

Reduced airway microbiota diversity is associated with elevated allergic respiratory inflammation

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ABSTRACT

Background: An increased prevalence of allergic disorders in developed countries has been associated with decreased exposure to environmental micro-organisms and an alteration of microbiota colonization. An appropriate model is needed to investigate the mechanisms by which hygiene environment-driven changes in microbiota could regulate allergic disorders.

Objective: To discover the correlation between the higher incidence and severity of allergies with the relative hygiene environment in a developed country.

Methods: Allergic respiratory inflammation was induced in specific pathogen-free and control rats by sensitization and challenge with ovalbumin. The diversity of lower airway bacteria community was analyzed by polymerase chain reaction denaturing gradient gel electrophoresis and sequencing before ovalbumin sensitization. Allergic respiratory inflammation resulting in cellular infiltrate was measured after the last challenge.

Results: The diversity of microbiota in the airway of specific pathogen-free rats decreased compared with the control rats; the more frequent microbiota in the control rats were Proteobacteria and Bacteroidetes. In addition, increased nasal rubbing and sneezing combined with exaggerated IgE production and leukocyte number was observed in ovalbumin-treated specific pathogen-free rats.

Conclusion: These data indicate that the excessive “hygienic” environment resulted in a decreased bacterial diversity in the airway during infancy, leading to an increased susceptibility to allergic disease.

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Introduction

Asthma is a chronic respiratory disease mainly caused by the interaction between genetic and environmental factors. It is usually characterized by airway hyperreactivity and airway remodeling.¹ Asthma was considered an immune system-related disease. Dysregulation of immune tolerance at a young age could affect allergen sensitivity and lead to the development of allergic diseases such as allergic rhinitis and asthma.² Epidemiologic studies have indicated that asthma affects more than 300 million people throughout the world, and its prevalence has been increasing

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during the past 2 decades.³ The use of inhaled corticosteroid combined with a long-acting β_2 -agonist is effective to alleviate the symptoms of asthma, but the incidence of asthma is still increasing, thus leading to a heavy burden on society.⁴

Large variations in the worldwide prevalence of asthma have been found in developed and developing countries. The global incidence of asthma in Scotland, Wales, England, and the United States was documented as higher than 10%, whereas the incidence in India, Nepal, Indonesia, and China was lower than 5%.^{5,6} This phenomenon could be explained by the “hygiene hypothesis.” It postulates that children living in a “hygiene environment,” which means too much cleanliness with a lack of exposure to microbes especially pathogens, are sensitive to allergens.^{7–9} Subsequent studies showed that neonatal microbial colonization plays a larger role than special pathogen infection in asthma, which supports the “maturing hygiene hypothesis.” The maturing hygiene hypothesis postulates that the dysbiosis of commensal microbiota plays a central role in the development of allergic diseases, which occurs in an environment without micro-organisms.¹⁰

To better understand this relation between microbiota and asthma and mechanistically study the impact of microbiota on the allergic response in relation to the hygiene hypothesis, a dysbiosis model induced by a “hygienic environment” should be established. Researchers have used germ-free (GF) mice to study sensitivity to allergens in mice without bacteria.¹¹ Although GF mice were a good model to study the function of microbiota on their host, this entirely sterile environment cannot simulate the hygienic environment in developed countries. To the authors’ knowledge, specific pathogen-free (SPF) rats are kept in an SPF condition under a strict managing system, and this relatively clean environment involves smaller amounts of bacteria than a normal environment. Therefore, the authors considered SPF rats might better simulate people living in more hygienic Western environments.

Increasing evidence has shown that the potential mechanism by which commensal bacteria modulate the immune system is through regulating the formation of immune tolerance during childhood.^{2,12} Immune tolerance is a protective immunity reaction to allergic disease. The balance of archetypal T-helper cell type 1 cytokine interferon- γ and prototypic T-helper cell type 2 cytokine interleukin-4 is important for maintaining such protective immunity. Recently, the importance of the T-regulatory cell population in modulating allergic inflammation has been demonstrated.¹³ Investigations of the relation between commensal bacteria and allergic disorders in addition to their possible immune tolerance mechanisms have focused mostly on the intestine in the past decade, although the effect of intestinal microbiota on respiratory inflammation was indirect.¹⁴ Compared with gut microbiota, the study trajectory of respiratory microbiota is slightly behind, which is due in part to the idea that the lower airway is sterile. Several studies have reported recently that bacteria are present in the lower respiratory tract, and a dysbiosis of airway microbiota has been observed in asthma.^{15,16} These findings suggest airway microbiota might be important in shaping the airway immune response.¹⁷

An allergic airway inflammation model in SPF rats was used to simulate the hygienic environment in developed countries; specifically, the authors focused on the airway microbiota and explored the influence and mechanism of hygienic environment-driven changes in microbiota on allergic respiratory inflammation. Differences in airway microbes, asthma symptoms, and inflammation caused by pulmonary cellular infiltrates were measured. The present research sought to interpret the phenomenon of a higher incidence of asthma in a relatively hygienic environment in developed countries and thus to develop novel strategies aimed at preventing and treating allergic disorders.

Glossary of Terms

Specific pathogen free (SPF): A term describing laboratory animals that are guaranteed free of particular pathogens.

Germ free (GF): A term describing animals that have no microorganisms living in or on them.

Dysbiosis: A microbial imbalance on or inside the body. Broadly defined, dysbiosis is any change to the composition of resident commensal communities relative to the community found in healthy individuals.

Methods

Animals

Three-week-old SPF Wistar rats were provided by the Experimental Animal Center of Dalian Medical University (Dalian, China) and maintained under SPF conditions with sterile food and water in individual ventilated cages. Three-week-old control Wistar rats were maintained under open conditions, that is, they were kept in

Plexiglas cages in a common laboratory with free access to water and food. All experiments were performed with approval from the animal ethics committees of Dalian Medical University.

Protocol for Sensitization and Challenge

The SPF and control groups were sensitized by a subcutaneous injection of 10 μ g of ovalbumin (OVA) and 10 mg of aluminum hydroxide on days 21 and 28. On days 35, 36, and 37, the rats were challenged with 0.1% OVA in an aerosol of phosphate buffered saline delivered using an ultrasonic nebulizer device (402B; Yu Yue, Nan Jing, China) for 30 minutes daily. On day 21, 3-week-old rats randomly selected from the 2 groups were sacrificed to obtain bronchoalveolar lavage fluid (BALF). On days 35, 36, and 37, the frequency of nasal rubbing and sneezing was observed. On day 38, 24 hours after the last OVA challenge, the animals were sacrificed with an intraperitoneal injection of 10% chloral hydrate, and BALF, lung tissue, and peripheral blood were obtained.

Analysis of Microbial Community in Lower Respiratory Tracts of 3-Week-Old Rats before Sensitization

Total bacterial DNA was extracted by the QIAGEN DNA mini-kit (QIAGEN, Hilden, Germany). The V3 region of the total bacterial 16S rRNA gene was amplified using primers GC338F and 518R in a polymerase chain reaction (PCR) mixture. PCR was performed using the following parameters: 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, and then an extension step at 72°C for 7 minutes.

The PCR products were analyzed on 8% polyacrylamide gel with a 25% to 55% denaturing gradient using a DCodeTM gel electrophoresis system (Bio-Rad, Hercules, California). Bands were excised from denaturing gradient gel electrophoresis (DGGE) and sequencing was performed (Invitrogen, Beijing, China). The 16S rRNA sequences of airway microbiota were performed for the BLAST search in the National Center for Biotechnology Information database to obtain the most similar species.

Assessment of Allergic Airway Inflammation in Sensitized Rats

To assess the severity of allergic respiratory inflammation, nasal rubbing and sneezing, IgE in serum, infiltrating lymphocytes, and eosinophils were measured. After every inhalation of OVA dissolved in phosphate buffered saline solution (0.1%), the frequency of nasal rubbing and sneezing was counted for 15 minutes by an observer for 3 consecutive days. Concentrations of total IgE in serum were measured by an enzyme-linked immunosorbent assay according to the manufacturer’s instructions (USCN Life Science, Inc, Hubei, China). The number of lymphocytes in blood was estimated immediately using automatic cell analyzer. Some lung tissues were removed immediately and fixed in 4% neutral buffered formalin and embedded in paraffin. Sections with a thickness of 5 μ m were stained with hematoxylin and eosin to observe inflammatory cell infiltration in the lung tissue. Total RNA was extracted from other parts of the lungs using RNAiso Plus (Takara Bio Inc, Shiga, Japan) to analyze the expression of eotaxin (forward 5'-CACITCTATTCTGCTGCTC-3', reverse 5'-CCCAGCTTGGTCTTGAAGACT-3') by real-time reverse transcription PCR.

Statistical Analysis

Data are expressed as mean $\pm$ SD, and group comparisons of airway microbiota colonization, total number of leukocytes in BALF, eotaxin level in lung tissue, frequency of nasal rubbing, number of sneezes, and total IgE levels in serum were statistically analyzed using independent-samples *t* test (SPSS 19.0; SPSS, Inc, Chicago, Illinois). A *P* value less than .05 was considered statistically significant. Diagrams were made with GraphPad Prism 5.0 (GraphPad,

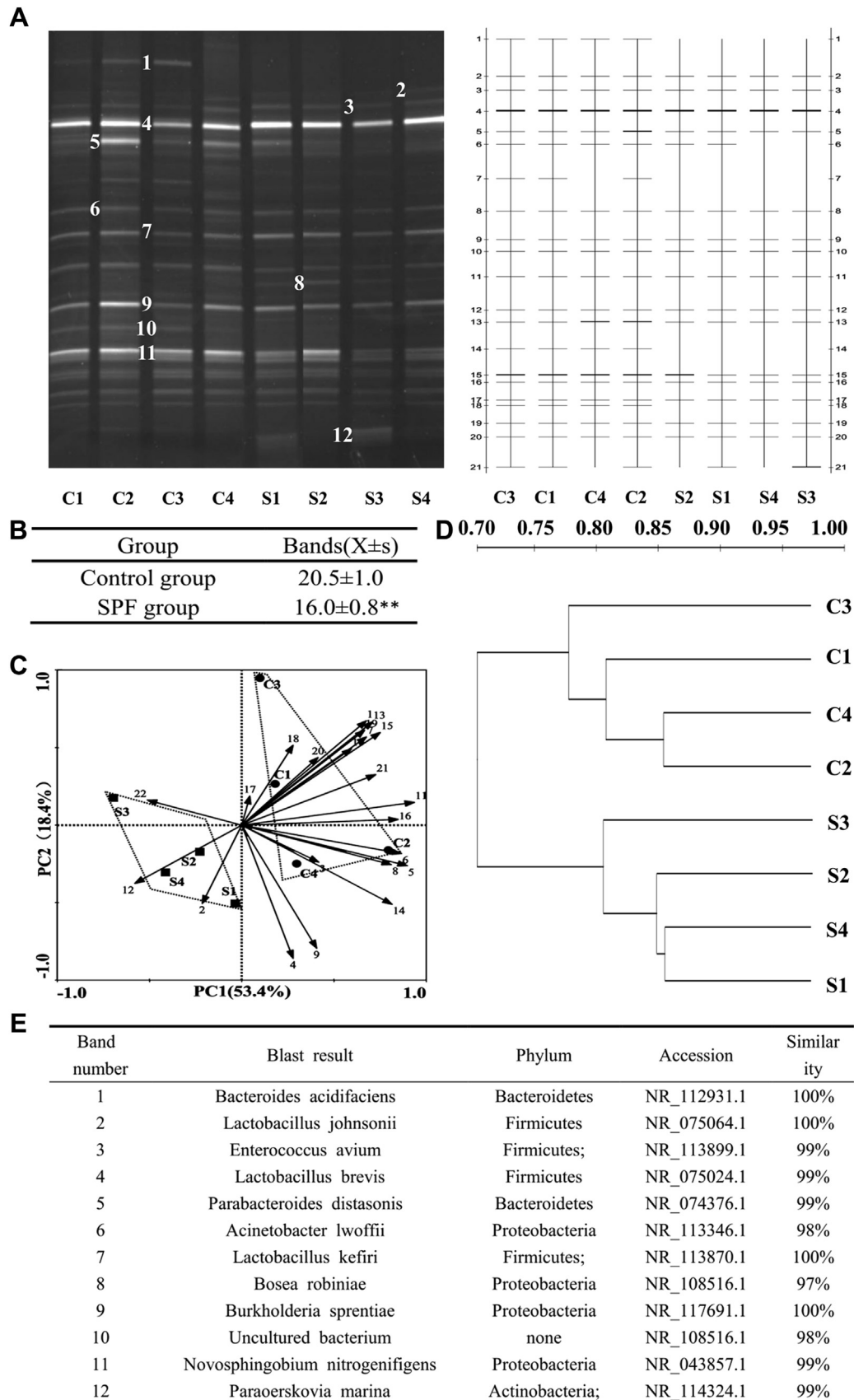


Figure 1. Diversity of the airway microbiota was decreased in a specific pathogen-free (SPF) environment. Microbiota in bronchoalveolar lavage fluid was analyzed by polymerase chain reaction denaturing gradient gel electrophoresis. (A) The denaturing gradient gel electrophoresis profiles and line diagrams of samples from the control and SPF group were compared. (B) The band number of each group is depicted and data are expressed as mean $\pm$ SD (** $P < .01$ compared with control group). (C) Principal component analysis. (D) Clustering dendrogram using the unweighted pair group method with arithmetic mean. (E) Sequence analysis of polymerase chain reaction amplicon isolated from denaturing gradient gel electrophoresis. SPF group, S1–S4; control group, C1–C4.

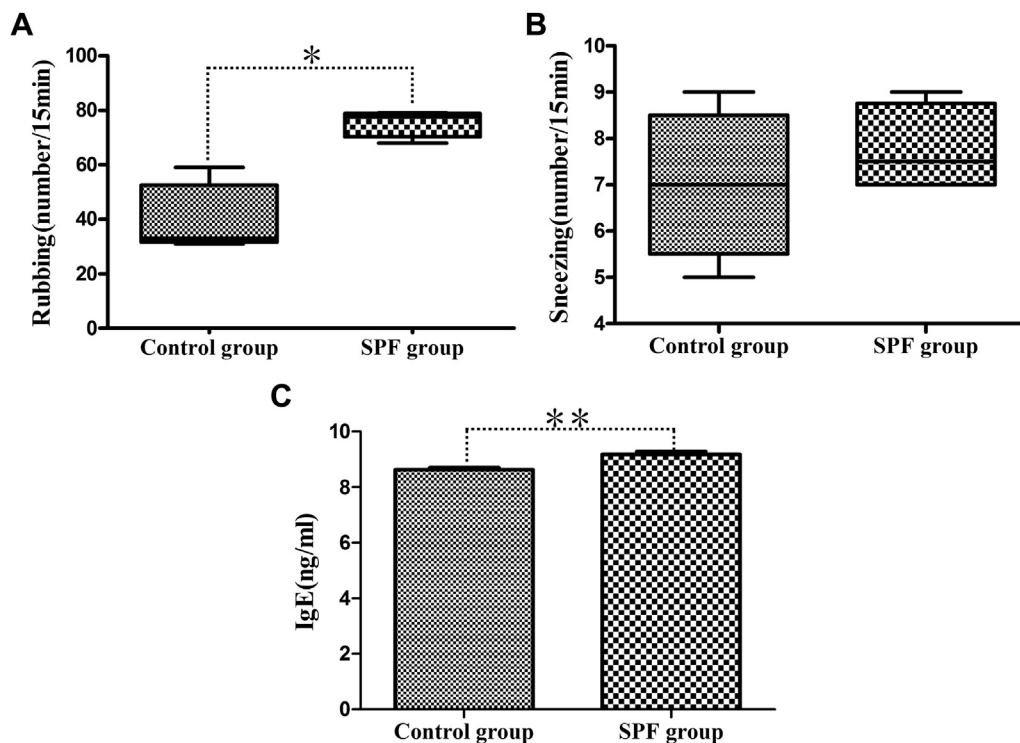


Figure 2. Ovalbumin-induced asthmatic symptoms were increased in a specific pathogen-free (SPF) environment. (A) The frequency of nasal rubbing and (B) the number of sneezes in the control and SPF groups were measured for 15 minutes after every inhalation of ovalbumin. (C) Total IgE levels in serum were measured by enzyme-linked immunosorbent assay. Data are expressed as mean $\pm$ SD (* P < .05, ** P < .01 compared with control group).

La Jolla, California). The DGGE images were observed by the Universal Hood II (UVItex Limited, Cambridge, United Kingdom) and then identified and analyzed by Quantity One 4.6.0 software (Bio-Rad). Principal component analysis of the DGGE bands was performed with Canoco 4.5 (Canoco; <http://canoco.software.informer.com/4.5/>).

Results

Changes of Airway Microbiota Colonization in OVA-Treated SPF and Control Rats

The PCR-DGGE analyses were performed to evaluate the diversity of airway microbiota in BALF. The DGGE profiles showed a variance band number in samples from the SPF and control groups (Fig 1A), with a mean of 16.0 ± 0.8 in the SPF group vs 20.5 ± 1.0 in the control group (P < .01; Fig 1B). These data suggested a lesser diversity of airway microbiota colonization in the SPF group compared with the control group.

The DGGE patterns of the airway microbiota in BALF were measured using analysis with the unweighted pair group method with arithmetic mean, which showed 2 main clusters. One cluster was formed by S1, S2, S3, and S4 (SPF cluster) and the other was generated by C1, C2, C3, and C4 (control cluster; Fig 1D). Principal component analysis of airway microbiota for the SPF and control groups is showed in Figure 1C; the first and second principal components explained 53.4% (PC1) and 18.4% (PC2) of the variance, and statistical analysis of the samples score for each principal component analysis axis showed a significant separation of the SPF and control groups along PC1. Twelve bands of interest were excised from DGGE gels for sequencing, and the BLAST search in the National Center for Biotechnology Information database was performed for 16S rRNA sequences of airway microbiota. All selected bands contained 4 phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Fig 1E). These data demonstrated that airway

microbiota colonization in the SPF group was distinct from the control group.

Regulation of Allergic Airway Inflammation by Airway Microbiota Colonization

To study the effect of airway microbiota colonization on the development of allergic airway inflammation, an OVA-specific T-helper cell type 2 inflammation was induced in the SPF and control rats, after which nasal rubbing and sneezing were observed. The average frequency of nasal rubbing (Fig 2A) and the number of sneezes (Fig 2B) in the SPF group were 75.5 and 7.75, which were increased compared with those in the negative control group (P < .05). Total IgE levels in the serum of SPF rats after the OVA inhalation challenge measured by enzyme-linked immunosorbent assay was significantly increased compared with the control rats (P < .01; Fig 2C).

The lungs of OVA-treated SPF rats and control rats were examined by histopathology and quantitative PCR to confirm the alteration of allergic inflammation. An increased number of cell infiltrates, increased epithelial thickness (Fig 3A), and an increased eotaxin level (Fig 3C) were observed in the SPF group compared with the control group. To establish the changes from the allergic response, BALF was evaluated for leukocyte accumulation, and the total number of leukocytes was significantly increased in the SPF group (P < .05; Fig 3B). All these results indicated more intense inflammation in the OVA-treated SPF rats.

Discussion

Lack of microbial exposure has been considered a major characteristic of people who live in a hygienic environment. Importantly, it has been linked to the development of asthma and other allergic diseases.^{7,18,19} However, owing to the lack of suitable animal models, the mechanistic study of how the decreased exposures to infectious micro-organisms modulate allergic diseases has been

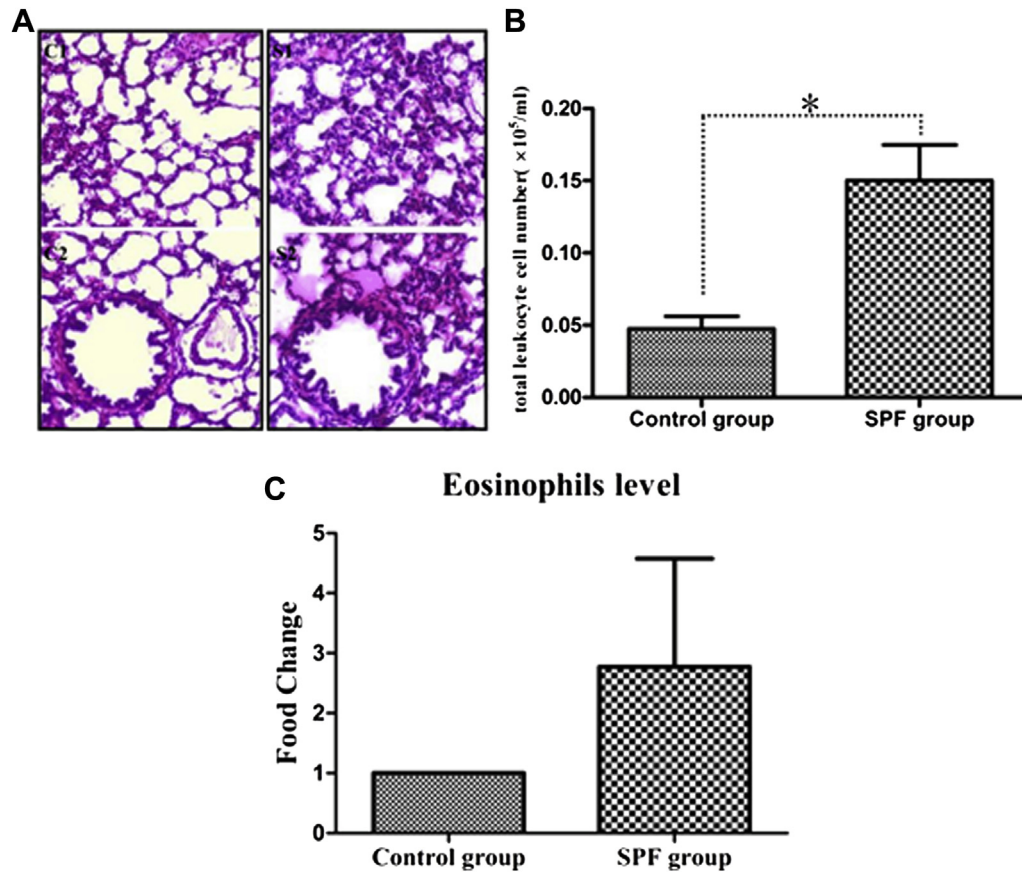


Figure 3. More intense respiratory inflammation was observed in ovalbumin-treated specific pathogen-free (SPF) rats. (A) Pathology analysis performed using common paraffin sections stained with hematoxylin and eosin. C1 and C2, control group; S1 and S2, SPF group. (B) Total leukocyte number in bronchoalveolar lavage fluid was estimated using an automatic cell analyzer. (C) Expression of eotaxin mRNA in lung tissue was analyzed by real-time quantitative polymerase chain reaction. Data are expressed as mean $\pm$ SD (* P < .05 compared with control group).

hampered. Herbst et al¹¹ used a GF model to demonstrate that the complete absence of microbial colonization affects susceptibility to allergic disease. It proved the hypothetical effect of commensal bacteria on allergic respiratory inflammation. The GF environment is distinct from a hygienic environment. There has been no good model to simulate a hygienic environment because many complex factors exist inside the environment. For the first time, the present study used an allergic respiratory inflammation rat model in an SPF environment, which could well simulate the Western hygienic environment and further elucidate the phenomenon of the high incidence of asthma in developed countries.²⁰

The DGGE results showed bacterial diversity in the airway was significantly decreased in the 3-week-old SPF rats compared with the control rats. The SPF environment likely had a significant impact on the colonization of commensal bacterium in the rat airway. Russell et al¹² reported that perinatal exposure to antibiotics profoundly altered gut microbiota, which was more serious than an SPF environment, and an SPF environment was considered closer to the hygienic environment of the modern industrial society. Thus, SPF rats can be used as a suitable model to assess the influence of decreased airway commensal bacteria caused by a hygienic environment on the susceptibility to allergic diseases. In the present study, the major microbial species in rat airways belonged to 4 phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria. Therefore, although some specific types were different, the phylum-level description of microbiota in the rat airway was similar to that in the human airway. The results showed that Proteobacteria and Bacteroidetes and some unknown bacteria were more frequent in the airway of control rats than of SPF rats,

which could help the development of immune tolerance and decrease sensitivity to asthma. The present results differ somewhat from those in adult humans, in which *Haemophilus*, *Moraxella*, and *Neisseria* species in the airways were very strongly associated with an increasing risk for allergic respiratory disease.¹⁵ This could be due to a special phase in the development of immune tolerance, so the timing and type of bacteria colonization could play different roles in the development of an allergic disorder.

The authors used SPF rats and normal rats to develop an OVA-induced asthma model, which exhibited increased serum IgE production combined with frequent nasal rubbing and sneezing that are hallmarks of allergic respiratory inflammation.²¹ Interestingly, the SPF rats, which had less bacterial diversity in the airway, showed significantly increased nasal symptoms after the OVA challenge compared with the control rats, suggesting that the decreased diversity of commensal bacteria in the airway increased sensitization to OVA. In addition, the SPF rats exhibited increased counts of lymphocytes in BALF and more abundant inflammation infiltration in the lung tissues. Together, these findings suggest that decreased flora diversity in the airway could promote allergic respiratory inflammation.

Some studies have reported that antibiotic use at a young age induces intestinal microbial dysbiosis and increased susceptibility to asthma.^{9,22,23} Those works showed that altering the natural colonization of gut microbes at a young age can have a long-term influence on the development of the immune system, and those changes were consistent with those in the present study. However, the intestinal microbiota shapes the immune respiratory response indirectly by cytokines and metabolites that are distributed

systemically. There is no research on the direct link between flora in the airway and immune development of the airway. The present study indicated that decreased commercial microbiota in the airway heightened the allergic respiratory response, which suggests the dysregulation can be reversed by supplementation with commensal microbes. It suggested that recolonization of normal bacteria, especially in newborns, is important for protecting against allergic disorders, which could be easier for children who have frequent contact with healthy farm animals.

In summary, the present study demonstrated that the excessive “hygienic” environment results in decreased bacterial diversity of the airway in infancy, which leads to an increased susceptibility to allergic diseases. In addition, this work applied a suitable model to explain the high prevalence of asthma in developed countries. It indicated that the possible therapeutic and preventive capacities of modulating commensal bacteria in the airway are of great importance for preventing asthma and other allergic diseases.

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