Bifidobacterium pseudocatenulatum is associated with atopic eczema: A nested case-control study investigating the fecal microbiota of infants

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Background: Exposure to specific bacterial bowel commensals may increase/reduce the risk of atopic diseases.
Objective: To compare fecal bacterial communities of young infants with/without eczema.
Methods: Nested case-control study. Infants age 3 to 6 months with eczema (cases, n = 37) and without (controls, n = 24) were matched for sex, age, feeding (breast/bottle/mixed/solids), ethnicity. Information was collected on maternal/infant antibiotic exposure, feeding, gastrointestinal symptoms, family history of allergy. Eczema severity scoring was used (Severity Scoring of Atopic Dermatitis index). Samples were taken for determination of allergen-specific serum IgE (cases) and urinary/fecal eosinophilic protein X. Gastrointestinal permeability was measured. The compositions of fecal bacterial communities were analyzed (culture-independent, nucleic acid–based analyses).
Results: There was no difference in overall profiles of fecal bacterial communities between cases and controls. Family history of allergy increased likelihood of bifidobacteria detection (history, 86%; no history, 56%; $P = .047$); breast-fed infants were more likely to harbor *Bifidobacterium bifidum* (odds ratio, 5.19; 95% CI, 1.47-18.36; $P = .01$). *Bifidobacterium pseudocatenulatum* was detected more commonly in feces of non–breast-fed children (odds ratio, 5.6; 95% CI, 1.3-24.3; $P = .02$) and children with eczema (eczema, 26%; no eczema, 4%; $P = .04$). There were no significant associations between clinical measurements and detection of *B pseudocatenulatum*.
Conclusion: Presence of *B pseudocatenulatum* in feces was associated with eczema and with exclusive formula-feeding; *B bifidum* was associated with breast-feeding. (J Allergy Clin Immunol 2008;121:135-40.)

Key words: *Eczema, atopic dermatitis, atopy, allergy, gastrointestinal, microbiota, microflora, bifidobacteria, fecal bacterial community*

Many affluent countries have experienced an increase in the prevalence of allergic diseases in recent decades, including atopic dermatitis, asthma, and atopic rhinitis.[1] Colonization of the infant bowel by specific species of bacteria may be important in the initial regulation of the developing immune system.[2] Alterations to neonatal exposure to particular bacteria may have occurred because of the effects of hospital deliveries, cesarian sections, special care baby unit admissions, smaller family size, widespread use of antibiotics, good hygiene, and differences in maternal diet in modern times. Changes in the composition of the bowel microbiota might result in exposure, or the lack of exposure, of infants to specific bacteria. This would alter the exposure of children to important bacterial antigens at a critical time in the maturation of the immune system.[3] Differences in the composition of the bacterial community in the feces (bowel) of children sampled in countries of high or low prevalence of atopic diseases have been reported,[4,5] although there is little agreement in bacterial species between studies. Members of the genus *Bifidobacterium* have been considered to be important bacteria for investigation, however, because they form a major portion of the fecal bacterial community in early life.[6]

Previous work[7] that we have conducted demonstrated that certain bifidobacterial species were characteristic of the fecal community of infants born in countries (United Kingdom, New Zealand) with a high prevalence of atopic diseases compared with babies born in a low-prevalence country (Ghana). We report now the results of a study in which the fecal microbiota of infants with or without atopic eczema were compared and associations with clinical parameters related to allergy were sought.

METHODS

Study population

Infants age 3 to 6 months were recruited to an intervention study to investigate the effect of probiotic administration on the prevalence of atopic dermatitis. A subset of these infants formed the basis of a nested case-control study that we report here. The infants were divided into 2 groups: those with physician-diagnosed eczema (cases)[8] and healthy infants without eczema (controls). The infants were recruited at community clinics, were in good general health, and had normal growth. Detailed information was obtained on the history of maternal exposure to antibiotics during pregnancy, exposure of the infant to antibiotics, feeding, gastrointestinal symptoms (colic, vomiting, stool characteristics), and family history of allergic disease. Exclusion criteria were gestation of 34 weeks or less, congenital abnormality, chronic disease, current administration of oral antibiotics, and current consumption of soya or

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Abbreviations used
Cy5: Indodicarbocyanine
EPX: Eosinophilic protein X
F-EPX: Fecal eosinophilic protein X
OR: Odds ratio
SCORAD: Severity Scoring of Atopic Dermatitis
TTGE: Temporal temperature gel electrophoresis
U-EPX: Urinary eosinophilic protein X

extensively hydrolyzed formula. The infants were examined by a pediatrician (C. G.) who evaluated skin changes (Severity Scoring of Atopic Dermatitis [SCORAD] index)[9] at 3 to 6 months of age. Urine was collected for determination of urinary eosinophilic protein X (U-EPX), and feces was collected for fecal eosinophilic protein X (F-EPX) assay and analysis of the fecal bacterial community. Gastrointestinal permeability was also measured. Blood was collected for measurement of allergen-specific serum IgE (cases only). Samples used in this study were collected before probiotic or placebo interventions. Infants with eczema (cases) were matched with infants without eczema (controls) according to the following variables: sex, age (days/weeks), feeding (breast/bottle/mixed), weaned to solids, and ethnicity (which might affect maternal diet). All aspects of the study were approved by the South Manchester Local Research Ethics committee (approval number 01/320), and informed consent was obtained from the parents.

U-EPX estimation

Urinary EPX was measured by immunofluorometric assay using a kit (Phadia, Uppsala, Sweden; lower detection limit <0.5 μg/L). Total urine volumes were not known; therefore, urinary creatinine was measured (Jaffé reaction; HiCo Creatinine; Boehringer Mannheim GmbH, Mannheim, Germany), and the results were expressed as the ratio U-EPX/creatinine (μg/mmol).

F-EPX estimation

Fecal extracts were prepared and F-EPX was measured by using a kit as described. EPX concentrations in feces were adjusted for water and were expressed as nanograms of EPX per gram of dried feces.[10]

Allergen-specific IgE measurements

Determination of specific serum IgE (cow's milk, egg, peanut, dust mite, cat, dog) was achieved using a kit (ImmunoCAP; Phadia). We defined sensitization as specific IgE >0.35 kU/L.

Gastrointestinal permeability

The lactulose/mannitol dual sugar test was used as described previously.[11,12] Briefly, infants were administered the test solution (5 g lactulose and 1 g mannitol in 50 mL water) after a 3-hour fasting period. Then water was allowed for 2 hours after the test drink. Urine was collected from at least 60 minutes after administration of the test solution. Lactulose and mannitol were quantified in the urine by using HPLC with pulsed amperometric detection, and the lactulose/mannitol excretion ratio was calculated.

Analysis of the fecal bacterial community by temporal temperature gel electrophoresis

Fecal samples were collected before the clinic visit (held refrigerated in the home) or at the clinic, then stored at –80°C until analysis. The majority of samples were frozen within 8 hours of collection. Bacterial DNA was extracted from feces by using a previously described protocol.[13] 16S rRNA gene sequences (V3 region) obtained by PCR[14] from the DNA were used to generate profiles of the fecal bacterial community with the DCode universal mutation detection system apparatus (Bio-Rad, Hercules, Calif) on a 6% polyacrylamide gel prepared in 1.25 × TAE buffer (50 mmol/L TRIS, 25 mmol/L acetic acid, 1.25 mmol/L EDTA), and 7 mol/L urea (Merck, Darmstadt, Germany). Forty microliters of tetramethylethylenediamine (Sigma, St Louis, Mo) and 400 μL 10% ammonium persulfate (Bio-Rad) were added to 40 mL of the polyacrylamide/urea solution before pouring the gels. Thirty-five–microliter samples of PCR product were loaded into each well of the gel. Voltage was fixed at 20 V for 15 minutes at the beginning of electrophoretic period and then run at a fixed voltage of 40 V for 16 hours with an initial temperature of 63°C and a final temperature of 70°C (ramp rate, 0.4°C/h). A reference sample with fragments distributed throughout the whole gel was included in gel runs to permit normalization and valid comparison of profiles. After electrophoresis, the gels were stained with ethidium bromide solution (5 μg/mL) for 15 minutes, washed with deionized water for 30 minutes, and viewed by UV transillumination. The temporal temperature gel electrophoresis (TTGE) profiles were compared by using the Dice similarity coefficient with the Bionumerics software package (version 3.0; Applied Maths, Austin, Tex) at a sensitivity of 2% to 3%.

Quantification of bifidobacterial populations in feces by fluorescence *in situ* hybridization

Approximately 0.5 g (wet weight) stool sample was suspended in 5 mL PBS (130 mmol/L NaCl, 3 mmol/L NaH_2PO_4, 7 mmol/L Na_2HPO_4, pH 7.2) and vortexed with glass beads for 3 minutes. The fecal suspension was subsequently centrifuged at 300*g* for 1 minute, and a volume of 300 μL of the supernatant was removed and added to 900 μL 4% paraformaldehyde. After overnight storage at 4°C, the fecal suspension was centrifuged at 9000*g* for 5 minutes at 4°C. The pellet was suspended in 1 mL PBS and centrifuged at 9000*g* for 5 minutes at 4°C. Finally, the pellet was suspended in 400 μL PBS and mixed with 400 μL ice-cold ethanol and stored at –20°C until analysis.

The EUB 338 probe, which targets a conserved 16S rRNA gene sequence within the domain *Eubacteria*,[15] was used as a positive hybridization control. The NON 338 probe[16] was used as the negative control. Both control probes were covalently linked at their 5′ ends either to fluorescein isothiocyanate or to the sulfoindocyanine dye indodicarbocyanine (Cy5). The bifidobacterial probe Bif164[17] was labeled with Cy5. The probes were purchased from MWG Biotech (Bangalore, India).

As described previously,[18,19] fixed cells were washed once in PBS and then resuspended in 1 mL TRIS-EDTA buffer (100 mmol/L TRIS-HCl, pH 8.0, 50 mmol/L EDTA). After centrifugation, pellets were resuspended in TRIS-EDTA buffer containing 1 mg mL^{-1} lysozyme (Serva, Heidelberg, Germany) and incubated for 10 minutes at room temperature. The cells were then washed in PBS and suspended in 1.0 mL hybridization solution (900 mmol/L NaCl, 20 mmol/L TRIS-HCl, pH 8.0, 0.01% SDS, 30% formamide). Aliquots of 50 μL were used for fluorescence *in situ* hybridization with control and group-specific probes. Protected from light, hybridizations were performed by using 1-mL 96-well microtiter plates overnight at 35°C in 50 μL hybridization solution per well, to which was added 4 μL of the appropriate labeled probe at 50 ng/μL (4 ng/μL final concentration). After hybridization, 150 μL hybridization solution was added to each well, and the cells were pelleted at 4000*g* for 15 minutes at room temperature. Bacterial cells were resuspended and incubated at 37°C for 20 minutes in 200 μL washing solution (65 mmol/L NaCl, 20 mmol/L TRIS-HCl, pH 8.0, 5 mmol/L EDTA, pH 8.0, 0.01% SDS) to remove nonspecific binding of the probe. The cells were finally pelleted and resuspended in 150 μL PBS. Aliquots of 100 μL were added to 500 μL PBS for data acquisition by flow cytometry.

Data acquisition was performed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) as described previously[18,19] equipped with an air-cooled argon ion laser providing 15 mW at 488 nm, combined with a 635-nm red-diode laser. The parameters were collected as logarithmic signals. The 488-nm laser was used to measure the forward angle light scatter (FSC in the 488-nm band pass filter), the side angle light scatter (SSC in the 488-nm band pass), and the green fluorescence intensity conferred by fluorescein isothiocyanate–labeled probes (FL1 in the 530-nm band pass filter). The red-diode laser was used to detect the red fluorescence conferred by Cy5-labeled probes (FL4 in a 660-nm band pass filter). The acquisition threshold was set in the side scatter channel. The rate of events in the flow was generally