Gut microbiota and development of atopic eczema in 3 European birth cohorts

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Background: Stimulation of the immune system by gut microbes might prevent allergy development.
Objective: The present study examined the hypothesis that sensitization to food allergens and atopic eczema are influenced by the infantile intestinal colonization pattern.
Methods: Infants were recruited perinatally in Göteborg (n = 116), London (n = 108), and Rome (n = 100). Commensal bacteria were identified to the genus or species level in rectal (3 days) and quantitative stool cultures (7, 14, and 28 days and 2, 6, and 12 months of age). At 18 months of age, atopic eczema and total and food-specific IgE levels were assessed. These outcomes were modeled in relation to time to colonization with 11 bacterial groups and to ratios of strict anaerobic to facultative anaerobic bacteria and gram-positive to gram-negative bacteria at certain time points. Study center, mode of delivery, parity, and infant diet were included as covariates.
Results: Neither atopic eczema nor food-specific IgE by 18 months of age were associated with time of acquisition of any particular bacterial group. Cesarean section delayed colonization by *Escherichia coli* and *Bacteroides* and *Bifidobacterium* species, giving way to, for example, *Clostridium* species. Lack of older siblings was associated with earlier colonization by *Clostridium* species and lower strict anaerobic/facultative anaerobic ratio at 12 months.
Conclusions: This study does not support the hypothesis that sensitization to foods or atopic eczema in European infants in early life is associated with lack of any particular culturable intestinal commensal bacteria.
Clinical implications: The nature of the microbial stimulus required for protection from allergy remains to be identified.
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Key words: *Allergy, atopic eczema, sensitization, IgE, intestine, commensal bacteria, infant*

The causes of the ''allergy epidemic'' in Western countries[1] are unknown. The hygiene hypothesis postulates that deprivation of microbial exposure in infancy predisposes to immune dysregulation and allergy development.[2,3] This is based on observations that children growing up in poor[3] or large[3] families or with close animal contacts[4,5] have reduced risk of allergy. Childhood infections are associated with reduced allergy development, especially gastrointestinal infections,[6-8] but no particular protective microbial stimuli have yet been identified.

The commensal intestinal microbiota could play a role in shaping the developing immune system and in protecting against allergy development. Acquisition of the commensal microbiota proceeds in a sequential manner, with facultative (oxygen-tolerant) anaerobic bacteria establishing early. Strict anaerobic bacteria establish successively until an adult-type microbiota dominated by strict anaerobic bacteria by a factor of 1000:1 is established.[9] Infants in industrialized countries are colonized later than those in developing countries by fecal bacteria[10] and have slow strain turnover in their microbiota.[11] This could lead to a lesser stimulation of the immune systems of westernized infants because intestinal bacteria evoke an immune response transiently on their establishment in the microbiota and further persistence of the same strain does not further stimulate immunity.[12] Development of immunologic tolerance to dietary antigens, which occurs normally in human subjects and animals,[13,14] is, at least in part, dependent on the presence of commensal microbes.[15] Because gram-positive and gram-negative bacteria affect antigen-presenting cells differently,[16] the composition of the microbiota could influence the immune response to innocuous environmental antigens.

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It has been hypothesized that the ''Western microbiota'' fails to support maturation of immunologic tolerance to environmental antigens because of the disappearance of key groups of microorganisms necessary for tolerance development or because of a secondary expansion of groups of bacteria that hamper the induction of tolerance.[17] Indeed, certain studies have reported differences in the early intestinal microbiota between infants developing and those not developing allergic disease, with more prominent colonization by *Bifidobacterium* species but less by *Clostridium* species in the latter group.[18-20] The results are, however, not univocal, and a recent large study saw no protective effect of bifidobacteria but reported that colonization by *Clostridium difficile* at 1 month of age was associated with later allergy development.[21]

In the present birth cohort study, including more than 300 infants, we investigated whether colonization by culturable fecal bacteria is related to the development of atopic eczema and sensitization to at least 1 of the most common food allergens by 18 months of age in Swedish, British, and Italian infants, taking into account the possible influence of lifestyle and dietary factors. This is, to date, the largest cohort followed longitudinally with respect to establishment of the intestinal microbiota. Furthermore, inclusion of infantile populations from 3 European countries increases the generalizability of the findings.

METHODS

Study design

We recruited newborn infants at Sahlgrenska University Hospital, Göteborg; St George's Hospital, London; and Sandro Pertini Hospital, Rome. A normal singleton pregnancy was the only inclusion criterion, whereas language difficulty, moving house soon, or prematurity (<37 weeks' gestation) were exclusion criteria. Atopic heredity, family structure, and exposure to animals were ascertained by questionnaire, and information on delivery and antibiotic treatment of the mother or baby was retrieved from hospital records. Parental interviews at 6 and 12 months of age probed for feeding pattern, infections, and medication. Maternal diet while breastfeeding was not considered.

At 18 months, all infants were examined through a standardized questionnaire and physical examination. Atopic eczema was diagnosed according to Williams' criteria,[22] and a SCORAD value was obtained by using validated software (SCORAD-Card; TPS Production, Rome, Italy).[23] Serum total IgE (IgE-FEIA; Pharmacia Diagnostics, Uppsala, Sweden) and specific IgE levels against common food antigens (FX5 food mix: egg white, cow's milk, fish, peanut, soya, and wheat; Immunocap FEIA, Pharmacia Diagnostics) were tested centrally.

Informed consent was obtained, and the study was approved by local ethics committees.

Culture and identification of bacteria in intestinal microbiota

A rectal swab was collected at 3 days of age and cultured semiquantitatively for facultative anaerobic bacteria within 24 hours, as described in detail elsewhere.[24] Fecal samples were collected at 7, 14, and 28 days and 2, 6, and 12 months of age and transported under anaerobic conditions to the laboratory, where serial dilutions were plated on selective media and cultured aerobically and anaerobically within 24 hours.[24] Free-lying colonies of different morphology were separately enumerated and subcultured for purity, including confirmation of inability to grow aerobically for strict anaerobes (except for gram-positive rods, for which scanty aerobic growth was accepted).[24] The limit of detection was 330 ($10^{2.52}$) colony-forming units/g of feces. Total strict anaerobic and facultative anaerobic bacterial counts were calculated from growth on nonselective media incubated anaerobically and aerobically, respectively.[24]

Bacteria were identified through Gram staining and biochemical/genetic tests.[24] *Enterobacteriaceae* were isolated from Drigalski agar and speciated with API20E (API Systems, Montalieu-Vercieu, France). *Staphylococcus aureus* and coagulase-negative staphylococci were isolated from *Staphylococcus* agar and identified by using catalase and coagulase tests. Enterococci were identified by means of esculine hydrolysis on Enterococcosel agar. *Bacteroides* species were isolated from *Bacteroides* Bile Esculin agar and speciated by using Rapid ID 32A (API Systems). *Clostridium* species were defined as straight gram-positive or gram-labile rods isolated from alcohol-treated samples (which kills vegetative cells but leaves spores intact), cultivated on Brucella blood agar, and speciated with Rapid ID 32A. *Bifidobacterium* species were isolated from Beerens' agar and identified by using genus-specific PCR.[25] *Lactobacillus* species were isolated from Rogosa agar and identified by means of PCR with species-specific primers.[26] Total gram-positive and gram-negative bacterial counts were calculated by combining the counts of each gram-positive and gram-negative bacterial group, respectively.

All centers used identical methods and reagents, and staff were trained centrally. Uniformity in analysis was checked repeatedly by means of blinded analysis of the same fecal sample in the 3 laboratories.

Data handling and statistics

Databases were transferred to STATA version 8.2 (Stata Corp, College Station, Tex) for linkage, cleaning, and statistical analysis.

For each major bacterial group, the presence or absence in the feces at each time point was determined. The cumulative incidence of colonization was derived by combining the data across the 7 time points (6 for strict anaerobic bacteria, which were not cultured at 3 days of age). The ratios of strict anaerobic to facultative anaerobic counts and gram-negative to gram-positive counts were evaluated at each time point.

Regression models were constructed by using time to colonization for each bacterial group as an ordinal 7- or 8-category explanatory variable (including never colonized) or log-transformed strict anaerobic/facultative anaerobic ratio or gram-negative/gram-positive ratio at specific time points as a continuous explanatory variable. Atopic eczema at 18 months of age was analyzed as a dichotomous outcome variable, as were food-specific IgE (no vs any positive reaction) and total IgE levels (dichotomized at 100 kU/L). Total IgE was also analyzed as a continuous variable after logarithmic transformation. Recruitment center was included as a 3-level covariate in all analyses. In a second model, mode of delivery, parity, and breast-feeding at 6 months were included as 2-level covariates. In a third model, timing of introduction of certain solid foods was introduced as a continuous covariate.

Results are presented as *P* values and direction of association (rather than estimates of magnitude of effect) because of the variety of exposure metrics and our preference for test for linear trend across all exposure categories rather than specific 2-group comparisons. Acknowledging the large number of interrelated hypotheses tested, only *P* values of .01 or less were considered significant.