

## Original Article

# Intestinal microflora at 4 months of age and the development of allergy

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### ABSTRACT

**Background:** Because microflora has been reported to have an important effect on the development of allergic disorders, we measured intestinal microflora levels in 4-month-old infants and studied the development of allergic disorders.

**Methods:** Blood samples from 18 4-month-old infants and 15 1-year-old infants were examined for total serum IgE and specific IgE antibodies. Stool samples from 18 4-month-old infants were examined for the presence of microflora.

**Results:** A positive correlation was observed between the ratio of breast-feeding at 1 month and the percentage of bifidobacteria in the intestine at 4 months (correlation ratio = 0.54;  $P = 0.022$ ). Atopic dermatitis was observed in 12 of 18 infants at 4 months and in five of 15 infants at 1 year. Egg white-specific IgE was positive ( $\geq 0.70$  U<sub>A</sub>/mL) in six infants at 4 months and in seven infants at 1 year. No relationship was observed between the percentage of bifidobacteria, lactobacilli or clostridia in the intestinal tract at 4 months and the development of allergy. However, all five infants who exhibited a percentage of bacteroides (compared with the total intestinal microflora level) of more than 10% at 4 months had positive egg white-specific IgE and higher levels of total IgE ( $>25$  IU/mL) at 1 year; these relationships were statistically significant ( $P = 0.01$ ).

**Conclusions:** Colonization with bacteroides at 4 months of age is suggested to be related to the allergic state at 1 year of age.

**Key words:** allergy, bacteroides, bifidobacteria, egg white-specific IgE, intestinal microflora.

### INTRODUCTION

The intestinal mucosa, which is exposed to various allergens, including foods, is important for host defense. The intestinal microflora has been reported to have a significant effect on the development of allergic disorders.<sup>1,2</sup> Furthermore, oral tolerance has been reported to be inducible in germ-free mice by the reconstitution of intestinal flora with *Bifidobacterium infantis*.<sup>3</sup> In humans, oral intake of *Lactobacillus* GG has been reported to prevent the development of allergic disorders<sup>4</sup> and *Lactobacillus* strains have proven effective in food allergy<sup>5</sup> and in atopic dermatitis.<sup>6</sup>

In reports from various countries, different kinds of bacteria, including clostridia and bifidobacteria, have been reported to affect the development of allergy.<sup>1,2</sup> We studied such relationships in Japan, ascertaining whether there may be any difference in intestinal microflora levels with different life styles. We measured the intestinal microflora levels at 4 months of age to investigate the relationship with the existence of atopic dermatitis at 4 months of age and with the development of allergy at 1 year of age.

### METHODS

Informed consent for participation in the present study, which was approved by the ethics committee of our hospital, was obtained from the parents of 20 1-month-old infants, all of whom had a family history of allergy (Table 1). We asked the mothers about the approximate ratio of breast and formula feeding, and asked them to visit our hospital with their infants at 4 months of age because atopic dermatitis frequently develops at that

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**Table 1** Development of allergic disorders and intestinal microflora levels

	Case																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Sex	M	F	M	F	F	F	F	F	M	M	M	M	M	M	F	M	M	F	
Allergy of family members*	bs	b	b	ms	mfs	b	mfb	s	m	mfb	mf	m	mf	m	f	ms	fb	bg	
Breast feeding (%)	100	100	100	100	100	100	100	100	100	90	83	75	67	50	50	17	0	0	
At 1 month																			
At 4 months	100	100	100	100	100	100	100	100	90	90	67	0	50	40	0	0	0	0	
Atopic dermatitis (at 4 months)	Mild	Mild	Mild	Mild	No	No	No	No	Mild	Mild	Mild	Mild	No	Mild	Moderate	No	Mild	No	
Total IgE (IU/mL; at 4 months)	1.67	22	10	6	0	13	6	3	6	7	0	7	8	24	39	4	21	3	
Egg white-IgE (U <sub>A</sub> /mL; at 4 months)	0	4.2	2.8	1.1	0	0	0	0	0	0	0	0	0	1.5	6.2	0	4.2	0	
Atopic dermatitis (at 1 year)	No	No	Moderate	NT	No	Mild	Mild	NT	NT	No	No	No	No	Mild	No	Mild	No	No	
Total IgE (IU/mL; at 1 year)	1.40	17	48	NT	3	27	78	NT	NT	15	2	11	23	19	39	26	45	6	
Egg white-IgE (U <sub>A</sub> /mL; at 1 year)	5.4	0.5	4.6	NT	0	0	0.8	NT	NT	0	0	0	0	2.5	3.2	1.4	7.1	0	
Ketotifen†	No	Yes	Yes	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	Yes	No	
Elimination diet‡	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No	No	No	No	No	No	Yes	No	
Total bacteria (× 10 <sup>10</sup> )	2.2	1.5	3.7	4.2	1.4	18	4.2	2.9	1.3	16	1.8	17	11	11	3.1	3.1	6.2	4.4	
Enterococcus (%)	1.8	2.9	Low	0	0.6	0.2	2.4	0.2	0.6	0.1	9.1	4.5	3.2	2.7	0.3	0.2	26	19	
Streptococcus (%)	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	4.5	0	2.3	
Enterobacteriaceae (%)	Low	1	2	1.4	0.4	0.5	11	0.9	62	0.3	0.3	2.9	3	0.8	1.3	0.6	8.1	12	
Bifidobacterium (%)	97	86	44	86	99	94	62	69	0	98	86	89	72	89	46	12	26	30	
Eubacterium (%)	0.3	2.9	16	3.6	0	3.3	9.6	2.1	11	0.6	4.6	2.4	14	5.5	7.2	25	16	34	
Lactobacillus (%)	0	0.2	0	0.6	0.3	0	0	0	0	Low	0	0	Low	0	0	Low	0	2.7	
Clostridium L(+) (%)	Low	0	Low	0	0	0	Low	0	0	Low	Low	Low	0	0.1	Low	0	0	Low	
Clostridium L(-) (%)	0	0.3	Low	0.4	0	Low	0.2	0.1	0	Low	0.3	Low	0	Low	Low	0.4	0	0	
Peptostreptococcus (%)	0	1.4	16	2.9	0	0.6	0	3.5	0	0.9	0	0.8	7.9	1.3	5.2	5.8	5.8	0	
Bacteroides (%)	1.1	4.3	22	4.8	0	1.5	14	24	27	Low	0	Low	0	0.2	39	52	16	0	

\*m, mother; f, father; b, brother; s, sister; g, grandparent.

†Yes, administered for more than 1 month.

‡Yes, continued until 1 year of age.

Low, detected, but less than 0.1%; NT, not tested.

age. At that time, medical check-ups, including assessments for the presence of atopic dermatitis and a blood sample examination, were undertaken. We also took stool samples from 18 infants, but could not take a sufficient amount of stool sample from two infants. At 1 year of age, medical check-ups and a blood sample examination were again administered to 15 of the 18 infants. Blood samples at 4 months and 1 year of age were examined for total serum IgE and specific IgE antibodies for house dust, *Dermatophagoides pteronissinus*, egg white, cow's milk, wheat, soy bean and rice.

The presence of microflora in stool samples was analyzed based on previously described methods<sup>7</sup> (supported financially by Nikken Chemicals, Tokyo, Japan). Briefly, approximately 1 g stool samples was mixed well and suspended in 9 mL recommended diluent. The suspension was serially diluted 10-fold with the same diluent to a 10<sup>-8</sup> dilution. These procedures were performed in an anaerobic chamber. The diluted suspensions (50 µL aliquots) were each streaked onto 15 different agar media and incubated in the respective conditions required for optimal growth of each organism.<sup>7</sup> The colonies grown on these agar plates were then used for bacterial identification and cell numbers counted.

Statistical significance was examined using StatMate III (ATMS, Tokyo, Japan).

## RESULTS

### Development of allergy

Atopic dermatitis was observed in 12 infants at 4 months of age and in five infants at 1 year of age (Table 1). The egg white-IgE titer was positive ( $\geq 0.70$  U<sub>A</sub>/mL) in six infants at 4 months and in seven infants at 1 year of age (Table 1). Specific IgE titer for cow's milk, wheat, soy bean or rice was negative in all infants at 4 months and 1 year of age. Case 11 had experienced wheezing and another subject (case 17) was diagnosed as having bronchial asthma. *Dermatophagoides pteronissinus*-specific IgE was positive in only one infant (case 2; 2.1 U<sub>A</sub>/mL at 1 year of age). No relationship was observed between the ratio of breast-feeding and the development of allergic manifestations (Table 1).

### Intestinal microflora at 4 months of age

A positive correlation was observed between the ratio of breast-feeding at 1 month and the percentage of

bifidobacteria (compared with the total intestinal microflora level) at 4 months of age (correlation ratio = 0.54;  $P = 0.022$ ; Fig. 1). Such a correlation was not observed between the percentage of bifidobacteria in the intestinal tract and the ratio of breast-feeding at 4 months of age (Table 1). No relationship was observed between the ratio of breast-feeding and any other intestinal microflora (Table 1). None of the 10 infants who exhibited a percentage of bifidobacteria greater than 70% and seven of eight infants with bifidobacteria of less than 70% showed a percentage of bacteroides of more than 10% (Table 1).

### Relationship between microflora at 4 months of age and allergic manifestations

No relationship was observed between the percentage of bifidobacteria, lactobacilli or clostridia in the intestinal tract at 4 months of age and the development of atopic dermatitis or the level of serum egg white-IgE at 4 months or 1 year of age (Table 1; Fig. 2). However, all five infants who exhibited a percentage of bacteroides greater than 10% at 4 months of age had a positive egg white-IgE titer at 1 year of age (Fig. 3) and had a total IgE level of more than 25 IU/mL at 1 year of age (Table 1); these results were statistically significant according to Fisher's exact test ( $P = 0.01$ ).

Three infants had wheezing/bronchial asthma or a house dust mite allergy, but there were no significant characteristics of intestinal microflora in these three cases.

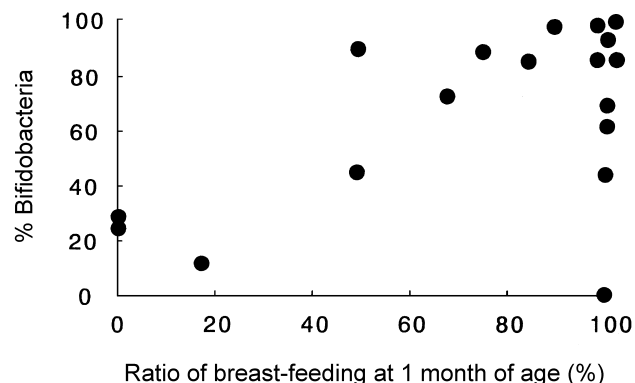
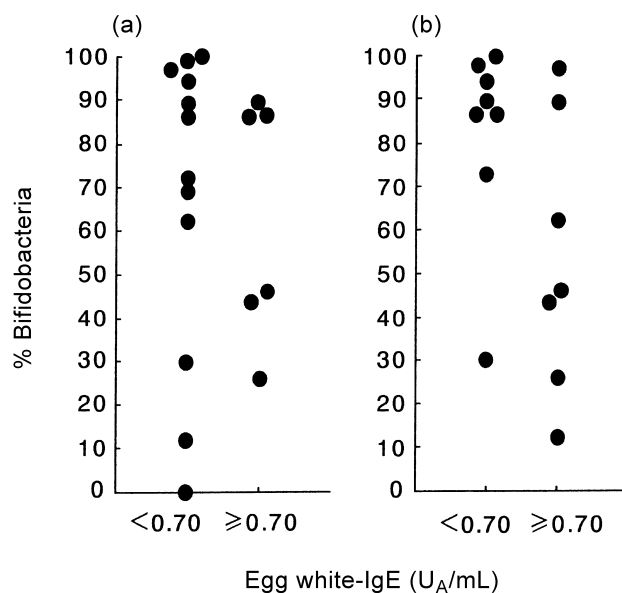
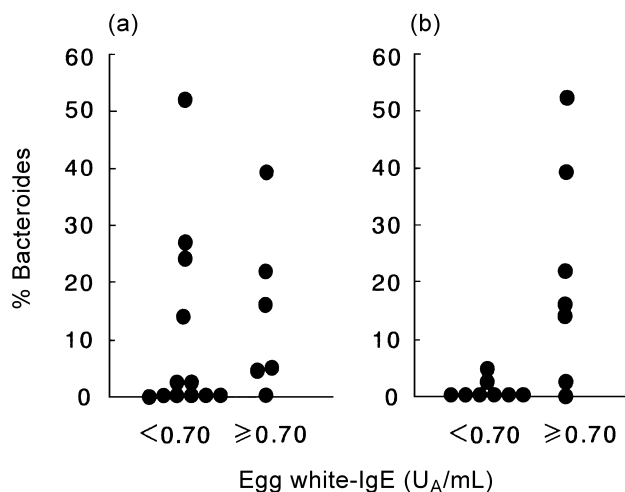


Fig. 1 Relationship between the ratio of breast-feeding at 1 month of age and the percentage of bifidobacteria in the intestine at 4 months of age.



**Fig. 2** Relationship between the percentage of bifidobacteria in the intestine at 4 months of age and the presence of egg white-IgE at (a) 4 months and (b) 1 year of age.



**Fig. 3** Relationship between the percentage of bacteroides in the intestine at 4 months of age and the presence of egg white-IgE at (a) 4 months and (b) 1 year of age.

### Administration of ketotifen and elimination diets

Ketotifen had been administered for more than 1 month in five infants to control atopic dermatitis. Four of these five infants given ketotifen and three of the 13 infants not given ketotifen exhibited more than 10% bacteroides in

their intestinal microflora at 4 months of age; however, this was not statistically significant ( $P = 0.055$ ).

Elimination diets for specific foods suspected as allergens were continued until 1 year of age in five infants: elimination of egg for all five infants and milk for one infant (case 1). Three of five infants with elimination diets and four of 13 infants without elimination diets exhibited more than 10% bacteroides in their intestinal microflora at 4 months of age; however, this was not statistically significant.

Six infants were given ketotifen and/or elimination diets. Five of the six infants had positive egg white-IgE and higher levels of total IgE at 1 year of age.

### DISCUSSION

In the present study, 4-month-old infants in whom bacteroides accounted for more than 10% of the intestinal microflora showed a higher prevalence of positive egg white-IgE and a higher level of total IgE at 1 year of age, suggesting that colonization of bacteroides at 4 months of age was related to an allergic state at 1 year of age. A percentage of bacteroides greater than 10% of the intestinal microflora at 4 month of age may be a predictive factor for allergy at 1 year of age.

The correlation between serum total IgE levels and bacteroides counts has been reported.<sup>8</sup> Feces of infants who were later diagnosed as cow's milk allergy have been reported to have a tendency to contain higher numbers of bacteroides than healthy infants.<sup>9</sup> In the present study, we suggested a positive correlation of bacteroides to allergy and possible mechanisms to explain such correlations can be speculated. In the present study, none of the infants who had a percentage of bacteroides greater than 10% showed a percentage of bifidobacteria greater than 70%. Bifidobacterial supplementation protects against an increase in bacteroides numbers in intestinal microflora.<sup>8</sup> Bifidobacteria seem to modify the intestinal microflora in a manner that may alleviate allergic inflammation.<sup>3,8,10</sup> *In vitro* experiments have shown that Gram-positive bacteria, including *Bifidobacterium adolescentis* and *Lactobacillus plantarum*, induce considerably higher levels of interleukin-12, which can induce a Th1 response, than Gram-negative bacteria, including *Bacteroides vulgatus*, in mononuclear cells.<sup>11</sup> Therefore, the percentage of bifidobacteria decreased in accordance with an increased percentage of bacteroides, which may indicate a potential mechanism of bacteroides for atopic sensitization, although

there was no relationship between the percentage of bifidobacteria and allergy in the present study. Another possible mechanism may be the direct effect of bacteroides on the intestinal mucosa. *Bacteroides fragilis* enterotoxin has been reported to have proteolytic activity that may cause intestinal damage.<sup>12</sup> Further investigation will be necessary to ascertain how bacteroides may affect allergic inflammation.

Bifidobacteria have been reported to become dominant in breast-fed infants.<sup>13–15</sup> In the present study, breast-fed infants had more bifidobacteria in their intestines, but intestinal bifidobacteria had no significant effect on allergic manifestations. Because breast-feeding may have an effect on the development of allergy, although its influence appears contradictory,<sup>16–18</sup> further investigation using infants fed formula only will be necessary to investigate the effect of intestinal microflora levels, including bifidobacteria and bacteroides, on allergic manifestations.

The results of the present study suggest that bacteroides have a positive effect on allergic sensitization. It has been reported that counts of bacteroides at 3 months of age did not differ between allergic and non-allergic children, and were even lower at 12 months of age in allergic children than in non-allergic children, in Estonia and Sweden.<sup>2</sup> However, the counts of clostridia have been reported to be higher in allergic children than in non-allergic in Finland, Estonia and Sweden.<sup>1,2</sup> In the present study, no significant relationship was observed between the percentage of clostridia (compared with the total intestinal microflora level) and allergic manifestations. The differences between our results and those of others regarding the relationship between intestinal microflora and the development of allergic manifestations may be caused by differences in the timing of stool collection and in the standards of living, including environment and diet.

Six infants were given ketotifen and/or elimination diets. Although ketotifen and elimination diets are thought to be effective in the treatment of allergy, five of the six infants had positive egg white-IgE and higher levels of total IgE at 1 year of age. However, one of these infants (case 2) showed a total IgE level of less than 25 IU/mL and a negative egg white-IgE at 1 year of age; this infant had a percentage of bacteroides of less than 10% at 4 months. It is unclear whether the decreased level of IgE found in this infant at 1 year of age was due to treatment with ketotifen and the elimination diet, or the small percentage of bacteroides at 4 months of age, or

both. Further investigation with more subjects will be needed to clarify the relevance of several factors, including not only these treatments, but also genetic backgrounds and familial life styles, to the clinical course of allergy in relation to the development of intestinal microflora.

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