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Bifidobacterial Species Differentially Affect Expression of Cell Surface Markers and Cytokines of Dendritic Cells Harvested from Cord Blood

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The gut microbiota may be important in the postnatal development of the immune system and hence may influence the prevalence of atopic diseases. Bifidobacteria are the most numerous bacteria in the guts of infants, and the presence or absence of certain species could be important in determining the geographic incidence of atopic diseases. We compared the fecal populations of bifidobacteria from children aged 25 to 35 days in Ghana (which has a low prevalence of atopy), New Zealand, and the United Kingdom (high-prevalence countries). Natal origin influenced the detection of bifidobacterial species in that fecal samples from Ghana almost all contained *Bifidobacterium infantis* whereas those of the other children did not. Choosing species on the basis of our bacteriological results, we tested bifidobacterial preparations for their effects on cell surface markers and cytokine production by dendritic cells harvested from cord blood. Species-specific effects on the expression of the dendritic-cell activation marker CD83 and the production of interleukin-10 (IL-10) were observed. Whereas CD83 expression was increased and IL-10 production was induced by *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Bifidobacterium pseudocatenulatum*, *B. infantis* failed to produce these effects. We concluded that *B. infantis* does not trigger the activation of dendritic cells to the degree necessary to initiate an immune response but that *B. bifidum*, *B. longum*, and *B. pseudocatenulatum* induce a Th2-driven immune response. A hypothesis is presented to link our observations to the prevalence of atopic diseases in different countries.

In recent decades, many affluent countries have experienced an increase in the prevalence of allergic diseases, including asthma, atopic dermatitis, and atopic rhinitis (11). Several aspects of lifestyle have changed in these countries over the same period, and theories have been advanced to explain the increased prevalence of allergic diseases. The "hygiene hypothesis" proposes that the prevalence of atopic diseases is inversely related to infection in childhood, because the neonatal immune system would be driven toward a T helper 1 (Th1) response, but no specific "infectious protective factor" has been identified (16, 23). Attention has turned to the gut microbiota (microflora) and the possibility that colonization of the gut ecosystem in infancy by specific bacterial species may be important in the initial regulation of the developing immune system (16). The environmental conditions under which babies are born and nurtured may affect which microbes they are exposed to and may subsequently influence the composition of their gut microbiota. For example, the composition of the gut microbiota has been reported to differ with regard to the numbers of lactobacilli and clostridia in the feces of Estonian and Swedish children (19). Members of the genus *Bifidobacterium* could be of particular interest because they are the numerically predominant bacteria during the first month of life, regardless of diet. Bifidobacteria form 60 to 91% of the total bacterial community in the feces of breast-fed babies and 28 to 75% (average, 50%) of that for formula-fed infants (9). Thus, these bacteria could have an important role in the ontogeny of the immune system associated with the gut mucosa.

Ouwehand and colleagues have reported that the prevalences of *Bifidobacterium adolescentis* differed in the feces of healthy and allergic Finnish children aged 2 to 7 months (17). Six out of seven allergic children harbored *B. adolescentis* in the feces, whereas this species was not detected in six healthy children. Another approach is to examine the bacteria colonizing the bowel in early infancy in relation to variations in the prevalence of allergic diseases (12). Comparative studies in the United Kingdom and New Zealand have shown high rates of atopy compared with those in developing countries in Africa and Asia. For example, with regard to four or more attacks of wheezing in the past 12 months (a specific indicator of clinically important asthma), the prevalence was more than 9% in New Zealand and the United Kingdom. A survey of the prevalence of exercise-induced brochospasm and atopy in Ghana, conducted during the same period, gave values of 3 and 4%,

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respectively (1, 12). Differences in neonatal gut microbiota might occur due to the effects of hospital deliveries, cesarean sections, special-care baby unit admissions, smaller family size, widespread use of antibiotics, good hygiene, and differences in maternal diet in affluent countries. Either the lack of exposure of babies to particular bifidobacterial species or elimination of bifidobacterial species from the gut through the use of antibiotics, or both, might alter the exposure of children to important bacterial antigens at a critical time in the maturation of the immune system (2).

We report here a study in which we compared the prevalence of bifidobacterial species in the feces of infants born in Ghana (with a low incidence of atopic diseases) to that of infants born in affluent countries with high incidences of asthma and allergies (New Zealand and the United Kingdom). These bacteriological investigations allowed us to make a rational choice of bifidobacterial species that could be investigated with respect to their impact on the expression of cell surface markers and the production of cytokines by dendritic cells. These cells are principal antigen-presenting cells that are present at mucosal surfaces, including that of the gut, and therefore are likely to have an important role in the interplay occurring between the gut microbiota and the immune system (17).

MATERIALS AND METHODS

Study population. To compare the prevalences of *Bifidobacterium* species in the feces of children of different natal origins, we recruited 78 children aged 25 to 35 days through maternity clinics in Ghana (32 children), the United Kingdom (17 children), and New Zealand (29 children). A single fecal sample was obtained from each child. The children had not been administered antibiotics, and the majority of the babies in each group were fed wholly or partially with breast milk (32 of 32 in Ghana, 12 of 17 in the United Kingdom, and 28 of 29 in New Zealand).

The recruitment of children and collection of fecal samples were approved by the Wellington Ethics Committee (reference 00/03/012) and the South Manchester Ethics Committee (reference SOU/00/139).

Detection of bifidobacterial species in feces of children. Fecal samples were obtained at the time of recruitment and stored frozen at −20°C until they were sent to the laboratory on dry ice. Upon receipt, the samples were thawed and diluted 10-fold in phosphate-buffered saline (PBS; pH 7.5), and 1-ml volumes were used for extraction of bacterial DNA as described previously (25). Bifidobacterial species present in the feces were detected by PCR (amplification of a region of the transaldolase gene showing different nucleotide base compositions in different species) and denaturing gradient gel electrophoresis, as described previously (18). Species were identified by reference to an identification ladder or by sequencing and alignment of sequences with those of reference strains (13).

Generation of dendritic cells from cord blood monocytes. The recruitment of neonates and the collection of cord blood as a source of dendritic cells were approved by the Otago Ethics Committee (reference CPD 03/03). Dendritic cells were generated from cord blood monocytes by using a modification of a previously published protocol (21). Briefly, heparinized umbilical cord blood (mean volume, 60 ml) was fractionated over Ficoll-Hypaque Plus gradient (Amersham Biosciences, Piscataway, N.J.), and leukocytes were isolated. Cells expressing the human monocyte marker CD14 were positively selected by using immunomagnetic microbeads (Miltenyi Biotec, Auburn, Calif.) according to the manufacturer's instructions. Cells were incubated for 15 min at 4°C with the CD14 microbeads (20 μl of beads per 10^7 cells). The cell-bead mixture was then washed in MACs buffer (Miltenyi Biotec), and the pellet was resuspended in approximately 1 ml of MACs buffer. This suspension was then added to a MiniMACs column, and the flowthrough was discarded. CD14-positive cells were collected by removing the column from the magnet and washing the column twice with buffer. These cells were then cultured for 5 days in RPMI 1640 medium (Invitrogen, Grand Island, N.Y.) supplemented with 10% fetal calf serum containing granulocyte-macrophage colony-stimulating factor and interleukin-4 (IL-4), each at a concentration of 25 ng per ml.

Preparation of bacterial-cell suspensions and stimulation of dendritic cells. Bifidobacterial cultures (4-h incubation at 37°C) were prepared anaerobically on brain heart infusion agar plates supplemented with yeast extract and a hemin-vitamin K solution (10). Culture collection strains, including type cultures, of *B. adolescentis* (DSM 20083^T, DSM 20086, and DSM 20087), *Bifidobacterium infantis* (DSM 20088^T and ATCC 15702), *Bifidobacterium bifidum* (DSM 20456^T), *Bifidobacterium longum* (ATCC 15707^T), and *Bifidobacterium pseudocatenulatum* (DSM 20438^T) were used. The bacterial cells were washed from the plates, harvested by centrifugation at 10,000 × *g* for 15 min, washed twice in 50 ml of ice-cold sterile deionized water, resuspended in 20 ml of sterile water, and then heat killed at 80°C for 2 h. The absence of viable cells was checked by inoculating brain heart infusion agar plates with cell suspensions that were then incubated anaerobically at 37°C for 48 h. The cell suspensions were lyophilized for 48 h and stored at 4°C.

Lipopolysaccharide (LPS) extracted from *Escherichia coli* O26:B6 was purchased from the Sigma Chemical Company (St. Louis, Mo.).

To determine the effects of bifidobacterial preparations on the expression of cell surface markers and on cytokine production by dendritic cells, day-5 cord blood monocyte-derived dendritic cells (CBDC; 5×10^5 to 1×10^6 per ml) were incubated with final concentrations of 1 μg of LPS per ml or 100 μg of bifidobacterial cells per ml, as used by others (5), for 18 h. Nonstimulated control preparations were exposed only to culture medium.

The number of dendritic cells that could be harvested from the cord blood was different for different samples. Therefore, not all bacterial preparations could be tested against dendritic cells from all of the infants. Nonstimulated and LPS-exposed preparations were assayed in all cases, however, and as many bifidobacterial preparations per sample as possible were used thereafter. Thus, data were obtained from 15 to 23 cord blood samples for each cell surface marker or cytokine.

Measurement of expression of cell surface markers on dendritic cells. CBDC harvested from cultures were washed in PBS and counted, and aliquots of 10^5 cells were suspended in PBS containing 0.01% sodium azide and incubated for 30 min at 4°C with 1 μg of phycoerythrin-conjugated monoclonal antibodies to the dendritic-cell activation markers CD83 (clone HB15e; IgG1; Caltag, Burlingame, Calif.), major histocompatibility complex class II (clone L243; isotype IgG2a; BD Biosciences Pharmingen, Chicago, Ill.), CD86 (clone BU63; isotype IgG1; Serotec, Kidlington, United Kingdom), and CD80 (clone L307.4; IgG1; BD Biosciences Pharmingen) or concomitant phycoerythrin-conjugated isotype controls. CBDC were then washed and analyzed by use of a FACScalibur flow cytometer and CELLQUEST software (BD Biosciences Pharmingen). Additionally, we checked that we were indeed studying immature CBDC in these cultures by analyzing for expression of the marker CD1a, which characterizes these cells (DAKO catalog no. F7141; fluorescein isothiocynanate conjugated).

Measurement of IL-10 and IL-12 produced by dendritic-cell preparations. Supernatants from CBDC cultures were collected on day 6 of culture and immediately frozen at −70°C. Supernatants collected from the cultures were tested in one batch for IL-10 and IL-12 (p70) content by using standard sandwich enzyme-linked-immunosorbent assays. The enzyme-linked-immunosorbent assays were performed by using antibodies (BD Biosciences Pharmingen) according to the manufacturer's instructions, 2 μg of a primary anti-cytokine capture antibody (clone JES3-19F1, isotype IgG2a, against IL-10; clone 20C2, isotype IgG1, against IL-12) per ml and 1 μg of a biotinylated cytokine-detecting monoclonal antibody (clone JES3-12G8, isotype IgG2a, for IL-10; clone C8.6, isotype IgG1, for IL-12) per ml, followed by streptavidin-horseradish peroxidase and 3′,3′,5′,5′-tetramethylbenzidine. The reaction was stopped with 1 M H_2SO_4, and the absorption of light at 450 nm was determined by using a spectrophotometer (Bio-Rad, Hercules, Calif.). The amount of each cytokine in the supernatant was extrapolated from the linear region of a standard curve. The standards were recombinant cytokines (BD Biosciences Pharmingen) ranging from 2,000 to 15 pg per ml for IL-10 and IL-12.

Statistical analysis. A nonparametric method (Mann-Whitney test) was used to compare the results of immunological assays.

RESULTS

Impact of natal origin on the prevalence of bifidobacterial species in feces. The majority of the fecal samples obtained from children in Ghana contained *B. infantis* (Table 1). In contrast, this species was not detected in the feces of New Zealand or United Kingdom children (Table 1).

TABLE 1. Bifidobacterial species detected in the feces of children

Bifidobacterial species	No. of children with the indicated species in the following country of birth:		
	Ghana (n = 32)	New Zealand (n = 29)	United Kingdom (n = 17)
B. infantis	23	0	0
B. longum	2	16	8
B. bifidum	0	6	4
B. adolescentis	0	1	3
B. pseudocatenulatum	0	4	2
None detected	7	2	0

Impact of exposure to bifidobacterial cells on the expression of cell surface markers by dendritic cells. The degree of expression of dendritic-cell surface markers involved in the activation of T cells was analyzed by flow cytometry. In general, exposure to LPS from *E. coli* stimulated greater expression of cell surface markers than that of nonstimulated cells, as shown by percent staining and mean fluorescence index, for all four markers that were assayed ($P < 0.01$). An exception was expression of major histocompatibility complex class II as indicated by mean fluorescence, where a lower level of statistical significance was obtained ($P = 0.05$).

The percentage of cells exposed to *B. adolescentis* DSM 20083^T, *B. adolescentis* DSM 20086, *B. adolescentis* DSM 20087, *B. bifidum* DSM 20456^T, *B. longum* ATCC 15707^T, or *B. pseudocatenulatum* DSM 20438^T that stained for CD83, considered a specific indicator of dendritic-cell activation, was higher than that of nonstimulated controls ($P < 0.01$ [Table 2]). Exposure to *B. infantis* ATCC 15702 or *B. infantis* DSM 20088^T did not affect CD83 expression ($P > 0.05$ [Table 2]).

The percentage of cells exposed to *B. adolescentis* DSM 20083^T, *B. adolescentis* DSM 20086, *B. adolescentis* DSM 20087, *B. pseudocatenulatum* DSM 20438^T, *B. infantis* ATCC 15702, or *B. infantis* DSM 20088^T that stained for CD86 was higher than that of nonstimulated controls ($P < 0.01$ [Table 2]). Exposure to *B. bifidum* DSM 20456^T or *B. longum* ATCC 15707^T did not affect CD86 expression ($P > 0.05$ [Table 2]).

Exposure of dendritic cells to *B. pseudocatenulatum* DSM 20438^T increased the mean fluorescence of CD80 relative to that for nonstimulated controls ($P < 0.01$).

All other measurements not recorded above were unaffected by exposure of dendritic cells to bifidobacterial preparations ($P > 0.05$).

Impact of exposure to bifidobacterial cells on the production of cytokines by dendritic-cell preparations. The production of IL-10 by dendritic cells is associated with both Th2 and regulatory T-cell development, whereas IL-12 is indicative of predominantly Th1 development. Exposure to LPS from *E. coli* resulted in increased production of IL-10 and IL-12 relative to that of nonstimulated cells ($P < 0.01$).

IL-12 (p70) production was not affected by exposure to any of the bifidobacterial preparations. The absence of detectable IL-12 could not be attributed to a reported deficiency in IL-12 gene expression in CBDC (8), because when we exposed these cells to LPS, IL-12 was detected.

Increased IL-10 production occurred in dendritic cells exposed to *B. bifidum* DSM 20456^T, *B. longum* ATCC 15707^T, or *B. pseudocatenulatum* DSM 20438^T ($P < 0.01$ [Table 2]). Other bifidobacterial species did not affect IL-10 production ($P > 0.05$).

DISCUSSION

The first aim of our study was to determine the impact of natal origin on the composition of the *Bifidobacterium* population detected in the feces of children during early life. There was a clear difference between the prevalences of *B. infantis* in the feces of babies in Ghana (with a low prevalence of atopic diseases) and in the feces of children born in more affluent countries (with high prevalences of atopic diseases). Thus, the bifidobacterial species to which the immune systems of the children were exposed in early life were different in Ghana than in New Zealand and the United Kingdom. The bacteriological part of our study thus identified bifidobacterial species that would be relevant to the second aim of the research project.

The second aim of our study was to measure the expression of cell surface markers and IL-10 and IL-12 production by dendritic cells harvested from the cord blood of neonatal infants. These dendritic cells had not been previously exposed to bifidobacterial antigens and provided naïve immune cells with which we could search for a differential impact exerted by bifidobacterial species that we had detected in infant feces. Of particular interest was the possibility that *B. infantis* cells would

TABLE 2. Differential impact of bifidobacterial strains on dendritic cells

Stimulus	% CD83$^+$ cells[a]	% CD86$^+$ cells[a]	IL-10 production (pg/ml)[a]
None	57.0 (1.0–88.0)	51.0 (3.5–96.0)	4.0 (ND–123.0)
LPS	**92.0** (18.0–99.0)	**95.0** (66.0–99.0)	**185.0** (ND–592.0)
Bifidobacterial strains			
DSM 20083^T	**77.0** (2.8–96.0)	**90.0** (16.0–97.0)	8.0 (ND–594.0)
DSM 20086	**72.0** (2.0–98.0)	**92.0** (22.0–99.0)	4.0 (ND–591.0)
DSM 20087	**79.5** (2.3–97.0)	**91.0** (16.0–99.0)	53.0 (ND–586.0)
ATCC 15702	65.5 (1.0–96.0)	**88.0** (19.0–98.0)	1.5 (ND–570.0)
DSM 20088^T	68.5 (1.0–97.0)	**91.0** (4.0–98.0)	3.0 (ND–587.0)
DSM 20456^T	**72.0** (9.0–92.0)	**79.0** (40.0–97.0)	**312.0** (ND–584.0)
ATCC 15707^T	**74.0** (1.0–98.0)	**91.0** (3.0–99.0)	**275.0** (ND–595.0)
DSM 20438^T	**85.0** (25.0–99.0)	**91.0** (46.0–99.0)	**395.0** (ND–565.0)

[a] Values are medians (ranges). Median values statistically different from those for nonstimulated cells are boldfaced ($P < 0.01$). ND, none detected.

affect the dendritic cells differently than the bifidobacterial species commonly detected in New Zealand and United Kingdom children. We did indeed detect differential expression of CD83. There was increased expression of this marker on dendritic cells exposed to *B. adolescentis*, *B. bifidum*, *B. longum*, or *B. pseudocatenulatum* preparations. These species were characteristic of children from the more affluent countries. Expression of CD86 was also affected, but in this case *B. infantis* preparations were influential as well as those of *B. adolescentis* and *B. pseudocatenulatum*. It is noteworthy that all three *B. adolescentis* strains and both *B. infantis* strains produced the same effect on dendritic cells, suggesting that these were species-specific rather than strain-specific influences. Perhaps most significantly, IL-10 production was stimulated only in dendritic cells exposed to *B. bifidum*, *B. longum*, or *B. pseudocatenulatum* preparations. One or more of these species were detected in the feces of 40 out of 46 New Zealand and United Kingdom children aged 25 to 35 days, but only one of these species (*B. longum*) was found in the feces of a minority (2 of 32) of infants of a similar age living in Ghana. These results suggest that the immune-stimulating properties of bifidobacteria detected in United Kingdom and New Zealand infants are different from those in Ghanaian infants.

We present the following hypothesis in an attempt to link our bacteriological and immunological observations with geographical variation in the prevalence of atopic disorders. This hypothesis is based on evidence that dendritic cells, once activated, influence the nature of an ensuing T-cell response. According to this evidence, the T-cell response differs depending on the microbial signal delivered to the dendritic cell, the cytokine milieu, the antigenic dose to which the dendritic cells were exposed, or the dendritic-cell subset that has been activated (DC1 or DC2) (6, 7, 20). We propose that, in susceptible infants (basis not yet known), *Bifidobacterium*-activated dendritic cells that produce IL-10 in the absence of IL-12 permit Th2 expansion and associated immunoglobulin E (IgE) responses to antigens. Th2 cells preferentially produce IL-4, which acts as an antibody isotype switch to IgE production. IgE bound to mast cells is cross-linked by an allergen, and the mast cells degranulate, resulting in an inflammatory response (3). When this occurs in the gut mucosa in response to, for example, cow's milk or other food antigens, gut permeability increases, allowing increased systemic exposure to these antigens and the development of atopic dermatitis. Transport of the allergen to other anatomical locations leads to inflammation in sites remote from the gut. Additionally, Th2 cells produce IL-5, which induces eosinophilia. There are two ways in which this process could be involved in asthma: IL-5 passes from the gut-associated lymphoid tissues into the blood circulation and contributes to respiratory distress (2), and/or an allergen transported from the leaky gut mucosa to the bronchus-associated lymphoid tissues induces local production of IL-5 (2).

We propose that *B. bifidum*, *B. longum*, and *B. pseudocatenulatum* activate dendritic cells to produce IL-10 and that the sequence of events outlined above occurs in susceptible children living in New Zealand and the United Kingdom. In contrast, *B. infantis*, characteristic of children in Ghana, fails to induce production of IL-10 by dendritic cells. Upregulation of CD86 expression by *B. infantis* indicates that a degree of activation of dendritic cells occurs, but apparently not to an extent sufficient to drive Th1 or Th2 responses. These incompletely matured cells thus effectively downregulate the immune response (15) and favor immunological tolerance. In support of this view, germfree mice tolerized to ovalbumin have been reported to produce IgE after subsequent systemic challenge with this antigen, whereas ex-germfree mice whose guts were colonized by *B. infantis* did not produce IgE (24).

We acknowledge that dendritic cells harvested from cord blood do not necessarily respond to antigens in the same way as those cells located in the gut mucosa, but difficulty in obtaining dendritic cells from gut tissue limits such research (14). Dendritic cells derived from rodent guts can be studied, but the mechanisms of anergy appear to be different from those of human cells (22).

Our hypothesis linking specific bifidobacterial species with atopy or tolerance has not been tested and is based on circumstantial observations. However, it provides a basis for future investigations of the molecular interplay among bifidobacteria, human dendritic cells, T cells, and allergens. Methodologies for this research are readily available (6), and determining the cytokine profile (including IL-4 and IL-5 production) of T cells will provide valuable information that may support our hypothesis. It will also be important to include studies of cell components of *B. bifidum*, *B. longum*, and *B. pseudocatenulatum* that trigger dendritic-cell activation after binding to pattern recognition receptors such as the Toll-like molecules and mannose receptors (4).

Our observations could provide a useful basis for human studies in which colonization of the neonatal gut by *B. infantis* in affluent countries could be attempted by means of a probiotic product, or purified substances extracted from *B. infantis* cells could be administered to infants in an attempt to reduce the prevalence of atopic diseases.

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