The dynamics of the developmental bacterial community in the Japanese neonatal gastrointestinal tract were examined by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples by PCR and denaturing gradient gel electrophoresis (DGGE). The results showed a certain pattern common in infants without antibiotic treatment, in which aerobes, e.g., Pseudomonas, appeared first and were then immediately replaced by facultative anaerobe, Enterococcus, Streptococcus, and Enterobacteriaceae through the first month, and finally strictly anaerobic Bifidobacterium appeared.

Key words: denaturing gradient gel electrophoresis (DGGE); gastrointestinal tract; infant; 16S rRNA; intestinal microbiota

Soon after birth, bacterial colonization starts in the previously germfree gastrointestinal tract and commensal-host-microbial relationships begin.1,2) The colonizing bacteria contribute to maintenance of the mucosal barrier, facilitate carbohydrate assimilation, and modulate the mucosal immune system. Thus, the initial development of intestinal microbiota is considered to have great influence on the health of the infant. In this study, the succession of the gastrointestinal bacterial community was examined for the first two months in nine Japanese infants by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples.

All infant subjects (infants nos. 1, 2, 5, 6, 10, 20, 24, 25, and 33) participated in this study were vaginally delivered. Infant no. 5 was fed formula milk and the other infants were fed both breast and formula milk during the sampling period. Infants no. 1 and no. 33 were subjected to antibiotic therapy, receiving cefalexin (50 mg/kg, 4 times a day) the first four days, whereas infant no. 5 was treated on day 0 only. Fecal samples were collected on day 0/1, day 3, day 5, month 1, and month 2 (there was no month-2 sample from infant no. 5). All the parents of our subjects gave written informed consent and the Ethics Committee of the Faculty of Medicine of Kyoto University approved this study protocol.

DNA was isolated from each fecal sample using a bead beating method essentially as previously described,3) except for 2–3 times washing of the fecal sample before the bead beating step. In order to construct 16S rDNA libraries, a V1–V3 region of 16S rDNA was amplified from each sample by PCR with 8UA (5'-AGGTTTGATCCTGGCTCAG-3')4) and 519B (5'-ATTACCGCGGTGCTG-3')5) primers, and cloned into a pGEM-T vector (Promega, Madison, WI), and transformed in E. coli JM109. About ten clones from each library were sequenced. In total, 357 clones were sequenced and the ribotypes found are summarized with the result of the database search in Table 1.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which allowed rapid and efficient molecular fingerprinting of gut microbiota,5,6) was performed in order to monitor the succession of the infant fecal bacterial community. The variable region V2–V3 of 16S rDNA was amplified by PCR using primers HDA1-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGC GGG GCG

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The PCR condition was as follows: 94°C for 5 min, 30 cycles consisting of 94°C for 40 s, 58°C for 20 s, and 72°C for 1 min, and finally 72°C for 5 min.

DGGE analysis was performed as described by Muyzer et al.8) and Heilig et al.9) using a Dcode System apparatus (Bio-Rad, CA). Each band in the DGGE gel was assigned one of the ribotypes in Table 1 either by sequencing of DNA fragments excised from the DGGE gel or by comparing band positions with those of reference clones derived from the 16S rDNA clone library.

Figure 1 shows the DGGE profile of nine subjects. Although each subject showed individual banding patterns, a stepwise development from aerobic to...
anaerobic microbial ecosystem was observed in the succession of bacterial composition in the seven subjects (nos. 2, 5, 6, 10, 20, 24, and 25) without successive antibiotic treatment in the first four days. In the beginning, bands corresponding to aerobic Gram-negative bacteria such as *Pseudomonas* appeared and they were then replaced by facultatively anaerobic bacteria such as *Streptococcus*, *Enterococcus*, or *Staphylococcus epidermidis* and *Enterobacteriaceae*. Particularly, strong bands closely related to *Streptococcus parasanguis*, *Streptococcus cremoris* and *Streptococcus thermophilus* appeared on day 3 in many subjects. It is interesting to note that a large majority of the bacteria types such as *Streptococcus parasanguis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Bifidobacterium dentium*, and *Veillonella parvula* detected in this period are regarded as oral-origin bacteria rather than intestinal species. This suggests that these oral-origin bacteria may transiently colonize the intestine during this period.

Bifidobacteria detected in the bottom part of the DGGE gel appeared within two months in most subjects. In infant no. 2, *Bifidobacterium pseudocatanulatum* colonized predominantly from day 3 and continued until 2 months of age. This subject was only the case which agreed with the finding of previous studies, showing that bifidobacteria usually appear and become dominant within a week after birth. The appearance of bifidobacterial bands in infant no. 2 was concomitant with a decrease in *Enterobacteriaceae* bands, which appeared as dominant on day 3. A concomitant decrease in *Enterobacteriaceae* with an increase of bifidobacteria in breast-milk fed infants has been reported. Bands related to *Clostridium butyricum* were also detected in infants nos. 6, 10, 20, and 24, in which they appeared earlier than bifidobacteria. The other strict anaerobes, *Veillonella parvula*-like bacteria, and *Ruminococcus* sp. were found only in infant no. 25. *Bacteroides uniformis* was detected only at day 5 in infant no. 5 (antibiotic treatment on day 0) who was the only subject brought up only on formula milk.

Infants nos. 1 and 33 treated with antibiotics in the first 3 days showed relatively simple microbiota, and the developmental patterns deviated remarkably from the trends observed in the other subjects without antibiotic treatment. In infant no. 1, a dominant band corresponding to *Micrococcus mucilaginosus*, which is not a common inhabitant of the intestine, appeared suddenly on day 5 and completely disappeared during month 1. In infant no. 33, a dominant band corresponding to *Enterococcus faecium* appeared on day 3 and disappeared during month 1. No bands corresponding to bifidobacteria and other strict anaerobes were found in the testing period in either baby and only bands corresponding to *Enterobacteriaceae* were found during month 1 and month 2, suggesting domination by *Enterobacteriaceae*. This was also indicated by the data of random sequencing of 16S rDNA clone libraries, which showed that all 30 clones sequenced from the month-1 and month-2 libraries of these two subjects belonged to *Enterobacteriaceae*. These data showed that antibiotic treatment at the beginning of life has strong...
influence on the establishment of a normal microbial ecosystem in the intestine.

In conclusion, this molecular study indicates the stepwise development from aerobic to anaerobic microbial ecosystem with a variety of bacterial groups, although the process differed among individuals at the species level. The step of the bacterial colonization in the gastrointestinal tract is most likely a key to the developmental process. Strong antibiotic treatment interrupted the development of normal microbiota, including bifidobacteria. Further studies with modern molecular methods are needed for understanding of the environmental and host factors affecting the developmental process of neonatal microbiota.

Acknowledgment

We thank all the families who provided fecal samples for this study. This research was supported in part by a Research Grant for Immunology, Allergy, and Organ Transplants from the Ministry of Health and Welfare of Japan, by a grant from Danon Institute for the Promotion of Health and Nutrition, and by a grant from Takeda Science Foundation.

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