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A Quantitative and Relative Increase in Intestinal *Bacteroides* in Allergic Infants in Rural Japan

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SUMMARY Recent studies have suggested that intestinal microbiota play a substantial role in the development of allergic diseases during infancy. We analyzed fecal microbiota in 18 Japanese infants with or without allergy at 6 months and 2 years of age using a cell culture technique. Allergy determination was based on doctor-diagnosed allergic diseases and skin prick tests. There were no differences between 9 allergic and 9 non-allergic infants at 6 months of age in the frequencies or counts of 13 genera and yeast-like organisms. *Bifidobacterium* was dominant in all infants irrespective of allergy status. At 2 years of age, 8 infants were non-allergic and 10 infants were allergic. Allergic infants at 2 years of age had higher counts of *Bacteroides* and higher ratios of *Bacteroides* to *Bifidobacterium* than non-allergic infants. Despite the small population size used in this study, the results support a significant role of *Bacteroides* in the pathogenesis of allergy during infancy.

Intestinal microbiota, which live in symbiosis with the human body, are a major bacterial stimuli received by neonates after birth, and are reported to be essential for the development and maintenance of the immune system.[1,2] Studies in northern Europe have shown a relationship between intestinal microbiota and allergic diseases, and aberrant intestinal microbiota may affect the development of allergy in infancy.[3-7] However, aberrances in the levels of intestinal microbiota vary among the published studies. Bjorksten's group found lower levels of *Bifidobacterium* and *Bacteroides* and higher clostridia,[3,4] while Isolauri's group showed lower levels of *Bifidobacterium* and higher *Bacteroides* and clostridia[5-7] in allergic infants in comparison with non-allergic infants. In contrast, Nambu *et al*.[8] reported that *Bacteroides* but not *Bifidobacterium* or *Clostridium* is associated with allergy in Japanese infants. Since intestinal microbiota may differ among genetic backgrounds,[9,10] it is important to conduct studies in areas outside northern Europe.

In the present study, we analyzed fecal microbiota of Japanese infants at 6 months and 2 years of age, and compared intestinal microbiota between infants with and without allergies.

MATERIALS AND METHODS

Study design

This study was conducted at a local hospital in Omigawa (population of approximately 25,000),

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located in a rural region of Chiba prefecture in Japan. All babies were born from May to December, 2001 and were examined at 1, 3, and 6 months of age whereupon the type of feeding was checked and physical examinations were performed by a pediatric allergist. Babies born preterm, those administered antibiotics before 6 months of age, or those diagnosed with non-allergic diseases were excluded from further participation. However, infants delivered by Caesarian section or those without atopic heredity were retained. At 6 months of age, a determination of allergy was conducted based on the diagnosis of allergic diseases and skin prick tests (SPTs). Infants with more than one allergic disease or one positive result on the SPT were considered allergic; infants without allergic symptoms and no positive reactions on the SPT were considered non-allergic. Among 45 infants that were monitored until the 6 month stage, 9 infants were determined to be allergic, and 9 non-allergic infants matched for feeding method were recruited to comprise the non-allergic group. A fecal sample was requested from parents within one month after the 6-month visit and again in the summer of 2003, when a reassessment of allergy was performed according to the same criteria.

Skin prick tests

Skin prick tests (SPT) were performed on the volar aspects of the forearms with standardized extracts (Torii Pharmaceutical Co., Ltd. Tokyo, Japan) from egg white, cow's milk, wheat, soy bean, rice, house dust mite, and house dust on infants at 6 months of age, and egg white, cow's milk, house dust mite, house dust, Japanese cedar pollen, cat extracts, alternaria, and orchard grass on infants at 2 years of age. Histamine hydrochloride (1 mg/ml) was used as a positive control and glycerol as a negative control. A result was considered positive if the diameter of the wheal was equal to or more than 3 mm compared to the negative control.[11]

Type of feeding during lactation

At the hospital where this study conducted, most babies were mixed-fed with breast milk and formula before 5 days of age.[12] The term breastfed is defined as infants who were exclusively breastfed from 1 month to the first fecal analysis. Formula-fed infants were those exclusively formula-fed from 1 month, and mixed-fed are those who were not exclusively breastfed or formula-fed from 1 month to the first fecal analysis. Babies were administered water containing 10% glucose at approximately 6 h after delivery, and were fed formula milk until receiving breast milk from their mothers in the maternity ward of the hospital until discharge.

Collection of fecal samples and bacterial analyses

None of the infants had received antibiotics for 4 weeks, or been fed yogurt or drinks containing lactic acid bacteria, or any other foods containing live bacteria for one week prior to fecal collection. Approximately 2 g of a fresh fecal sample was collected in an anaerobic transport container (seed tube, Eiken Chemical Co, Ltd, Tokyo, Japan) as soon as possible after defecation. It was stored in a styrofoam box and kept at 4°C during transportation. Bacteriological analysis was performed within 24 hours following collection. Fecal microbiota was analyzed according to the method of Mitsuoka, as reported previously.[13] The detection limit of bacteria was 2.3 $\log_{10}$ colony-forming units per gram of wet feces.

Statistical evaluation

Counts and the ratio of *Bacteroides* to *Bifidobacterium* between allergic and non-allergic infants were compared by the Mann-Whitney test. Frequency of intestinal microbes was analyzed by Fisher's exact test. Data were analyzed using computer software included in the Statistical Package of Social Sciences (SPSS standard version 11.0; SPSS, Inc., Cary, North Carolina, USA). A p value less than 0.05 was considered statistically significant.

Ethical aspects

This study was performed according to the Declaration of Helsinki, and written informed consent was obtained from the mothers at birth. This study was approved by the ethical committee of Omigawa General Hospital.

RESULTS

Characteristics of the participants

Of the 18 participants, 83% had atopic heredity, the average weight at birth was 3,012 g, and

the percentage delivered by Caesarean section was 17.7%. Sex, type of feeding during lactation, allergic diseases, and the results of SPTs in the participants are shown in Table 1. Other characteristics such as frequency of eating fermented foods and the number of episodes requiring the administration of antibiotics prior to the second fecal analysis were not statistically different between the allergic and non-allergic groups.

Fecal microbiota in allergic and non-allergic infants at 6 months of age

The comparison of fecal microbiota in 9 allergic and 9 non-allergic infants at 6 months of age is shown in Table 2. Regardless of allergic status, *Bifidobacterium* was found in all infants and was the most dominant genus. There was no statistical difference in the fecal microbiota between allergic and non-allergic infants. Counts of *Bifidobacterium* and *Lactobacillus* were positively correlated with the amount of breast milk at 6 months of age, and the prevalence of *Lactobacillus* at 6 months of age was also correlated with the amount of breast milk (data not shown).

Fecal microbiota in allergic and non-allergic infants at 2 years of age

Of the 9 non-allergic infants at 6 months of age, 3 were determined to be allergic at 2 years of age, while of the 9 allergic infants at 6 months of age, 2 infants were determined to be non-allergic at the time of reassessment at 2 years of age. Thus, fecal microbiota between allergic (n = 10) and non-allergic (n = 8) infants were compared at 6 months and 2 years of age (Table 3). There was no a statistical difference at 6 months of age. In contrast, allergic infants had higher counts of *Bacteroides* and lower counts of *Staphylococcus* than non-allergic infants at two years of age ($p = 0.027$, and $p = 0.043$, respectively).

Increased ratio of *Bacteroides* to *Bifidobacterium* in allergic and non-allergic infants at 2 years

Using the assumption that *Bacteroides* perform offensively and *Bifidobacterium* perform pro-

Table 1 Participant background and allergic status at 6 months and 2 years of age

Sex	Atopic heredity	Feeding during lactation	6 months		2 years	
			Allergic disease	SPTs	Allergic disease	SPTs
F	+	Breast	AD	m	AD	d
F	+	Breast	-	e	AD	-
F	+	Mix	AD	emd	AD, BA, FA	emdcoh
M	+	Mix	AD	e	AD	djcoh
M	+	Mix	AD	-	AD	-
F	+	Formula	-	e	-	dh
M	+	Formula	AD	e	AD, AR	dah
M	+	Formula	AD	e	AD, FA	e
M	+	Formula	AD	m	-	-
F	-	Breast	-	-	-	d
F	+	Breast	-	-	AD	djch
F	+	Mix	-	-	-	-
M	-	Mix	-	-	-	-
M	+	Mix	-	-	-	-
F	+	Formula	-	-	-	d
F	+	Formula	-	-	-	-
M	-	Formula	-	-	-	-
F	+	Formula	-	-	-	-

AD, atopic dermatitis; BA, bronchial asthma; FA, food allergy; AR, allergic rhinitis
e, egg white; m, cow's milk; d, dermatophagoides; c, cat extract; j, Japanese cedar pollen
o, orchard grass; h, house dust; a, alternaria.

tectively in the development of allergy, we considered the balance between *Bacteroides* and *Bifidobacterium* to be of significance. Thus the ratio of *Bacteroides* to *Bifidobacterium* was compared between allergic and non-allergic infants at 6 months and 2 years of age (Fig. 1). The ratio was not different at 6 months of age (Fig. 1A); however, it was significantly higher in allergic than non-allergic infants at 2 years of age (Fig. 1B; $p = 0.027$).

DISCUSSION

Our results show that prevalence and count of lactic acid bacteria, *Bifidobacterium* and *Lactobacillus*, do not differ between allergic and non-allergic infants. Since we did not examine fecal microbiota prior to 6 months of age, it might be possible that infants who later developed allergies had a lower frequency of *Bifidobacterium* in an earlier age than those who remained non-allergic. However, this is unlikely in our study subjects since we did not find any differences in the frequency of *Bifidobacterium* between allergic or non-allergic infants at 1, 3 months of age using a PCR method of detection.[12] Our results support the previous study by Nambu *et al*.,[8] who found that allergic infants do not necessarily have reduced amounts of lactic bacteria in Japan. These results are in contrast to the studies from Northern Europe,[3-7] but in accordance with a large cohort study from 3 European countries published recently.[13] Thus, discordance of the results among studies can not be fully explained by differences in food customs and genetic background. Another explanation for the discordance lies in the differences in the severity of allergic infants, since intestinal lactic acid bacteria have not been detected in infants with severe atopic dermatitis,[14] and an inverse correlation between the ratio of *Bifidobacterium* and the severity of atopic dermatitis in children has been reported in Japan.[15] Therefore, a large population study on intestinal microbiota in allergic infants in the future should refer to this point.

The intestinal microbiota of allergic infants showed higher counts of *Bacteroides* and a higher ratio of *Bacteroides* to *Bifidobacterium* at 2 years of age. *Bacteroides* differ from lactic acid bacteria in that they do not improve allergic colitis[16] and may induce intestinal or systemic inflammation.[17,18] An increase in intestinal *Bacteroides* counts has been found in Japanese infants prior to sensitization to hen's egg at 1 year of age,[8] and in Finish infants with milk allergy during breast-feeding.[6] In addition, a positive correlation with the serum IgE levels and

Table 2 Fecal microbiota of allergic and nonallergic infants at 6 months of age

	Allergic (n = 9)		**Non-allergic** (n = 9)	
	Count	**Prevalence**	**Count**	**Prevalence**
Bifidobacterium	10.53 ± 0.47	100%	10.18 ± 0.68	100%
Eubacterium	9.49 ± 0.49	67%	9.30 ± 0.49	44%
Lactobacillus	5.12 ± 2.70	67%	8.49 ± 1.26	44%
Lecithinase(+) *Clostridium*	4.60 ± 1.54	44%	6.97 ± 1.59	22%
Lecithinase(-) *Clostridium*	7.86 ± 1.49	78%	6.92 ± 1.32	67%
Peptostreptococcus	9.13 ± 0.52	67%	8.97 ± 0.44	44%
Veillonella	7.68 ± 2.38	67%	8.36 ± 0.57	78%
Bacteroides	9.40 ± 1.48	100%	9.00 ± 1.55	89%
Staphylococcus	4.04 ± 0.76	100%	4.86 ± 0.78	89%
Enterococcus	8.90 ± 0.39	89%	8.54 ± 0.71	100%
Streptococcus	8.64 ± 0.71	67%	8.50 ± 1.01	56%
Bacillus	3.74 ± 0.51	22%	ND	0%
Yeast like organism	0.75 ± 1.49	22%	ND	0%
Total counts	10.72 ± 0.41		10.45 ± 0.48	

Counts are presented as means ± SD and as log CFU per gram of wet feces
ND, not detected
[1]Counts were statistically analyzed by the Mann-Whitney U test
[2]Prevalence values were statistically analyzed by the Fisher's exact test.

Bacteroides counts has been shown in non-allergic Estonian children.[19] These reports show that intestinal *Bacteroides* may play a significant role in the pathogenesis of allergic diseases, independent of ethnic group. It might further be important to define what kind of *Bacteroides* species need to be reduced. The discordance in the counts of *Bacteroides* to allergies between Isolauri's group,[5-7] Bjorksten's group,[3,4] and Nambu *et al.*[8] and the present study may be due to the difference in *Bacteroides* species, since only an increase in the *Bacteroides fragilis* group has been found to be associated with the symptoms of Japanese cedar pollinosis in a report by Odamaki *et al*.[20] They also found that the cell number ratio of the *B. fragilis* group to *Bifidobacterium* increased significantly during the pollen season in the placebo group but not in the probiotic group. These results indicate that the balance of 'inflammatory' bacteria such as *Bacteroides* to 'anti-inflammatory' bacteria such as *Bifidobacterium* is

Table 3 Fecal microbiota of allergic and nonallergic infants at 6 months and 2 years of age

6 months	Allergics (n = 10)		Nonallergics (n = 8)	
	Count	Prevalence	Count	Prevalence
Bifidobacterium	10.54 ± 0.54	100%	10.11 ± 0.61	100%
Eubacterium	9.71 ± 0.23	60%	8.97 ± 0.37	50%
Lactobacillus	7.06 ± 2.74	60%	5.56 ± 2.89	50%
Lecithinase (+) *Clostridium*	5.58 ± 0.50	30%	5.49 ± 3.66	25%
Lecithinase (-) *Clostridium*	8.01 ± 0.85	80%	6.48 ± 1.79	63%
Peptostreptococcus	8.92 ± 0.30	60%	9.27 ± 0.65	50%
Veillonella	8.45 ± 0.77	70%	7.55 ± 2.27	75%
Bacteroides	9.29 ± 1.40	100%	9.13 ± 1.71	88%
Staphylococcus	4.55 ± 0.84	100%	4.24 ± 0.89	88%
Enterococcus	8.91 ± 0.49	100%	8.42 ± 0.64	88%
Streptococcus	9.01 ± 0.28	60%	8.06 ± 0.96	63%
Bacillus	3.75 ± 0.52	20%	ND	0%
Yeast like organism	3.15	10%	3.58	13%
Total counts	10.78 ± 0.38		10.40 ± 0.44	

2 years	Allergics (n = 10)		Nonallergics (n = 8)	
	Count	Prevalence	Count	Prevalence
Bifidobacterium	9.85 ± 0.37	100%	9.77 ± 0.37	100%
Eubacterium	9.97 ± 0.31	100%	9.64 ± 0.49	100%
Lactobacillus	8.01 ± 2.22	100%	7.28 ± 2.22	88%
Lecithinase(+) *Clostridium*	3.55 ± 1.07	20%	4.91± 0.91	25%
Lecithinase(-) *Clostridium*	8.20 ± 0.48	40%	8.89 ± 0.23	50%
Peptostreptococcus	8.91 ± 1.11	50%	8.35 ± 0.14	50%
Veillonella	7.31 ± 1.61	40%	6.90 ± 0.75	75%
Bacteroides	10.16 ± 0.39*	100%	9.62 ± 0.46	100%
Staphylococcus	3.83 ± 1.15	70%	4.72 ± 1.27*	100%
Streptococcus	7.84 ± 1.22	100%	7.54 ± 0.72	100%
Bacillus	8.82 ± 0.77	20%	ND	0%
Enterococcus	7.90 ± 0.92	100%	7.97 ± 0.53	100%
Yeast like organism	3.87 ± 1.03	70%	3.58 ± 0.56	38%
Total counts	10.59 ± 0.32		10.25 ± 0.32	

Counts are presented as means ± SD and as log CFU per gram of wet feces; ND: not detected.
1) Counts were statistically analyzed by Mann-Whiteney's U test.
2) Prevalence were statistically analyzed by Fisher's exact test.
*$p < 0.05$.

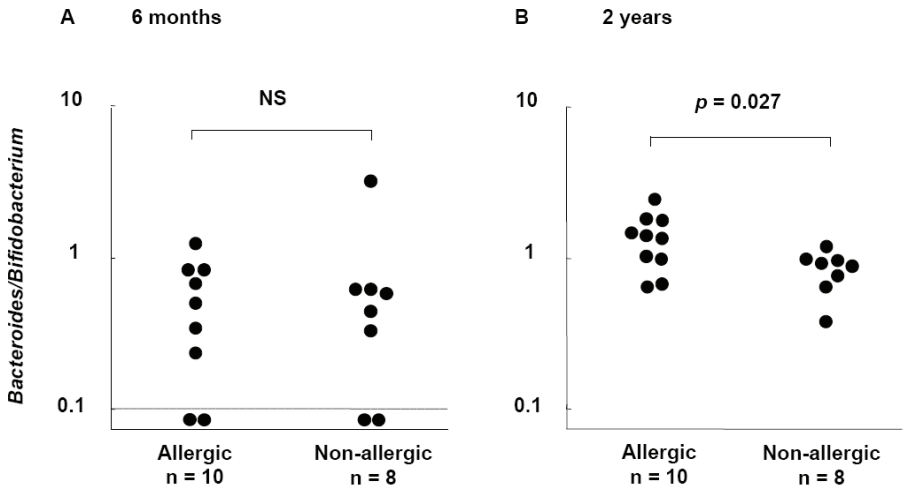

Fig. 1 The ratio of *Bacteroides* to *Bifidobacterium* at 6 months of age (A) and 2 years of age (B) in infants evaluated as allergic and non-allergic at 2 years of age. The data are shown in logarithmic scale. NS: not significant.

critical in the development of allergy, encouraging the administration of lactic acid not only for treatment but also for the prevention of the allergic diseases.

In summary, although the sample size smaller than required to provide conclusive proof, a quantitative and relative increase in intestinal *Bacteroides* was found in infants with allergy at 2 years of age. Dietary, probiotic or prebiotic intervention would elucidate the clinical significance of suppressing potentially inflammatory bacteria for the treatment or prevention of allergic diseases in infancy.

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