milk, as diagnosed by using double-blind, placebo-controlled, cow's milk challenge.

A maternal atopic history was observed in 19 (86%) of 22 atopic subjects and 45 (83%) of 54 nonatopic subjects ($P = .74$). Atopic and nonatopic subjects were comparable for birth characteristics: length ($P = .24$), weight ($P = .94$), and head circumference ($P = .98$, data not shown). At the ages of 3 weeks and 3 months, dietary characteristics were comparable between atopic and nonatopic subjects (Table I).

Neonatal gastrointestinal microflora

By 3 weeks, 3 (6%) of 54 nonatopic subjects but none of the atopic subjects had received a course of antibiotics ($P = .26$). There was a statistically significant difference in the bacterial cellular fatty acid profile of stool samples ($P = .005$, Fig 1) between infants in whom atopy was and was not developing, which was not detected by use of bacterial cultivation (Table II). To detect the bacteria possibly responsible for the discrepancy, FISH of fecal bacteria was applied to the remaining 29 fecal samples. The results demonstrated that the ratio of bifidobacteria to clostridia was reduced in atopic subjects (geometric mean [95% confidence interval]: 19 [3-122]) compared with that in nonatopic subjects (185 [57-604], $P = .03$). The difference was caused by a tendency toward lower counts of bifidobacteria and higher counts of clostridia in atopic subjects (Table III).

Gastrointestinal microflora at the age of 3 months

There were no statistically significant differences in gut microflora between infants in whom atopy was and was not developing at the age of 3 months. The respective bacterial cellular fatty acid spectra were comparable (Fig 1). Nor did results of bacterial cultivation differ between the groups, although nonatopic subjects tended to have yeasts in their stools more frequently than atopic subjects (Table II). After omitting the infants who had received a course of antibiotics, this tendency was reduced (4/40 [10%] vs 0/20, respectively; $P = .14$).

TABLE I. Dietary characteristics in infants at 3 weeks and 3 months

	Atopic subjects* (n = 22)	Nonatopic subjects* (n = 54)
3 wk†		
Exclusively breast-fed	16/22 (73%)	37/54 (68%)
Breast-fed and formula-fed	6/22 (27%)	15/54 (28%)
Formula-fed	0/22	2/54 (4%)
3 mo‡		
Exclusively breast-fed	11/22 (50%)	29/54 (54%)
Breast-fed and formula-fed	6/22 (27%)	14/54 (26%)
Formula-fed	5/22 (23%)	11/54 (20%)

Values are numbers (percentages) of infants exclusively breast-fed, both breast-fed and formula-fed, or solely formula-fed.
*Infants with at least one positive reaction in skin prick testing at 12 months were considered atopic, and those without positive reactions were considered nonatopic.
†$P = .65$ and ‡$P = .95$, χ^2 test (atopic vs nonatopic subjects).

TABLE II. Cultured fecal microflora in infants at 3 weeks and 3 months

	Yeasts and fungi*	Gram-negative rods*	Anaerobics*	*Clostridium difficile**	Lactobacilli*	Enterococci*
3 wk						
Atopic subjects†	0/20	15/20	18/20	0/20	16/20	18/20
Nonatopic subjects†	2/51	39/51	45/51	2/51	41/51	45/51
χ^2 Test	0.37	0.90	0.83	0.37	0.97	0.83
3 mo						
Atopic subjects†	0/20	20/20	18/20	1/20	18/20	20/20
Nonatopic subjects†	7/49	45/49	40/49	6/49	44/49	47/49
χ^2 Test	0.07	0.19	0.39	0.37	0.98	0.36

*Numbers in columns represent the number of infants having yeasts and fungi/bacterium-bacteria in fecal samples analyzed by cultivation.
†Infants with at least one positive reaction in skin prick testing at 12 months were considered atopic, and those without positive reactions were considered nonatopic.

TABLE III. Bacterial counts in fecal samples analyzed by means of FISH at the age of 3 weeks

	Atopic subjects* (n = 12)	Nonatopic subjects* (n = 17)	Unpaired *t* test‡
Clostridia† ($\times 10^7$)	9.3 (3.8-22.9)	3.3 (1.8-6.1)	.04
Bifidobacteria† ($\times 10^9$)	1.8 (0.4-7.6)	6.1 (2.5-14.6)	.11
Lactobacilli/enterococci† ($\times 10^8$)	2.4 (1.1-5.2)	3.4 (1.5-7.6)	.53
Bacteroides† ($\times 10^8$)	1.1 (0.3-4.4)	0.5 (0.2-1.4)	.30
Total cell count† ($\times 10^9$)	8.9 (4.0-19.4)	9.6 (5.3-17.3)	.87

*Infants with at least one positive reaction in skin prick testing at 12 months were considered atopic, and those without positive reactions were considered nonatopic.
†Geometric mean (95% confidence interval) of fecal number of bacteria per gram.
‡Unpaired *t* test was applied after logarithmic transformation of bacterial counts.

DISCUSSION

The data obtained here demonstrate for the first time that differences in gut microflora precede the development of atopy and characterize the difference as a reduced ratio of bifidobacteria to clostridia at neonatal age.

At birth, the gastrointestinal tract of the newborn is sterile, and the evolution of gut-associated lymphoid tissue (GALT) is in its infancy. During the first years of life, an adult-type pattern of stable indigenous gut microflora is established[24] concomitantly with the development of GALT in the most important effector organ of the adaptive immune system.[25] The successful maturation of the gut mucosal immune system requires constant microbial stimulus from the developing gastrointestinal microflora. Numerous experimental studies have shown that the lack or inadequacy of such a stimulus results in decreased intestinal surface area, altered mucosal enzyme patterns, defects in the nonimmunologic barrier of the intestine, reduced capacity for inflammatory responses, a defective mucosal IgA system,[10,25-27] and abrogation of oral tolerance[28] unresponsiveness to nonpathogenic antigens previously encountered on mucosal surfaces.

A recent study reported that oral tolerance was achieved in germ-free mice only if the intestinal microflora was reconstituted with bifidobacteria during the neonatal period.[28] The findings in the present study provide the first clinical demonstration of the potential crucial regulatory role of the neonatal gut microflora for the development of protective mechanisms against atopy. It is tempting to speculate on the extension of the hygiene hypothesis: the very early commensals in gut microflora might provide essential initial priming signals against atopy, which are further strengthened by environmental microbes later in life.

Although the exact mechanisms responsible for the protective effect remain to be elucidated, recent studies suggest that the favorable effect of the gut microflora on oral tolerance, and more generally against enhanced T_H2 activity, may be at least partly mediated by 2 structural components of bacteria, the lipopolysaccharide portion of gram-negative bacteria,[3] and a specified CpG motif in bacterial DNA,[29] which promote T_H1-dominant immune responses. T_H1 immunity is further strengthened by immune responses induced by microbes in the gut, including yeasts and fungi,[30] and by microflora bacteria eliciting the production of anti-inflammatory cytokines (eg, IL-10 and transforming growth factor [TGF] β).[31,32]

The classical bacteriologic culture methods have proved insensitive in studying changes in the gut microflora,[10] and new approaches are clearly needed to improve our knowledge of gut microbial diversity, which in cells exceeds that in the body by a factor of 10.[10] The cellular fatty acids analyzed by GLC are structural components of bacterial cell membranes, and each bacterial species has its own unique fatty acid composition. In a fecal sample the fatty acid profile represents all the bacteria present in the sample, thus allowing sensitive comparison of different samples.[23] In a previous study the profile of human fecal microflora was altered by an uncooked vegan diet, as analyzed by GLC of bacterial cellular fatty acids, whereas conventional quantitative bacterial culture did not detect any significant change in the same samples.[33] FISH of bacterial cells uses specific 16S rRNA-based oligonucleotide probes to enumerate all bacteria, culturable or not, in stool samples.[14,17] Previous studies have shown approximately 10-fold differences in the number of bacteria detected, depending on whether FISH or classical bacterial cultivation are used in analysis.[17,21] A recent study concluded that the molecular 16S rRNA-based techniques, as FISH in this study, provide more accurate quantitative data on gut microflora in newborns than do conventional culture techniques.[34] The introduction of these culture-independent methods may thus offer new sensitive tools for the study of host-microbe interactions operative in the development of both physiologic immunity and disordered states.

Neonatal intestinal microflora changes precede the development of atopic sensitization during infancy. This finding calls for further research to characterize the mechanisms by which specific intestinal microbes interact with the development of GALT at an early age. Studies are also needed to improve our understanding of the myriad factors interfering in the bacterial colonization of human subjects in economically developed countries[9,13,35] and of their effect on the recent rapid increase in the frequency of the diseases of the modern world, one of which is allergy.

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