Establishment of the intestinal microbiota and its role for atopic dermatitis in early childhood

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Background: Perturbations in the intestinal microbiota may disrupt mechanisms involved in the development of immunologic tolerance. The present study aimed to examine the establishment of the infant microbiota and its association to the development of atopic dermatitis (AD).
Methods: Within a randomized, placebo-controlled trial on the prevention of AD by oral supplementation of a bacterial lysate between week 5 and the end of month 7, feces was collected at the ages of 5 weeks (n = 571), 13 weeks (n = 332), and 31 weeks (n = 499) and subjected to quantitative PCRs to detect bifidobacteria, bacteroides, lactobacilli, *Escherichia coli*, *Clostridium difficile*, and *Clostridium* cluster I.
Results: Birth mode, breast-feeding but also birth order had a strong effect on the microbiota composition. With increasing number of older siblings the colonization rates at age 5 weeks of lactobacilli ($P < .001$) and bacteroides ($P = .02$) increased, whereas rates of clostridia decreased ($P < .001$). Colonization with clostridia, at the age of 5 and 13 weeks was also associated with an increased risk of developing AD in the subsequent 6 months of life (odds ratio$_{adjusted}$ = 2.35; 95% CI, 1.36-3.94 and 2.51; 1.30-4.86, respectively). Mediation analyses demonstrated that there was a statistically significant indirect effect via *Clostridium* cluster I colonization for both birth mode and birth order in association to AD.
Conclusion: The results of this study are supportive for a role of the microbiota in the development of AD. Moreover, the "beneficial" influence of older siblings on the microbiota composition suggests that this microbiota may be one of the biological mechanisms underlying the sibling effect. (J Allergy Clin Immunol 2013;132:601-7.)

Key words: *Microbiota, atopic dermatitis, birth mode, siblings, mediation analysis*

The intestinal microbiota is a key source of immune development and regulation early in life. Deprivation of microbial exposure is thought to predispose to immune dysregulation and the development of atopic diseases.[1] Animal studies have found that oral tolerance is difficult to achieve in germ-free animals[2] and that administration of lipopolysaccharides (constituents of the outer membrane of gram-negative bacteria) together with food antigens increases the tolerizing effect of feeding.[3] In addition, a complex intestinal microbiota, rather than colonization with a single microorganism, seems to be required to support oral tolerance development.[4]

Numerous epidemiologic studies showed indeed that the microbiota of infants with allergies differs from the microbiota of infants without allergies.[5] Although most of these studies were case–control studies, some, but not all, of the longitudinal studies found that these differences in the composition and diversity of the microbiota actually preceded the development of allergic manifestations.[5-7] Thus, the immune modulation by gastrointestinal (GI) microbiota is still one of the key candidates that may explain the increase of allergies (and other immune disorders) in terms of the hygiene hypothesis.

The fetal intestine is sterile and bathed in swallowed amniotic fluid. After delivery, the colonization of the intestines by a variety of microorganisms begins.[8] Intestinal colonization involves a succession of bacterial populations waxing and waning as the diet changes and the host develops.[9]

Factors that influence the intestinal microbiota composition can be divided into host factors (such as pH, bile acids, pancreatic enzymes, mucus composition, and transit time), nonhost factors (such as diet, medication, and environmental factors), and bacterial factors (such as adhesion capacity, enzymes, and metabolic capacities).[10] Especially changes in nonhost factors due to Western lifestyle (antibiotic use, diet, smaller family sizes, increased hygiene) may result in perturbations in the GI microbiota composition and thus may interfere with the mechanisms involved in the development of immunologic tolerance.[11]

In the present study, we investigated the influence of nonhost factors on the establishment of the intestinal microbiota in

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Abbreviations used
AD: Atopic dermatitis
C-section: Cesarean section
GI: Gastrointestinal
OR: Odds ratio
CFU: Colony-forming units

infancy, within a randomized, placebo-controlled trial of primary prevention of atopic dermatitis (AD) by oral supplementation of a bacterial lysate in very early infancy. Furthermore, we prospectively examined the composition of the infant intestinal microbiota in association to the subsequent development of AD and sensitization to common food allergens.

METHODS

Study population

The present study was conducted within the context of a randomized, placebo-controlled trial (registration no. ISRCTN60475069) on the primary prevention of AD by an orally applied bacterial lysate that contained heat-killed *Escherichia coli* Symbio DSM 17252 and *Enterococcus faecalis* Symbio DSM 16440 (Pro-Symbioflor). The study was approved by the Charité Ethics Committee in 2002, and all parents gave informed consent. The design of this trial has been described in detail elsewhere.[12]

Briefly, 606 healthy newborns (at term and birth weight ≥ 2500 g) with a single or double heredity for atopy (AD, allergic rhinitis, and/or asthma) were included in the study. Exclusion criteria were antibiotic treatment or other medication directly after birth, lymphocytopenia or thrombocytopenia, intensive care after birth, or parents lacking knowledge of the German language.

After an initial screening phase (age birth to 4 weeks), enrolled infants were randomly assigned at 4 to 5 weeks of age. From week 5 until the end of week 31 postpartum, infants were orally supplemented with the bacterial lysate or placebo daily.

Parents were asked to sample the infant's feces at the age of 5 weeks (start of intervention; n = 571), at 13 weeks (in a random subgroup only; n = 332), and at 31 weeks (end of intervention period; n = 499). Participants were provided with standard stool tubes with spoons attached to the lid (Sarstedt, Hilden, Germany) and were instructed to collect the fecal sample before the next visit during which times samples were handed to the researchers.

During the intervention period and thereafter until the age of 3 years, children were clinically examined at a regular basis by a pediatrician for signs of AD.

DNA purification from feces

At the laboratory 1 spatula of feces (approximately 200 mg) was diluted in 2 mL of Crowser-Medium (5 g of Lab Lemco [meat extract 3.0 g/L and Pepton 5 g/L] + 50 mL of Gycerol and 450 H_2O; ~pH 7.3) and stored at −80°C until further analysis.

For DNA isolation, 0.2 mL of the diluted feces was added to a 2-mL vial that contained approximately 300 mg of glass beads (diameter, 0.1 mm) and 1.4 mL of ASL buffer from the QIAamp DNA stool minikit (Qiagen, Hilden, Germany), and the samples were disrupted in a mechanical bead beater at 5000 rpm for 3 minutes. Subsequently, the bacterial DNA was isolated from the samples with the QIAamp DNA stool mini kit, according to the instructions provided by the manufacturer. The DNA was eluted in a final volume of 200 μL. DNA yields (ng/μL) were measured with an Eppendorf Photometer.

Microbial analysis of fecal samples

DNA from the fecal samples was subjected to quantitative real-time PCR assays for the quantification of bifidobacteria, *E coli*, *Clostridium difficile*, *Clostridium* cluster I (*Clostridium sensu stricto*), *Bacteroides fragilis* group, and lactobacilli targeting 16S rDNA gene sequences (see Table E1 for primer and probe sequences in this article's Online Repository at www.jacionline.org) as described previously.[13]

Counts of the bacterial groups and species were calculated for each stool sample from the threshold cycle values by using constructed standard curves and were expressed as the $\log_{10}$ colony-forming units (CFU) per milliliter of diluted feces. The prevalence of colonization was expressed as the percentage of infants colonized with a specific bacterial group or species.

Diagnosis of AD

Infants were clinically examined by a pediatrician during the intervention phase at the ages of 13, 21, and 31 weeks (end of the intervention phase). In the follow-up phase, participants were seen for additional visits at 1, 2, and 3 years of age. AD was clinically assessed.

Sensitization to food allergens

Sensitization to common food allergens (soy, peanut, cow's milk, hen's egg, wheat, and cod fish) was tested by panel ImmunoCAP fx5 on blood samples taken at 31 weeks, 1 year, and 2 years of age. Children who tested positive to any of the food allergens (>0.35 IU/mL) at any time point were labeled sensitized. Children were regarded nonsensitized when they were tested at least at age 2 years and were found negative at this time point and were negative at the other time points at which they were tested (31 weeks and/or 1 year).

Statistical analysis

Effects of birth characteristics, environmental factors, and intervention on gut microbiota. The following potential determinants were examined in association to the GI microbiota at the age of 5 and 13 weeks: sex (male/female), birth mode (spontaneous vaginal, assisted vaginal [forceps/vacuum extraction], cesarean section [C-section]), number of siblings (0, 1, 2 or more), atopy mother, or atopy father. For GI microbiota at the age of 31 weeks, this list was complemented with duration of breast-feeding (0-3 months, 3-6 months, or >6 months) and day care attendance (group size ≥3 children) during the first 6 months of life.

The Mann–Whitney rank sum test was used for the associations between these determinants and the counts of the bacteria under study (including the noncolonized infants with counts defined as zeros). The same method was used to examine the influence of the intervention on the bacterial counts in those infants who completed follow-up until the end of the treatment (age 31 weeks).

GI microbiota composition in association with AD and sensitization. Logistic regression analyses were used to test for associations between colonization with gut bacteria (colonized or noncolonized) under study and the development of AD or sensitization to food allergens respectively.

The following covariates were taken into account in the logistic regression models: sex, birth weight, maternal and paternal atopy, (duration of) breast-feeding, number of siblings, mode of delivery, and treatment group (placebo vs active group).

Logistic regression analyses were also used for associations between the concentrations (counts) of the gut bacteria and AD. Here, we additionally adjusted for the DNA concentrations of the samples to normalize the data. To test for trend bacterial counts were categorized (noncolonized infants were used as a reference category, and the remaining colonized infants were accommodated in 3 equal groups). These analyses were all limited to the completers group for the specific end points.

Survival analysis by Cox regression was used to examine the effects of the GI microbial composition on AD-free survival time.

Follow-up time for subjects who developed AD was calculated as the number of days between birth and the date of the visit at which AD was first diagnosed. The follow-up time of children who had not developed AD (yet) until they were lost to follow-up during the follow-up was the age in days at the moment of the last performed study visits.

To check the potential effect-modifying role of the treatment groups (placebo vs active group), we initially incorporated interaction terms between