

Intestinal Microbiota and the Development of Allergy in Infants

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We analyzed fecal microbiota in Japanese infants with or without allergy at 6 months using a culture technique. Determination of allergy was based on clinical symptoms, signs, and skin prick test. There were no differences in frequencies or counts of 13 genera and yeast-like organisms at 6 months of age between allergic and non-allergic infants. *Bifidobacterium* was dominant in all infants irrespective of the presence or absence of allergy. We next investigated the composition of fecal *Bifidobacterium* species in allergic and non-allergic infants using *Bifidobacterium* species-specific or group-specific primers based on 16S rDNA sequences at 1, 3 and 6 months of age. At 1 month of age, allergic infants had a higher prevalence of *B. catenulatum* than

non-allergic infants (60.0% vs. 6.3%, $P < 0.01$). At 6 months of age, allergic infants had a higher prevalence of *B. bifidum* than non-allergic infants (70.0% vs. 12.5%, $P < 0.01$). These differences were not related to feeding methods. Finally, we studied the activity of *B. bifidum* in comparison with *B. adolescentis* to induce TNF- α and IL-10 from cord blood mononuclear cells. The results suggest *B. bifidum* has lower activity to induce both cytokines compared to *B. adolescentis*, suggesting that colonization of lower cytokine producing *Bifidobacterium* species or strains may be a risk factor for the development of allergy in infancy. (Korean J Asthma Allergy Clin Immunol 2007;27:220-225)

Key words: Intestinal microbiota, *Bifidobacterium*, PCR, Cord blood mononuclear cells, Cytokines

INTRODUCTION

Intestinal microbiota, which live in symbiosis with the human body, are major bacterial stimuli received by neonates after birth, and are reported to be essential for the development¹⁾ and maintenance of the immune system.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 intestinal microbiota vary among the published studies. Bjorksten's group found lower *Bifidobacterium*, *Bacteroides* and higher *Clostridia*,^{3,4)} while Isolauri's group showed lower *Bifidobacterium*, higher *Bacteroides* and *Clostridia*⁵⁻⁷⁾ in allergic infants in comparison with non-allergic infants. In contrast, Nambu, et al. reported that *Bacteroides* but not *Bifidobacterium* or *Clostridium* is associated with allergy in Japanese infants.⁸⁾ Since intestinal microbiota may differ among genetic

backgrounds,^{9,10)} it is important to conduct studies in areas outside northern Europe. Thus we set up a small birth cohort and analyzed fecal microbiota in infants with or without allergy at 6 months using a culture technique in Japan.

In addition to aberrance at genus level in intestinal microbiota, aberrance at species level was also reported in allergy; *B. bifidum* was found to colonize in most non-atopic healthy infants whereas *B. adolescentis* was found in most allergic infants.^{11,12)} Therefore, we studied nine major species of *Bifidobacterium* in allergic infants and non-allergic controls at 1, 3 and 6 months of age by a recently-developed sensitive PCR technique in our cohort. We then studied activity of *Bifidobacterium* species that were different between allergic and non-allergic infants to induce cytokines to understand the relation between intestinal microbiota and development of allergy in infancy.

MATERIALS AND METHODS

1. Subjects

Babies born a local hospital in Omigawa town which is located in a rural region of Chiba prefecture in Japan was enrolled. Babies were examined at 1, 3, and 6 months of age, when the type of feeding was checked and physical examinations were performed by

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a pediatric allergist. Babies born preterm, those treated with antibiotics before 6 months of age, or those diagnosed with non-allergic diseases were excluded. Infants delivered by caesarian section and those without atopic heredity were not excluded. At 6 months of age, a determination of allergy was performed based on the diagnosis of allergic diseases and skin prick test (SPT). 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, and house dust mite, at 6 months of age. Histamine hydrochloride (1 mg/mL) was used as a positive control and glycerol as a negative control. A result was regarded as positive if the diameter of the wheal was more than 3 mm.¹³⁾ 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. Mothers were asked to bring fresh feces from their babies in a diaper at 1, 3, and 6 months of age. Of 26 infants followed until 6 months of age, 10 were allergic and 16 were non-allergy and defined as healthy controls.

2. Collection of fecal samples and bacterial analyses by culture method at 6 months of age

We selected 9 allergic and 9 non-allergic babies by adjusting feeding pattern. None of the infants had 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 after collection. Fecal microbiota were analyzed according to the method of Mitsuoka, as reported previously.¹⁴⁾ The detection limit of bacteria was 2.3 log₁₀ colony-forming units per gram of wet feces.

3. PCR analysis of *Bifidobacterium* species at 1, 3 and 6 months of age

Fecal samples were frozen at -20°C until analysis. Samples of 100 mg were washed three times by suspension in 1.0 mL of PBS and centrifugation at 14,000 × g to reduce the level of PCR inhibitors. The fecal pellets were resuspended in 450 µL of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 µL of 10% sodium dodecyl sulfate. Then, 300 mg of glass beads (diameter, 0.1 mm) and 500 µL of buffer-saturated phenol were added to the

suspension, and the samples were mixed vigorously for 30 s using a FastPrep FP 120 (Bio101 Inc., Vista, CA, USA) at a power level of 5.0. After centrifugation at 14,000 × g for 5 min, the supernatants were collected, and extracted with phenol-chloroform. DNA was obtained by isopropanol precipitation, and suspended in 100 µL of TE Buffer. The presence of *Bifidobacterium* species was determined using nine *Bifidobacterium* species-specific or group-specific primers based on the 16S rDNA sequences as reported by Matsuki, et al.¹⁵⁾

4. Mononuclear cells

Umbilical cord blood samples were obtained from normally delivered full-term babies at Kawatetsu Chiba Hospital in Chiba city. The samples were aspirated into the heparinized tubes. Mononuclear cells (MNCs) were isolated using Ficoll density gradient centrifugation within 24 hours after the aspiration and were cryopreserved at -80°C for subsequent studies of in vitro immunological responsiveness. After thawing, viability was more than 90% for all samples tested. Long-term cryopreservation does not significantly alter the pattern of cellular function of cytokine profiles (unpublished data).

5. Preparation of bacteria

B. bifidum JCM 1255^T and *B. adolescentis* JCM 1275^T were purchased from Japan Culture Collection (Riken, Wako, Japan). Strains of *Bifidobacterium* were anaerobically grown on GAM broth (Nissui, Tokyo, Japan) using AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 18 h. After fermentation, the cells were harvested in a refrigerated centrifuge (10,000 × g, 15 min), washed twice with saline followed by once with water. The cells were resuspended in distilled water, heat-inactivated at 75°C for 60 min, and lyophilized. Each lyophilized cell was resuspended in phosphate buffer saline (PBS), pH 7.2 at the concentration of 200 µg/mL and was used for in vitro assays.

6. Cell cultures

The MNCs were cultured in RPMI1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum, L-glutamine, 50 µg/mL of streptomycin sulfate, 10 µg/mL of gentamycin sulfate, and 20 mM of mercaptoethanol, at a concentration of 1×10⁶ cells/mL in flat-bottomed 96-well tissue culture plates. The MNCs were stimulated with 10 µg/mL of *Bifidobacterium* species for 24 hours at 37°C with 7% CO₂. Culture supernatants were collected, and duplicates were pooled and kept in -20°C until analyzed by enzyme-linked immunosorbent assay (ELISA). Repeated thawing and freezing were avoided.

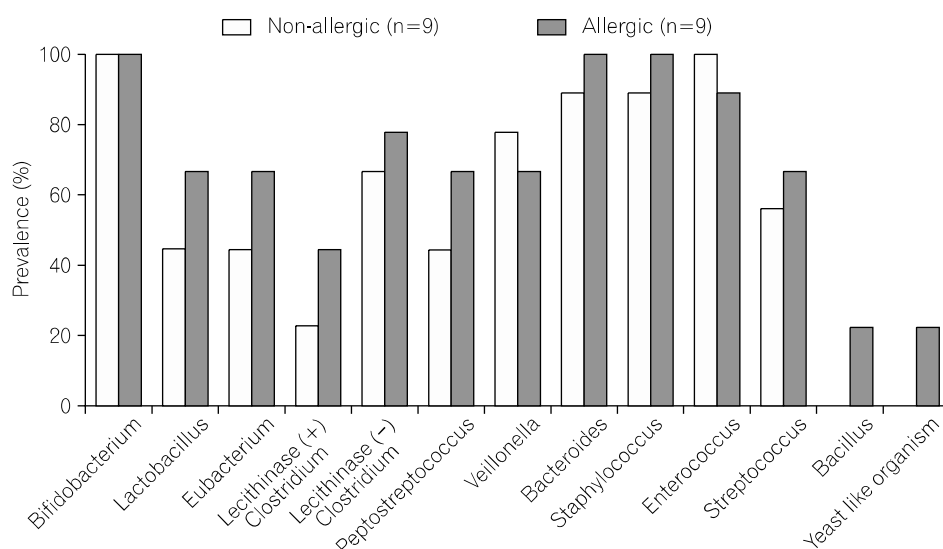


Fig. 1. Bacterial genera analysis of intestinal microbiota in allergic and non-allergic infants at 6 months of age.

7. Cytokine assay

The release of the cytokines TNF- α and IL-10 into the culture supernatants was measured by commercial ELISA kit (Amersham Bioscience, Tokyo, Japan) according to the manufacturer's instructions. All samples were run in duplicate. The detection limit was 5 pg/mL for TNF- α , and 3 pg/mL for IL-10.

8. Statistical evaluation

Data were analyzed using computer software in the Statistical Package for Social Sciences (SPSS standard version 11.0; SPSS Inc., Cary, NC, USA). Maternal age, gestational age and body weight at birth were compared between allergic infants and non-allergic infants using the Student's *t* test. Proportion of sex, mode of delivery, atopic heredity, distribution of feeding method, and frequencies of *Bifidobacterium* species between groups were evaluated using Fisher's exact test. Differences between the two bacterial bodies in regard with the supernatant TNF- α and IL-10 levels released from cord blood MNCs were assessed by the Wilcoxon signed rank test. Differences between eczema group and non-eczema group in regard with those parameters were assessed by the Mann-Whitney's *U*-test. A level of $P=0.05$ was considered statistically significant for all analyses.

9. Ethical considerations

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. Kawatetsu Chiba Hospital and

Chiba University.

RESULTS

1. 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 Fig. 1. There was no difference between 2 groups in maternal age, gestational age, body weight at birth, sex, mode of delivery and feeding pattern but atopic heredity (100% vs. 66.7%, $P<0.05$). 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).

2. Frequencies of *Bifidobacterium* species in allergic infants and non-allergic infants

Fig. 2 shows the frequencies of 9 fecal *Bifidobacterium* species in 10 allergic infants and 16 non-allergic infants at 1 month of age. The most frequently detected species belonged to the *B. adolescentis* group in non-allergic infants (43.8%), whereas it was the *B. catenulatum* group in allergic infants in whom the frequency was significantly higher than in non-allergic infants (60.0% vs. 6.3%, respectively, $P<0.01$). At 3 months of age, the most frequently

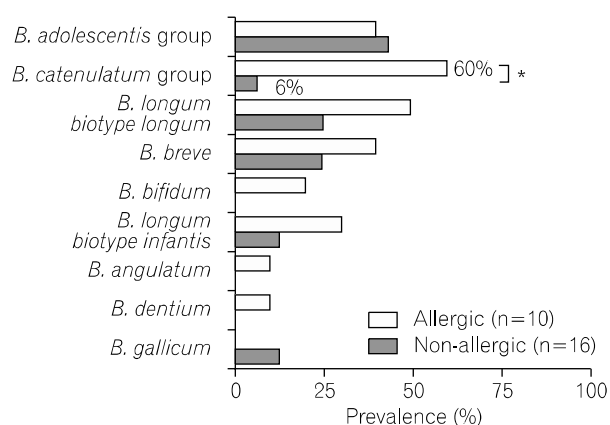


Fig. 2. PCR analysis of *Bifidobacterium* species in allergic and non-allergic infants at 1 months of age. * $P < 0.01$ in Mann-Whitney's U-test.

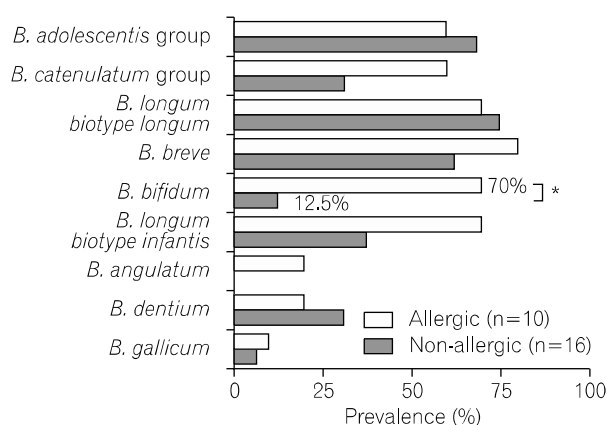


Fig. 3. PCR analysis of *Bifidobacterium* species in allergic and non-allergic infants at 6 months of age. * $P < 0.01$ in Mann-Whitney's U-test.

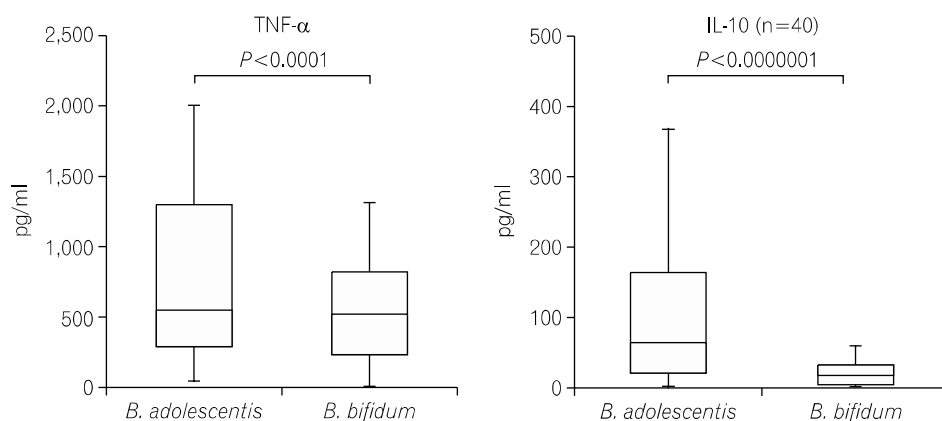


Fig. 4. TNF- α and IL-10 production from cord blood MNCs stimulated with *B. adolescentis* or *B. bifidum*. Cord blood mononuclear cells were cultured with heat-inactivated bacteria for 24 hours, and TNF- α in the culture supernatant was measured using a commercial ELISA kit. Statistical significance was calculated by Wilcoxon's rank test.

detected species were those of the *B. adolescentis* group in non-allergic infants (68.8%), whereas *B. breve* was the most frequently detected species in allergic infants (70.0%). The frequency of *B. catenulatum* group species in allergic infants decreased dramatically and they became less frequent than in non-allergic infants (20.0% vs. 31.3%, respectively). Allergic infants had a tendency to have a higher frequency of *B. bifidum* than non-allergic infants (50.0% vs. 12.5%, respectively, $P = 0.069$). At 6 months of age, *B. adolescentis* group species, *B. breve*, and *B. longum* biotype longum were found with high frequencies in both groups. In contrast, *B. bifidum* was found with high frequency only in allergic infants, with a significant difference as compared to non-allergic infants (70.0% vs. 12.5%, respectively, $P < 0.01$) (Fig. 3).

3. Cytokine release from cord blood MNCs stimulated with *Bifidobacterium* species.

TNF- α and IL-10 release from cord blood MNCs ($n = 40$) by

stimulation of *B. adolescentis* and *B. bifidum* are shown in Fig. 4. TNF- α release was significantly lower in the stimulation with *B. bifidum* than with *B. adolescentis* ($P < 0.0001$). IL-10 release also was significantly lower in stimulation with *B. bifidum* than with *B. adolescentis* ($P < 0.0000001$) (Fig. 4). The ratio of TNF- α /IL-10 in the culture supernatant stimulated with *B. bifidum* was significantly higher than that that with *B. adolescentis* (Fig. 5).

DISCUSSION

Our results show that prevalence and count of lactic acid bacteria, *Bifidobacterium* and *Lactobacillus* in feces do not differ between allergic and non-allergic infants, a result that supports 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⁾ This discordance may be due to differences in food customs and genetic

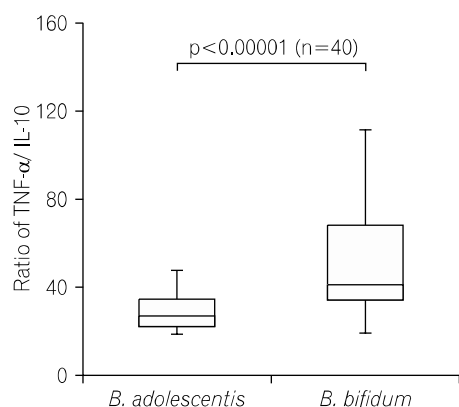


Fig. 5. Ratio of TNF- α to IL-10 in the culture supernatant in the Fig. 4. Statistical significance was calculated by Wilcoxon's rank test.

background. Another explanation lies in the mild symptoms of the allergic participants in Nambu's study and ours, since intestinal lactic acid bacteria have not been detected in infants with severe atopic dermatitis,¹⁶⁾ and an inverse correlation between the ratio of *Bifidobacterium* and the severity of atopic dermatitis in children has been reported in Japan.¹⁴⁾ 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 PCR method (data not shown).

Despite no difference in numbers the composition of fecal *Bifidobacterium* was found to differ between allergic infants and non-allergic infants. Allergic infants had a higher prevalence of *B. catenulatum* group species at 1 month of age and *B. bifidum* at 6 months of age than non-allergic infants. Feeding method was not related to the differences in the composition of fecal *Bifidobacterium*. The finding of a difference in *Bifidobacterium* species between allergic subjects and non-allergic controls is consistent with the report of Ouwehand from Finland.^{11,12)} However, the dominant species in allergic and non-allergic infants in the present study differs somewhat from those in their data, which showed *B. bifidum* to colonize most non-allergic infants whereas *B. adolescentis* colonized most allergic infants. *B. catenulatum* is generally categorized as an adult-type *Bifidobacterium* species; thus the findings in the two studies are basically the same in terms of the presence of an adult-type *Bifidobacterium* species in allergic subjects. However, the rate of detection of *B. bifidum* is opposite between the Finnish study and our present study. One explanation for this difference involves

genetic and environmental differences between Finland and Japan, since the implantation and development of intestinal *Bifidobacterium* in the neonate are greatly influenced by prematurity, method of birth, early feeding regime, endogenous substrate availability from breast milk or formula and the environment.¹⁷⁾ In fact, there is a clear difference in the composition of *Bifidobacterium* species other than members of the *B. bifidum* and *B. adolescentis* groups between infants in the report by Ouwehand and those in the present study. Young, et al reported a significant species difference in *Bifidobacterium* among residents of Ghana, New Zealand and the United Kingdom,¹⁸⁾ suggesting significant differences in the prevalence of *Bifidobacterium* species among countries and races. The results indicate some correlation between species differences in intestinal *Bifidobacterium* and allergic diseases in infants, although our data are in contrast with the report from Finland.

Since *B. bifidum* was detected in allergic infants compared to non-allergic infants, we compared production of proinflammatory cytokine, TNF- α and anti-inflammatory cytokine, IL-10 from cord blood MNCs stimulated with two *Bifidobacterium* species, *B. bifidum* JCM 1255^T and *B. adolescentis* JCM 1275^T. The results suggest that production of two cytokines, especially IL-10 from cord blood MNCs were lower upon *B. bifidum* JCM 1255^T stimulation than *B. adolescentis* JCM 1275^T. Ratio of TNF- α to IL-10 was higher for *B. bifidum* than *B. adolescentis*, suggesting that for *B. bifidum* has less anti-inflammatory than *B. adolescentis*. The present data is compatible with the observations that species in the same genus such as *Bifidobacterium*^{19,20)} and *Lactobacillus*²¹⁾ have different cytokine stimulating activity. However, TNF- α and IL-10 inducing activity of 2 *Bifidobacterium* species was opposite to the findings previously reported by He et al¹⁹⁾ and Young et al.¹⁸⁾ At present we do not know the reason for the difference between others and ours. Although our data was in contrast to previous studies, our observation at least suggests importance of species or strains of *Bifidobacterium* in the property of immune regulatory activity. This is quite important in terms of selecting probiotics for the treatment of allergic diseases in infants.

In summary our studies suggest relation between aberrance in microbiota and allergic diseases in infancy in Japan.

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