Administration of Antibiotics during Infancy Promoted the Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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This study aimed to determine whether oral antibiotic administration during infancy is associated with the spontaneous development of atopic dermatitis-like skin lesions by modulating intestinal microbiota. Female NC/Nga mice at 3 weeks of age were orally administered kanamycin or polymyxin B. Clinical symptoms, scratching behavior, and serum antibody levels were evaluated. Changes in intestinal microbiota were determined by culture-independent analysis and cultural analysis. The kanamycin-treated mice showed higher clinical scores and scratching frequency than the control mice. IgE levels were significantly higher in the kanamycin-treated mice than in the control mice. Transient changes in intestinal microbiota were observed under kanamycin treatment. Polymyxin B treatment failed to affect scratching behavior. These results suggest that oral administration of kanamycin during infancy promoted the development of atopic dermatitis-like skin lesions in NC/Nga mice and was associated with a transient change in intestinal microbiota.

Key words: antibiotics; intestinal microbiota; atopic dermatitis-like skin lesions; allergy; NC/Nga mice

The incidence of allergy in the form of atopic diseases such as atopic dermatitis (AD), atopic eczema, allergic rhinitis, and allergic asthma has steadily increased during the last several decades in developed countries. AD is a clinical syndrome characterized by pruritic eczematous skin lesions in characteristic locations, along with other major and minor clinical signs. Among the many cell types involved in the pathogenesis of AD, T-helper (TH) 2 cells producing interleukin (IL)-4, -5, -10, and -13 play critical roles in the initial phase of disease progression. Hence AD is caused primarily by overproduction of TH2-mediated cytokines, chemokines, and IgE.

NC/Nga mice develop an eczematous condition when kept in conventional surroundings but not when kept under specific pathogen-free conditions. Clinical signs begin with scratching behavior and IgE elevation starting from the age of 8 weeks, followed by the onset of eczematous conditions along with infiltration of various inflammatory cells into the skin lesions.

Elevated expression of TH2 cytokines and chemokines has been observed in lesional skin areas. These findings resemble the various characteristics of AD patients, suggesting that NC/Nga mice are an excellent animal model for human AD.

The drastic increase in the prevalence of allergies, including AD, in a short genetic interval suggests the influence of environmental determinants. One difference that has been proposed to underlie the increased prevalence of allergy is the hygiene hypothesis, which proposes that environmental factors associated with a western lifestyle promote the development of allergy. Indeed, a wide body of epidemiological literature has shown an inverse association between exposure to intracellular infections, including tuberculosis, measles, and hepatitis A, and allergic disease.

In addition to such microbial pathogens, intestinal microbiota play a key role in the postnatal development of the immune system in infants. Approximately 400 different microbial species, mostly bacteria, inhabit the intestinal tract of mammals, where the bacterial density reaches more than 1011 cells/g contents. These indigenous bacteria play major roles in the maturation of the immune system, maintenance of homeostasis of the gut-associated immune system, and the induction of oral tolerance. Because oral administration of broad-spectrum antibiotics depletes normal commensal microbiota as they target microbial pathogens, it is hence speculated that depletion of microbiota by antibiotics is associated with increased risk for the development of allergy. Oyama et al. reported that oral administration of kanamycin during infancy, but not in adulthood, promoted a shift in the TH1/TH2 balance toward TH2-predominant immunity in mice. Moreover, Wickens et al. reported that antibiotic use during infancy is associated with an increased risk for the development of allergic asthma in humans.

In this study, we examined to determine whether oral administration of the Gram-positive antibiotic kanamycin and the Gram-negative antibiotic polymyxin B in
infancy would influence the spontaneous development of AD-like skin lesions by modulating intestinal microbiota in NC/Nga mice.

**Materials and Methods**

**Animals.** Three-week-old female NC/Nga mice were purchased from Japan SLC (Hamamatsu) and housed in standard plastic cages in a temperature-controlled room (23 ± 2 °C) with a dark period from 2000 to 0800 h. They were allowed free access to commercial chow (Labo MR Stock; Nihon Nosan Kogo, Yokohama) and water. This study was approved by the Hokkaido University Animal Use Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

**Oral administration of antibiotics.** To determine the effects of kanamycin administration, the mice were divided into two groups (n = 5–6/group) and orally administered either 100 µl of distilled water containing kanamycin sulfate (0.6 mg/mouse/d, Sigma, St. Louis, MO) or distilled water alone once a day for 7 consecutive d from the age of 3 weeks. Fresh feces were collected 1 d after completion of treatment. Eight weeks after completion of treatment, the mice were anesthetized by inhaling of diethyl ether, blood was drawn from the carotid artery, and the cecal contents were collected. In a separate experiment, mice were divided into three groups (n = 6/group) and orally administered either 100 µl of distilled water containing kanamycin sulfate (0.6 mg/mouse/d) or polymyxin B sulfate (0.4 mg/mouse/d, Wako Pure Chemical, Osaka) or distilled water alone once a day for 7 consecutive d.

**Evaluation of the severity of atopic dermatitis-like skin lesions.** Eight weeks after completion of antibiotic administration, the severity of AD-like skin lesions was evaluated by a clinical score and observation of scratching behavior. The total clinical score for dermatitis severity was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness).22 Scanning behavior was observed for 10 min per mouse to evaluate scratching frequency. Observers blinded to the experimental groups performed these observations.

**Evaluation of antibody levels.** The level of serum total IgE was determined by enzyme-linked immunosorbent assay (ELISA). All assays were performed in 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ). The wells were coated overnight at 4 °C with rat anti-mouse IgE (clone ME-01-DE; Yamasa, Choshi) diluted to 5 µg/ml in carbonate buffer (pH 9.6). The plates were blocked with PBS containing 5% v/v horse blood (Nihon Biotest, Tokyo) and the plates were incubated at 37 °C for about 24 h by the anaerobic gas pack method. The number of colonies was counted after incubation.

**Profiling of fecal microbiota by polymerase chain reaction-denaturing gradient gel electrophoresis.** DNA was extracted from fresh feces and cecal contents using a Fecal DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA) following the manufacturer’s instructions. DNA samples were used as a template to amplify the fragments of 16S rDNA with universal primers, U968-GC (AAC CCG AAC AAC G) and L1401. Polymerase chain reaction (PCR) was performed in a reaction volume of 25 µl that contained 500 nM each of U968 and L1401, 1 x PCR buffer, 0.2 mM each of dNTPs, and 1.25 U of Taq HS polymerase (Takara, Ohtsu). The reaction conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 3 min, and a final extension at 72 °C for 7 min. The amplicons were purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bioscience) and cloned into pGEM-easy T vectors (Promega, Madison, WI). Transformation was performed with competent Escherichia coli XL-1 Blue cells, and the transformants were spread on Luria-Bertani agar plates supplemented with 25 µg/ml of ampicillin, 30 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 20 µg/ml of isopropyl-β-D-thio-galactopyranoside, and incubated overnight at 37 °C. White colonies were picked out and grown on Luria-Bertani agar. Plasmid DNAs were amplified with an Illustra TempliPhi DNA Amplification Kit (GE Healthcare Bioscience) following the manufacturer’s instructions. The resulting amplicons were sequenced using an ABI3730XL or an ABI3730 automatic sequencer (Applied Biosystems, Foster City, CA) with M13-F (GTT TTC CCA GTC ACG ACG TT) as the sequencing primer.

**Analysis of 16S rDNA sequences of intestinal bacteria.** The fecal DNA samples from each group were pooled and used as templates to amplify the fragments of 16S rDNA with universal primers, U968 (AAC CCG AAC AAC G) and L1401 (CGG TGT GTA CAA GAC CC), and denaturing gradient gel electrophoresis (DGGE) analysis of the amplicon was performed as described previously.25 Quantity One software (version 4.6.0, Bio-Rad) was used for band identification and normalization of band patterns from the DGGE gels. Subsequently, a dendrogram of the DGGE-band profile was constructed using Pearson’s curve-based correlation and the unweighted pair-group method by the arithmetic average (UPGMA) clustering method of the Quantity One program, as previously described.25

**Quantification of intestinal bacteria by the culture method.** Bacteriological analysis of mouse feces was performed by the method of Mitsuoka et al.23 Briefly, fresh fecal samples were immediately diluted in 10-fold steps with anaerobic phosphate buffer, and 50 µl of each dilution was inoculated on the following plates. For quantification of total aerobes, trypticase-soy agar (Becton Dickinson, Franklin Lakes, NJ) containing 5% v/v horse blood (Nihon Biotest, Tokyo) was used, and the plates were incubated at 37 °C for 24 h. For quantification of total anaerobes, BL agar (Eiken Chemical, Tokyo) containing 5% v/v horse blood (Nihon Biotest) was used, and anaerobic incubation was carried out at 37 °C for about 24 h by the anaerobic gas pack method. The number of colonies was counted after incubation.

**Quantification of lactobacilli by real-time PCR.** Amplification and detection of fecal DNA were performed with a Smart Cycler II (Cepheid, Sunnyvale, CA), as described previously.31 Briefly, a Lactobacillus genus-specific primer pair (forward, TGG AAA CAG (A/G)TG CTA ATA CCG; reverse, GTC CAT TGT GGA AGA TTC CC)21 was used. Real-time PCR was performed in a reaction volume of 25 µl, containing 12.5 µl SYBR Premix Ex Taq (Takara), 200 nM each of the forward and reverse primers, and 1 µl of fecal DNA samples. The reaction conditions were 95 °C for 30 s, followed by 43 cycles at 95 °C for 5 s, 57 °C for 20 s, and 72 °C for 15 s. Lactobacillus murinus (JCM 17172) were cultivated in MRS broth (Becton Dickinson), and genomic DNA was extracted with Epiplast II (Wako Pure Chemical). Fragments of 16S rDNA were amplified by PCR with the genus-specific primer pair listed above. The amplicons were cloned
in pGEM-Easy T vectors, and the resulting plasmids were used as a standard for real-time PCR.

Statistical analysis. The results are presented as means ± SEM. A unpaired or paired *t* test or Tukey-Kramer’s test following one-way analysis of variance was used to compare mean values. Data analysis was performed with StatView for Macintosh (version 5.0, SAS Institute, Cary, NC). *p* values of less than 0.05 were considered statistically significant.

Results

Effects of kanamycin administration on AD-like skin lesions

At 8 weeks after completion of oral administration of kanamycin, the clinical scores of the kanamycin-administered mice tended to be higher than those of the water-administered control mice (Fig. 1A, Supplemantal Fig. 1; see Biosci. Biotechnol. Biochem. Web site). The scratching frequencies for the kanamycin-administered mice were significantly higher than those for the control mice (Fig. 1B).

At 4 weeks after completion of oral kanamycin administration, the serum total IgE levels of kanamycin-treated and control animals were virtually the same (7.00 ± 1.79 μg/ml vs. 5.13 ± 1.66 μg/ml). Although total IgE levels were elevated in both the kanamycin-treated and the control animals at 8 weeks after completion of treatment, the IgE levels of the kanamycin-treated mice (69.99 ± 3.94 μg/ml) were significantly higher than those of the control mice (44.04 ± 7.02 μg/ml).

Quantification of fecal bacteria by the culture method

At 1 d after completion of kanamycin administration, the numbers of total aerobic bacteria tended to be lower in the feces from the kanamycin-treated mice than in those from the control mice (Fig. 2A). The numbers of total anaerobic bacteria were significantly lower in the feces from the kanamycin-treated mice than in those from the control mice (Fig. 2B).

Quantification of bacterial diversity by culture-independent methods

PCR-DGGE analyses of bacterial 16S rDNA extracted from fresh feces and from cecal contents were performed to compare the microbiota of the kanamycin-treated mice with those of the control mice. Representative DGGE gel images are shown in the left-hand panels of Fig. 3. The intensities and positions of the detected bands were subjected to cluster analysis. At 1 d after completion of kanamycin administration, the dendrogram showed two large clusters for the kanamycin-treated and control groups (Fig. 3A), but distinct clusters were not observed in the dendrogram at 8 weeks after completion of treatment (Fig. 3B).

For further analysis of the intestinal microbiota, 16S rDNA libraries were constructed from fecal DNA of the mice at 1 d after completion of treatment using universally conserved 16S rDNA-targeted PCR primers. Thirty-six and 41 clones were randomly selected from the libraries of the kanamycin-administered and the control mice respectively, and their sequences were determined. Based on sequence similarities, the clones were classified into several clusters corresponding to the classes of bacteria (Table 1). The number of clones classified into the class Bacilli was lower in the libraries from the kanamycin-administered mice than in those from the control mice (Table 1). The clones classified into the class Bacilli were closely related to Lactobacillus acidophilus (M99704) and L. murinus (AF157049) (data not shown).

Quantification of fecal lactobacilli

Quantification of fecal lactobacilli was performed by real-time PCR using Lactobacillus genus-specific primers. At 1 d after completion of kanamycin administration, the fecal lactobacilli levels in the kanamycin-treated mice were significantly lower than those in the control mice (Fig. 4A), but the cecal lactobacilli levels of the kanamycin-treated and the control mice were the same at 8 weeks after completion of treatment (Fig. 4B).

Comparison of the AD-exacerbating effects of kanamycin and polymyxin B

At 8 weeks after completion of oral antibiotic administration, scratching frequency was significantly higher in the mice administered kanamycin than in the control mice (Fig. 5), but scratching frequencies were not significantly different between the polymyxin B-administered and the control groups (Fig. 5).
The prevalence of allergies has drastically increased in the last several decades. Although a large increase in a short genetic interval suggests the influence of environmental determinants, the causative mechanisms are not clearly understood. Epidemiological studies indicate that antibiotic usage during infancy increases the risk for asthma and allergic diseases. The present study investigated the mechanisms behind the exacerbation of atopic diseases by antibiotic use in a NC/Nga mouse model. NC/Nga mice are widely used as a model for human AD because aspects similar to those of AD patients are observed in these mice.

In the present study, NC/Nga mice 3 weeks of age were orally administered a clinical dose of antibiotics for 7 d, and the spontaneous development of AD-like skin lesions was then evaluated. The clinical score tended to be higher and the scratching frequency was significantly higher in the mice treated with kanamycin than in the control mice at 8 weeks after completion of treatment (Fig. 1, Supplemental Fig. 1), clearly indicating that kanamycin treatment in infancy promotes the development of skin lesions in NC/Nga mice.

It is generally accepted that commensal bacteria in the intestinal tract play a major role in the maturation and maintenance of the immune system. Oyama et al. have reported that kanamycin treatment during infancy promoted a shift in the TH1/TH2 balance toward TH2-dominant immunity in BALB/c mice. In the present study, total IgE levels in the mice treated with kanamycin were higher than those in the control mice at 8 weeks after completion of treatment. Because TH2 cells are necessary to produce IgE, it is suggested that kanamycin treatment induces TH2-skewed immune responses in NC/Nga mice. TH1 responses were reported to be diminished in germ-free mice when compared as specific pathogen-free mice. Several strains of bacteria belonging to the genus Lactobacillus reportedly enhanced TH1 responses. Thus it is possible that an alteration in the numbers, the composition, or both of commensal microflora, especially the decrease in lactobacilli, induced by kanamycin treatment is involved in the promotion of skin lesions through a TH2-skewed immune response in NC/Nga mice. Inoue et al. have reported that AD-like skin lesions in NC/Nga mice were prevented by oral administration of Lactobacillus johnsonii NCC533 at 20–22 d of age. In the present study, kanamycin treatment was started at 3 weeks of age. Thus it is possible that intestinal lactobacilli were decreased by kanamycin during the phase at which lactobacillus treatment
induced preventive effects on AD development. In contrast to kanamycin, treatment with polymyxin B failed to promote the development of scratching behavior (Fig. 5). Kanamycin selectively affects Gram-positive bacteria, whereas polymyxin B shows antibacterial activity toward Gram-negative bacteria. Tsukahara et al. have reported that oral administration of polymyxin B decreased Escherichia coli in the large intestine of pigs, whereas the numbers of lactobacilli were unchanged. These results also confirm our speculation described above.

Decreases in fecal total aerobes and anaerobes were observed at 1 d after completion of kanamycin treatment (Fig. 2). DGGE and 16S clone library analyses revealed that changes in the composition of intestinal microbiota, especially a decrease in lactobacilli, occurred at this point (Fig. 3A and Table 1). However, differences in bacterial composition were no longer observed at 8 weeks after completion of treatment (Fig. 3B). This suggests that the changes in intestinal microbiota induced by kanamycin treatment are transient.

Although it remains unclear whether antibiotic treatment at an older age exacerbates skin lesions, the possibility appears small. Oyama et al. have reported that antibiotic treatment at 52 weeks failed to affect the TH1/TH2 balance in mice. TH2-skewed responses to common environmental allergens are reportedly present in human neonates at both high and low risk for atopic diseases. It has also been found that the key causative factor in atopic diseases might be the efficiency of immune deviation mechanisms, which in non-atopic individuals redirect the fetal immune responses toward the TH1 cytokine phenotype. Therefore, intestinal microbiota in infancy but not at an older age might be associated with the development of skin lesions.

Kanamycin might exert direct effects on immune responses independently of indigenous microflora, but this is unlikely, because neither kanamycin nor polymyxin B is absorbed into the body via the intestine when administered orally. In addition, it has been reported that the addition of a sufficiently high concentration of kanamycin to the culture media did not change the cytokine production of mouse splenocytes.

Given that changes in intestinal microbiota, especially a decrease in lactobacilli, due to antibiotic treatment in infancy is associated with the development of AD, administration of probiotics in infancy might prevent the development of AD. Dietary supplementation with indigestible oligosaccharides stimulates the growth of intestinal lactobacilli and bifidobacteria. Moro et al. have reported that a higher number of fecal bifidobacteria was associated with a lower incidence of AD in infants administered a mixture of galacto- and fructooligosaccharides as compared with control infants. In addition, Kukkonen et al. have reported that administration of probiotics plus galactooligosaccharides to pregnant women and their infants prevented atopic diseases in the offspring until the age of 2 years. Furthermore, we recently reported that intestinal microbiota in infant mice were modified by ingestion of fructooligosaccharides by the pregnant and lactating dams. These findings suggest that modulation of intestinal microbiota in infancy due to administration of prebiotics to pregnant and lactating mothers and their offspring is a promising method of preventing atopic diseases.

In conclusion, spontaneous development of AD-like skin lesions in NC/Nga mice was promoted by kanamycin treatment in infancy and was associated with a transient change in the numbers and the composition of intestinal microbiota. Hence we propose that disruption of indigenous microbiota by antibiotic use during infancy and childhood promotes the development of atopic diseases in high-risk subjects.

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