Microbial exposure early in life regulates airway inflammation in mice after infection with *Streptococcus pneumoniae* with enhancement of local resistance

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Yasuda Y, Matsumura Y, Kasahara K, Ouji N, Sugiura S, Mikasa K, Kita E. Microbial exposure early in life regulates airway inflammation in mice after infection with *Streptococcus pneumoniae* with enhancement of local resistance. *Am J Physiol Lung Cell Mol Physiol* 298: L67–L78, 2010. First published September 25, 2009; doi:10.1152/ajplung.00193.2009.—The immunological explanation for the “hygiene hypothesis” has been proposed to be induction of T helper 1 (Th1) responses by microbial products. However, the protective results of hygiene hypothesis-linked microbial exposures are currently shown to be unlikely to result from a Th1-skewed response. Until now, effect of microbial exposure early in life on airway innate resistance remained unclear. We examined the role of early life exposure to microbes in airway innate resistance to a respiratory pathogen. Specific pathogen-free weanling mice were nasally exposed to the mixture of microbial extracts or PBS (control) every other day for 28 days and intratracheally infected with *Streptococcus pneumoniae* 10 days after the last exposure. Exposure to microbial extracts facilitated colonization of aerobic gram-positive bacteria, anaerobic microorganisms, and *Lactobacillus* in the airway, compared with control exposure. In pneumococcal pneumonia, the exposure prolonged mouse survival days by suppressing bacterial growth and by retarding pneumococcal blood invasion, despite significantly low levels of leukocyte recruitment in the lung. Enhancement of airway resistance was associated with a significant decrease in production of leukocyte chemokine (KC) and TNFα, and suppression of matrix metalloproteinase (MMP-9) expression/activation with enhancement of tissue inhibitor of MMP (TIMP-3) activation. The exposure increased production of IFN-γ, IL-4, and monocyte chemoattractant-1 following infection. Furthermore, expression of Toll-like receptor 2, 4, and 9 was promoted by the exposure but no longer upregulated upon pneumococcal infection. Thus, we suggest that hygiene hypothesis is more important in regulating the PMN-dominant inflammatory response than in inducing a Th1-dominant response.

The prevalence of allergic diseases has currently risen in high-income countries (8). This phenomenon is thought to be associated with the modern westernized lifestyle, smaller families, and cleaner homes. The “hygiene hypothesis” (54) has indicated that the lack of intense infections in industrialized countries may induce inappropriate immune responses to innocuous substances including pathogenic microbes (63). However, there is little consistent evidence that these factors could account for the increase in atopic diseases.

Most clinical and epidemiological studies have provided strong evidence inversely linking the prevalence and severity of atopic disorders with early-life exposure to microbes in various settings (6, 9, 11, 38, 47). Limited exposure to microbes early in life does not induce a sufficient stimulation of Th helper type 1 (Th1) cells, whereas early life under an environment rich in a variety of microbes primes the immune system in the Th1 direction (34). Although Th1 and Th2 responses are counterregulatory (16, 43), asthmatic inflammation, characterized by an increase in Th2, is not necessarily associated with a concomitant reduction in Th1 responses. Furthermore, respiratory infections are potent triggers of asthma exacerbations, particularly in atopic asthmatics (7, 24, 41, 46).

In the airway, anatomical and physical factors such as mucociliary system and secretion of IgA play an important role in innate immunity. In addition, the transition from fetal to neonatal life includes the rapid colonization of microflora on skin and mucosal linings; the residential flora is beneficial to innate immunity by providing “colonization resistance” (58, 59). In this connection, weaning is considered to induce the change in the composition and density of flora in the gut and the airway, which may strengthen the mucosal immune system. In the airway, anatomical and physical factors such as mucociliary system and secretion of IgA play an important role in innate immunity. In addition, the transition from fetal to neonatal life includes the rapid colonization of microflora on skin and mucosal linings; the residential flora is beneficial to innate immunity by providing “colonization resistance” (58, 59). In this connection, weaning is considered to induce the change in the composition and density of flora in the gut and the airway, which may strengthen the mucosal immune system. In the airway, anatomical and physical factors such as mucociliary system and secretion of IgA play an important role in innate immunity. In addition, the transition from fetal to neonatal life includes the rapid colonization of microflora on skin and mucosal linings; the residential flora is beneficial to innate immunity by providing “colonization resistance” (58, 59). In this connection, weaning is considered to induce the change in the composition and density of flora in the gut and the airway, which may strengthen the mucosal immune system.
(TLR) 2 and 4. We propose that early-life microbial exposure in the airway may confer beneficial effect on local resistance by not only promoting microbial colonization but also preventing the excess of PMN-dominant response in association with the low responsiveness of TLRs, and regulation of tissue protease/protease inhibitor systems, while it does not induce Th1-skewed responses.

**MATERIALS AND METHODS**

**Mice**

Weanling C57BL/6 mice (specific pathogen-free 3-wk-old male) were purchased from Charles River Japan (Kanagawa, Japan). Immediately after mice were transported to our animal center, they were housed in sterilized polycarbonate boxes on sterilized PaperClean Bedding (Japan SLC) and given γ-irradiated sterile food (CE-7; CLEA Japan, Tokyo, Japan) and autoclaved water. The mice were housed four to five per cage and kept in a laminar flow rack (SK-Bio International) where HEPA-filtered air was supplied (flow rate 17 m/s). Room lights were on a 12-h light/dark cycle beginning at 6 a.m., and temperature and relative humidity were maintained between 21 and 24°C and 40–55%, respectively. For daily care and treatment, they were aseptically handled. All mice were kept under sterile conditions throughout experiments. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University School of Medicine.

**Fungi and Bacteria**

*Aspergillus fumigatus* [American Type Culture Collection (ATCC) 14109], *Penicillium marneffei* (ATCC 201013), *Cladosporium herbarum* (ATCC 201090), *Candida albicans* (ATCC 14053), *Staphylococcus epidermidis* (clinical isolate) and *Escherichia coli* (clinical isolate) were used for preparation of the mixture of microbial extracts (MME). In infection study, type 3. *pneumoniae* strain NMU112 (62) was used; the intratracheal LD50 for C57BL/6 mice was ~500 colony-forming units (cfu).

**Preparation of Microbial Extracts**

*C. albicans* was grown in Sabouraud liquid medium (BD Diagnostic Systems, Franklin Lakes, NJ) with rotation at 400 rpm at 28°C for 48 h. Yeast cells were harvested, washed by centrifugation, and suspended in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, pH 7.5, 1.5 mg/ml Zymolyase-20T (Seikagaku Biobusiness), and 0.5 mM PMSF (Wako Pure Chemical Industries, Osaka, Japan). The suspension was incubated at 37°C for 2 h and sonicated for 5 min at an output of 3.5 at 1-min intervals (Ultrasonic Disruptor UD 200, Tomy Seiko, Tokyo). The broken cells were centrifuged at 13,000 g for 30 min at 4°C, and the supernatant was collected, filtered through fluoplastic membranes (5 µm pore size) using a vacuum filtration system N1650 (Sysmatec, Eyholz, Switzerland), and extensively dialyzed against distilled water. The dialyzed extract was lyophilized and stored at −20°C.

*A. fumigatus*, *P. marneffei*, and *C. herbarum* were cultured in Potato Dextrose broth (Difco Laboratories, Detroit MI) at 25°C for 6 days for *A. fumigatus* and *C. herbarum* and 8 days for *P. marneffei*, respectively. Fungal biomass was harvested by filtration and lyophilized (Nichionrika, Tokyo). The homogenate was used as the sample from the trachea and right bronchus was minced with sterile scissors and homogenized in 1 ml of sterile ADS with a sterile tissue homogenizer (Nichionrika, Tokyo). The broken mass suspension was sonicated for 5 min at an output of 4.0 at 30-s intervals. The broken mass suspension was centrifuged at 13,000 g for 15 min at 4°C, and the supernatant was collected, membrane-filtered, and concentrated using a dialysis membrane with 3.5-kDa molecular weight cutoff Slide-A-Lyzer Dialysis Cassette; Pierce Biotechnology, Rockford, IL) to achieve a 50-fold concentration. The dialyzed extract from each fungus was lyophilized and stored at −20°C.

*S. epidermidis* cells grown in tryptic soy broth (Difco) at 37°C overnight were suspended in digestion buffer (30% raffinose and 0.145 M NaCl in 0.05 M Tris, pH 7.5) supplemented with 100 µg of lyostaphin (Sigma-Aldrich) and 1 µg of DNase type I. The cell suspension was incubated for 1 h at 37°C in a shaking incubator at 24 rpm and then sonicated for 5 min at an output of 4.0 at 30-s intervals (Ultrasonic Disruptor UD 200). The sonicated suspension was centrifuged at 13,000 g for 20 min at 4°C, and the supernatant was collected and extensively dialyzed against distilled water. After the dialyzed filtrate was membrane-filtered, it was lyophilized and stored at −20°C.

Dry weight of each lyophilized extract was measured, and 300 µg of each extract was suspended in 1 ml of 10 mM PBS (pH 7.2). The 1-ml volume of each microbial suspension was mixed together to make a preparation of MME, which contained 50 µg/ml of each microbial extract.

**Sample Collection**

Feces were suspended (50 mg of sample/ml) in 1 ml of sterile anaerobic dilution solution (ADS: 5 mg/ml NaCl, 2 mg/ml glucose, and 0.3 mg/ml cysteine-HCl, pH 7.3) in a Gloveless Anaerobic Chamber (Wolf Laboratories, York, UK) with a gas mixture consisting of 5% CO2, 5% H2, and 90% N2. Sampling of microbes from the nasopharyngeal passage was performed according to the method of Heritage et al. (21). Mice were anesthetized by intraperitoneal injection of pentobarbital (Abbott Laboratories, North Chicago, IL) at 62.5 mg/kg of body weight. Tracheas were exposed by dissection and ligated with a 3.0 silk suture (Ethicon, Somerville, NJ), and PE-50 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ) was inserted via the oropharynx into the nasopharyngeal cavity. Contents of the nasopharyngeal passages from individual mice were washed out of the nares with 0.5 ml of sterile ADS. The nasopharyngeal passages were washed twice, and two washings (total vol: 0.7–0.9 ml) were used as the nasopharyngeal sample. Thereafter, the chests were opened, and the entire respiratory tree was rapidly removed and immediately immersed in ADS. The trachea and the right main bronchus were quickly excised and dissected free from adhering tissues. The whole organ comprising tracheal and right bronchus was minced with sterile scissors and homogenized in 1 ml of sterile ADS with a sterile tissue homogenizer (Nichionrika, Tokyo). The homogenate was used as the sample from the lower respiratory tract. These procedures for all sample preparations from the stool and the respiratory tracts were performed in the Anaerobic Gloveless Chamber. Sample collection for bacteriological assay was done before MME exposure (day 0) and 10 days after the final MME exposure (day 38).

For blood collection from infected mice, a small incision was performed on the tail vein after being wiped with 70% ethanol, and blood was collected in sterile sample tubes containing 10 µl of sterile EDTA solution. The 20- to 50-µl volume of blood sample was directly plated on blood agar for enumeration of pneumococcal numbers.

**Bacteriological Analyses**

All samples were serially diluted down to 10−6−10−10 with ADS and were plated in duplicate on universal and differential media for the growth of anaerobes and aerobes. Anaerobic cultures were grown...
in the anaerobic box, and colonies were counted after incubation at 37°C for 48 h (aerobes) and 96 h (anaerobes). Media used for bacterial isolation were as follows: brain heart infusion agar (Difco), blood agar, Endo agar (BD), Leeds acinetobacter medium (23), mannitol salt agar (BD), Columbia agar (Difco) with 5% horse blood containing 10 µg/ml of colistin and 5 µg/ml of oxolinic acid, Columbia agar with 5% horse blood, colistin and nalidixic acid, Columbia agar supplemented with glucose, cysteine hydrochloride, neomycin sulfate, propionic acid, lithium chloride, paromycin sulfate (52), Bacteroides bile esculin agar (BD), Tinsdale agar with Supplement SR0065 (Oxoid Kantoukagaku, Tokyo), fastidious anaerobe agar (FAA; Lab M, Lancashire, UK) with 5% sheep blood, Schaedler K-V agar with 5% sheep blood (BD), Rogosa agar (Oxoid), and Sabouraud dextrose agar (Oxoid) with penicillin (50,000 U/l) and streptomycin (40,000 U/l). After counting, colonies were picked and identified by biochemical analysis, morphological appearance, and gram staining; the counts of clostridia were estimated on FAA after ethanol treatment (33). The microorganisms were identified primarily at the genus level, and some were at the species level. For identification, several Api kits (bioMerieux Japan, Tokyo) were used: Api 20A for obligatory anaerobes, Api 20 Strep for facultatively anaerobic Streptococcus, Api Staph for Staphylococcus, Api 50CH and Api Coryne for facultatively anaerobic gram-positive rods, Api 20E for facultatively anaerobic and aerobic gram-negative rods, and API 20C AUX for yeast form fungi.

**Exposure to MME**

A group of mice was intranasally inoculated with 50 µl of MME containing 2.5 µg of each microbial extract (defined as MME group) or the same volume of sterile PBS (control group) once a day, starting from day 0 to day 28 every other day (Fig. 1). These two groups were used for flora assay before exposure and infection (4–6 mice/group, respectively), determination of survival rate (10 mice/group), and postinfection analyses (15–30 mice/group) at 10 days after the final exposure to MME or PBS. The same experiments were repeated two or three times.

**Infection with S. pneumoniae**

Pneumococcal pneumonia was induced by intratracheal inoculation of type 3 S. pneumoniae strain NMU112 in a 20-µl volume (5 x 10⁴ cfu, equivalent to 100 LD₅₀) as previously described (62). The exact inoculated dose was verified by plating out 500–1,000-fold dilutions onto blood agar plates. Survival of mice was determined during a week follow up.

**Bronchoalveolar Lavage**

Mice were anaesthetized with intraperitoneal injection of pentobarbital and then killed by extracting the blood from the heart. To collect bronchoalveolar lavage fluid (BALF), volumes (1 ml) of PBS were infused into the exposed lungs and withdrawn three times for a total infusion of 3 ml, with the average of 2.5 ml recovered. Collected BALFs were combined as a BALF sample, and cellular components were sedimented by centrifugation at 400 g for 10 min. Cell-free BALF samples were frozen at −80°C for subsequent assays after the volume and total protein of each sample were measured. Protein content was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). The sedimented cells were resuspended in a volume of 0.5 ml (0.4 ml PBS and 0.1 ml 0.4% Trypan blue), and total infiltrating cells were counted in a hemocytometer. For cytological examination, 500 cells were counted to obtain differential staining with May-Grünwald Giemsa stain. Lungs were either processed for histological examination or immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction or preparation of tissue extract.

**Cytokines and Chemokines**

The production of TNFα, IL-1β, IL-4, IL-10, IFN-γ, transforming growth factor (TGF)-β1, keratinocyte-derived chemokine (KC), and monocyte chemotactic protein (MCP)-1 in BALF samples was measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s recommendations.

**RT-PCR**

The frozen lungs were crushed using a sterile, nitrogen-cooled homogenizer (62). Total RNA was extracted using TRIZol (Life Technologies, Gaithersburg, MD), and 1 µg of total RNA was transcribed to cDNA by using the Preamplification system (Life Technologies) and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Cytokine- and chemokine-specific mRNA accumulation was estimated by PCR on a Perkin-Elmer Gene Amp 2400 (Perkin-Elmer Cetus, Norwalk, CT). A 1/10 dilution of cDNA was incubated with 1.25 µl of each sense primer in a total reaction volume of 50 µl. PCR was performed for 1 min at 95°C at 57–60°C, 30 s at 72°C for 30–35 cycles (depending on the target genes to be amplified), followed by one cycle of 7 min at 72°C. PCR products were assessed on 2% agarose gel, and the products were densitometrically analyzed with NIH image v1.62 software to quantitate their intensities. The expression level of cytokine mRNA was expressed as the ratio of intensity of cytokine bands to that of GAPDH bands of the same cDNA sample (62). Primer sets for IL-1β, IL-10, TNFα, IFN-γ, and IFN-β1 were purchased from Clontech (Palo Alto, CA) and IL-4 from Stratagene (LaJolla, CA), respectively. Primer sets for chemokines and GAPDH, synthesized according to the published sequences (17, 29), were as follows: KC (product size 354 bp), sense: 5'-CACCTCAGAAGACATCT-CAAGA TGCTG-3', and antisense: 5'-CAAGCAACTGAATTACCATCA AGCGG-3'; MCP-1 (411 bp), sense: 5'-TCCAGCTTCGCTGATTTGCT-3', and antisense: 5'-AAAGGCTGTTAGGTGTTTGGAA-3'; and GAPDH (451 bp), sense: 5'-ACCAGACCCCTGAGCCATAAC-3', and antisense: 5'-TCCACACCACTGTGCTGTA-3'. When tissue inhibitor of metalloprotease (TIMP)-3 mRNA was assessed, the primer sets were as follows (60): sense: 5'-CTG GCT TGG TCTG CGT GCTT-3'; antisense: 5'-TGG TCTG GCT TGG TCTG CGT C-3'. Primer sets for cytokines and chemokines were purchased from R&D Systems, Minneapolis, MN, in accordance with the manufacturer's recommendations.

**Fig. 1. Protocol of mouse exposure to mixture of microbial extracts (MME).** Weanling C57BL/6 mice were kept under sterile conditions and fed a sterilized diet and water after arriving at our animal center. A group of mice was intranasally inoculated once a day with 50 µl of MME containing 2.5 µg of each microbial extract (MME group) or 50 µl of sterile PBS (control group) every other day over a period of 28 days. These 2 groups were used for infection study or other analyses 10 days (day 28) after the final exposure. The number of mice in each group varied with experiments, ranging from 4 to 20 mice.
CCT GA-3', antisense: 5'-GGG AAG GAG GTG AGG TGG GGC AGG TC-3' (product size: 659 bp). The cycling conditions were one cycle at 95°C for 5 min; the reactions were cycled 30 cycles of 94°C for 15 s, followed by 72°C for 15 s, 72°C for 30 s, and 1 cycle at 4°C to end. Primer sets for mouse TLR2, 4, and 9 were purchased from InvivoGen (San Diego, CA), and PCR was performed following the recommendations exactly as given by the manufacturer.

**Assay for Matrix Metalloproteinase and TIMP**

The frozen lung was crushed and homogenized using a sterile, nitrogen-cooled homogenizer in 10 volumes (volume to weight) of a buffer containing 50 mM Tris·HCl, pH 7.5, 1% Triton X-100, and protease inhibitors (2 mg/ml leupeptin, 1 mg/ml pepstatin, and 100 mM PMSF). Samples were centrifuged at 800 g, and the pellet was then extracted with 5 volumes of a buffer containing 50 mM Tris·HCl, pH 7.5, 200 mM NaCl, 1% SDS, and protease inhibitors. Following centrifugation at 16,000 g, protein content of the supernatant was determined using BCA protein assay kits. Equal amounts of protein (50 μg each/5 μl) were mixed 1:1 with nonreducing SDS loading buffer (final concentration: 50 mM Tris protein (50 m), and the pellet was then extracted with 5 volumes of a buffer containing 50 mM Tris·HCl, pH 7.5, 200 mM NaCl, 1% SDS, and protease inhibitors. Following centrifugation at 16,000 g, protein content of the supernatant was determined using BCA protein assay kits. Equal amounts of protein (50 μg each/5 μl) were mixed 1:1 with nonreducing SDS loading buffer (final concentration: 50 mM Tris·HCl, pH 7.5, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue) and loaded on a 10% polyacrylamide gel with 1 mg/ml gelatin for zymography or a 10% polyacrylamide gel with 1 mg/ml gelatin plus 5 mg of recombining mouse MMP-9 (AnaSpec, San Jose, CA) for reverse zymography (42). The MMP-specific gelatinolytic activity on zymogram was confirmed by incubation of a sample lane in 10 μg GM6001 (Chemicon International, Temecula, CA). To identify each of TIMP-1, -2, and -3 on reverse zymography, purified mouse TIMP-1 (R&D Systems, Minneapolis, MN) and TIMP-2 and -3 (Cederlane Laboratories, Ontario, Canada) were used in each gel. After electrophoresis, gels were treated as described previously (18, 30); gels were washed in 2.5% Triton X-100 and incubated for 16 h at 37°C before being stained with 0.5% (wt/vol) Coomassie R-250. After staining, gels were destained with the destaining solution (methanol:acetic acid:water = 50:10:40). Areas of MMP protease activity appeared as clear bands against a dark blue background, whereas the inhibitory activity of TIMPs showed in a dark blue band on a clear background. Density of the dark blue was measured using Gel Pro Analysis software (Media Cybernetics, Silver Spring, MD). The sum of optical densities of the band was reported as relative density units.

The concentration of active MMP-9 and MMP-2 in BALF was analyzed using Amersham Matrix Metalloproteinase Biotrak Activity Assay System (GE Healthcare Life Science, Buckinghamshire, UK) (56) following the manufacturer’s instructions. These kits measure total activity of each MMP (zymogens and active forms) following activation of individual pro-forms.

**Western Blot Assay**

Protein levels of MMP-9 and TLR2, 4, and 9 in lung extract were assessed by Western blot. A 100-μg protein extract from frozen lungs was separated on a 10% SDS-PAGE gel under reducing or nonreducing conditions and then transferred to a nitrocellulose membrane. The membranes were blocked with 10% milk in TBST (10 mM Tris, 0.15 M NaCl, 0.1% Tween 20), followed by probing with goat anti-mouse MMP-9 antibodies (Ab) (GT15020, 1:5,000; Neuromics Antibodies, Edina, MN), goat anti-mouse TLR2 Ab (D-17, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse TLR4 Ab (M-16, 1:500; Santa Cruz), or rabbit anti-mouse TLR9 Ab (H-100, 1:250; Santa Cruz) at 4°C for 16 h. Secondary antibodies were donkey anti-goat IgG-HRP (sc-2020, 1:5,000; Santa Cruz) for MMP-9, TLR2, and TLR4 Ab, and goat anti-rabbit IgG-HRP (sc-2004, 1:5,000; Santa Cruz) for TLR9 Ab. Detection was performed using the ECL chemiluminescence kit (GE Healthcare). To demonstrate changes in MMP-9 protein levels in lung tissue, actin was also probed using sheep anti-actin antibody (1.0 μg/ml, R&D Systems) and 1:2,000 dilution of HRP-donkey anti-sheep Ig (R&D Systems). Signals of MMP-9 were normalized to the corresponding actin signal: the increase in MMP-9 protein levels after infection, defined as % change, was determined by densitometry scanning (62) comparing to the levels at time 0.

**Assay for Leukocyte MPO and Elastase**

Levels of PMN accumulation in lungs were determined by measuring the MPO activity in the extract of frozen lungs. MPO activity was measured using MPO assay kit (Cytostore, Alberta, Canada) following the manufacturer’s instructions as described previously (62). The MPO activity was expressed as U/mg tissue protein. Leukocyte elastase activity in samples of cell-free BALF was determined using EnzChek Elastase Assay Kit (Life Technologies Japan, Tokyo) according to the manufacturer’s instructions. The samples were ultra-centrifuged to concentrate their protein content to 200 μg/ml before assay. The elastase activity was expressed as U/ml of BALF.

**Measurement of IgA in the Airway Tract**

The cell-free BALF samples were prepared from five mice from the MME-treated group and the control group before infection (day 38). Protein content of individual samples was quantified using a BCA protein assay kit, and all samples were concentrated to a protein concentration of 200 μg/ml by ultracentrifugation. Total IgA in BALF samples was quantified using a mouse IgA ELISA Quantitation kit (GenWay Biotech, San Diego, CA) following the manufacturer’s instructions. The same experiment was repeated three times.

**Statistical Analysis**

All data are presented as means ± SD. Differences in data of cytokine and chemokine quantity, MPO and elastase activity, tissue enzyme activity (MMP/TIMP), and Western immunoblotting between groups were evaluated using ANOVA. Mann-Whitney U-tests with the Bonferroni correction was used to compare percent values of quantitative bacterial cultures before and after MME exposure, and also percent values of infiltrating cell population between groups at each time point. For comparison of survival curves, Kaplan-Meier analysis with a log-rank test was used. In all analyses, P values <0.05 considered to be statistically significant.

**RESULTS**

**Effect of MME Exposure on Microbial Flora**

MME exposure did not change either population or density of bacteria in the intestinal flora to a significant extent, compared with control exposure (Supplemental data for this article is available online at the AJPLung web site), although bacteria belonging to the altered Schaedler flora were not examined. On the other hand, nasal exposure to MME significantly increased the number of total bacteria in the nasopharyngeal tract (P < 0.05 vs. control) (Table 1); especially, cfu numbers of aerobic gram-positive bacteria (P < 0.05), strict anaerobic bacteria (P < 0.05), and facultative anaerobic *Lactobacillus* (P < 0.01) increased in this area. The latter two bacterial groups also increased in the lower respiratory tract (trachea and bronchia) (P < 0.05 vs. control) after MME exposure. The number of bacteria obtained in the bacterial groups was used to calculate the relative frequency of each of them and is shown as percentage values (Fig. 2). Among strict anaerobic bacteria, *Peptostreptococcus*, *Peptococcus*, and *Veillonella* were predominant in the nasopharyngeal and lower respiratory tracts of both mouse groups, whereas the relative frequency of these bacteria slightly increased, although not at a significant extent, after MME exposure (Fig. 2). *Clostridium* and *Bacteroides*.
were present only in the nasopharyngeal tract of both groups, and the frequency of these two bacterial groups was not changed by MME exposure. In contrast, the relative frequency of facultative anaerobic *Lactobacillus* in the nasopharyngeal and lower respiratory tracts increased approximately twofold after MME exposure (\(P < 0.05\) vs. control). The \(\alpha\)-hemolytic *Streptococcus* represented the dominant group of microorganisms from the nose to the lower respiratory tract of both groups, and its relative frequency slightly decreased, but not at a significant level, after MME exposure. *Staphylococcus* and *Micrococcus* represented the second dominant bacteria in the airway, but the frequency of these organisms did not change to a significant extent by MME exposure.

### Table 1. Quantification of the different groups of bacteria in the respiratory tract

<table>
<thead>
<tr>
<th></th>
<th>Before exposure (day 0)</th>
<th>PBS (control)</th>
<th>MME</th>
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<tbody>
<tr>
<td></td>
<td>Naso-pharyngeal tract</td>
<td>Lower respiratory tract</td>
<td>Naso-pharyngeal tract</td>
</tr>
<tr>
<td>Total cfu/organ</td>
<td>3.85±0.74</td>
<td>3.55±0.64</td>
<td>4.23±0.31</td>
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<tr>
<td>Gram positive</td>
<td>3.04±0.19</td>
<td>2.28±0.38</td>
<td>3.22±0.56</td>
</tr>
<tr>
<td>Gram negative</td>
<td>1.41±0.32</td>
<td>0.37±0.16</td>
<td>1.64±0.39</td>
</tr>
<tr>
<td>Strict anaerobe</td>
<td>2.27±0.36</td>
<td>1.04±0.32</td>
<td>2.93±0.54</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>1.04±0.26</td>
<td>1.08±0.22</td>
<td>1.18±0.22</td>
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The number of the different groups of bacteria in the nasopharyngeal tract and the lower respiratory tract was determined after mice were exposed to mixture of microbial extracts (MME) or PBS. Samples were obtained from mice (4–6 mice/group) before exposure (day 0) and 10 days after the final exposure (day 38); individual samples were processed and cultured in triplicate for determination of cfu numbers, and identification of bacteria was done as described in MATERIALS AND METHODS. Data represent means ± SD of 3 separate experiments. *\(P < 0.05\), †\(P < 0.01\) vs. control exposure.
days after infection, whereas 50% of MME-exposed animals survived at 5 days postinfection (Fig. 3A). The infection induced severe bronchopneumonia in control mice with evidence of PMN accumulation in the airways and interstitial spaces. Macroscopically, red congestion spreading over the lobe was noted between 24 and 36 h of infection (Fig. 3B); histologically, a marked infiltration of PMNs, erythrocytes, and fibrinous exudate were observed in the alveolar spaces at 36 h of infection. Control mice became bacteremic during the initial phase of infection (Fig. 3C). *S. pneumoniae* was recovered from the blood as early as 12 h after infection, followed by linear increase in the blood until death. By contrast, infection induced macroscopically moderate lesions in the lung of MME-exposed mice (Fig. 3B); congested lesions were observed only at the limited area of the lung, and histologically, low numbers of PMNs were noticed in the alveolar spaces at 36 h of infection. Furthermore, the exposed mice were not bacteremic until day 3 of infection (Fig. 3C); thereafter, cfu numbers gradually increased in the blood. The number of pneumococci rapidly increased in the lungs of control mice after 8 h of infection until death (Fig. 3D); cfu numbers in the lung were significantly greater in control mice than in MME-exposed mice from 8 h through 48 h of infection (at 8, 36, and 48 h; $P < 0.05$, at 12 and 24 h; $P < 0.025$). The activity of elastase released from leukocytes was also significantly greater in BALF of control mice at 12 h ($P < 0.05$) and 24 h ($P < 0.025$) postinfection compared with MME-exposed mice (Fig. 4C). In the lung, PMN infiltration was significantly greater in control mice than in MME-exposed mice after 12 h of infection (at 12, 24, 36, and 48 h; $P < 0.05$), as assessed by measuring MPO activity in lung tissue (Fig. 4D).

### Analyses of infiltrating cells and chemokines/cytokines in the airway.

The number of total infiltrating cells in BALF increased linearly during the first 24 h in mice of both groups and steadily declined in the following time course (Fig. 4A). At any time point, numbers of infiltrating cells in BALF were greater in control mice than in MME-exposed animals. Among the cells, PMNs were predominant in both groups at any time point (Fig. 4B); the ratio of PMN to mononuclear cell (monocytes + lymphocytes) was significantly higher in control mice than in MME-exposed mice from 8 h through 48 h of infection (at 8, 36, and 48 h; $P < 0.05$, at 12 and 24 h; $P < 0.025$).

### Fig. 3. Exposure to MME enhanced mouse resistance to intratracheal infection with *Streptococcus pneumoniae*.

Mice were infected intratracheally with $5 \times 10^4$ cfu of *S. pneumoniae* strain NMU112. A: Kaplan-Meier survival curves for the MME group and the control group are shown. Data are obtained from 3 separate experiments (10 mice/group in an experiment). $*$ $P < 0.05$ vs. control group. B: photographs of infected lungs are shown. Control mice developed severe pneumonia between 24 and 36 h of infection. In the lung of MME-exposed mice, moderately affected lesion was observed from 24 to 36 h of infection. Representative results obtained from 3 separate experiments with similar results are shown. Numbers of cfu in the blood (C), left lung (D), and BALF (E) were determined at indicated time points. At each time point, samples were obtained from 5 mice with 3 different measurements from each sample. Data are means ± SD of 3 separate experiments. In D, $*P < 0.005$; in E, $*P < 0.01$ vs. control mice.
In parallel to such differences in PMN response, ELISA levels of KC and TNF were significantly lower in BALF of MME-exposed mice at 12 and 24 h postinfection ($P < 0.05$ vs. control mice) (Fig. 5A). In contrast, levels of IFN-$\gamma$, IL-4, and MCP-1 were significantly higher in the MME group at 24 h postinfection ($P < 0.05$ vs. control group), although only MCP-1 levels were significantly higher in the treated group at 12 h of infection ($P < 0.05$ vs. control group). Quantity of IL-10 was at similar levels between both groups at these two time points. These findings were comparable to the expression level of mRNAs for respective cytokines/chemokines at 16 h postinfection (Fig. 5B). Both the message levels (at 16 h of infection) in lung tissue and the protein levels (at 12 and 24 h) in BALF of IL-1$\beta$, GM-CSF, and TGF-$\beta$ remained at the same levels between the two groups (data not shown).

**MMP activity and TIMP-3 expression.** Since elevated MMP expression is thought to play an important role in regulating the lung inflammatory response to injury (3), we examined the expression level and the activity of MMP-2 and MMP-9 in the airway. Levels of MMP-2 in BALF did not change significantly at the investigated time points of pneumococcal infection in both groups (data not shown). On the other hand, the infection induced in BALF of control mice significantly high levels of MMP-9 production/release ($P < 0.05$ vs. MME-exposed mice) at 6 h postinfection, and thereafter, levels of the enzyme linearly increased until 36 h of infection (Fig. 6A). In control mice, the active form of the enzyme was detected in BALF by zymography at 6, 36, and 60 h of infection (Fig. 6A). After 36 h of infection, MMP-9 levels fell rapidly in control mice. In MME-exposed mice, levels of MMP-9 did not significantly change during the first 24 h of infection. Thereafter, MMP-9 concentrations gradually increased until 72 h of infection, although they were significantly lower in MME-exposed mice compared with control animals (from 12 to 48 h; $P < 0.01$). At 72 h postinfection, the time when pneumococcal bacteremia occurred for the first time in the exposed mice (Fig. 3C), the MMP-9 concentration in the exposed mice increased to the same levels as observed for control animals at 24 h of infection. The active form of the enzyme was detected in BALF of the exposed mice at 36 and 60 h of infection by zymography (Fig. 6A). These findings were in agreement with results obtained from Western blot assay (Fig. 6B): at 6 and 36 h postinfection, % change of MMP-9 expression, as determined by densitometry assay on immunoblot bands, was significantly lower in the MME group than in the control group (at 6 h, $P < 0.025$; at 36 h, $P < 0.01$) (Fig. 6C).

The infection induced TIMP-1, -2, and -3 in lungs as demonstrated by reverse zymography. Among TIMPs, TIMP-3 was most prominently expressed in both groups, and TIMP-3 activity was apparently greater in MME-exposed mice as early
as 6 h postinfection (Fig. 7A). When TIMP-3 activity on reverse zymography was analyzed by densitometry assay, TIMP-3 levels were significantly greater in MME-exposed mice than in control mice ($P < 0.05$ at 6, 24, and 48 h) (Fig. 7A).
7B). Consistent with the results from reverse zymography, mRNA expression for TIMP-3 was apparently increased in MME-exposed mice at 6, 12, and 24 h postinfection compared with that in control mice (Fig. 7C). Levels of TIMP-1 and -2, as assessed by densitometry assay of reverse zymography, were not significantly different between both groups of mice (data not shown).

TLRs play an important role in initiating host responses upon stimulation by a wide variety of pathogens. We examined whether MME exposure altered the expression of TLRs in lung tissue by RT-PCR. In this study, mRNAs for TLR2 and TLR4 were targeted, since pneumococcal cellular components activate these two receptors (32, 50). As shown in Fig. 8A, MME exposure induced high levels of mRNA expression for TLR2 and TLR4 before infection, whereas the expression of these receptors was apparently lower in control mice than in MME-exposed animals. Enhancement of these receptors was also observed at protein levels in the exposed mice (Fig. 8B). Unexpectedly, pneumococcal infection neither enhanced to a further extent nor lessened the mRNA expression for these receptors (at 8 h), whereas protein levels of the receptors were apparently decreased compared with their preinfection levels (at 12 h). These changes were true for the mRNA and protein of TLR9. In control mice, the infection apparently upregulated the expression of TLR2 and TLR4, but not TLR9, at both message and protein levels.

Total IgA in BALF. The total protein in BALF of individual mice was \(0.33 \pm 0.05\) mg/ml in the MME group and 0.24 \(\pm 0.03\) mg/ml in the control group. All BALF samples were concentrated to a protein concentration of 0.5 mg/ml by ultrafiltration before ELISA determination. Total IgA in BALF of MME-exposed mice was 3.84 \(\pm 0.42\) mg per mouse, which was significantly greater (\(P < 0.05\)) compared with that of control mice (1.68 \(\pm 0.23\) mg/mouse).

**DISCUSSION**

In this study, we investigated the effects of early microbial exposure on airway innate resistance to pneumococci. Our major observation was that nasal exposure to MME in weaning mice induces a significant impact on the control of PMN-dominant inflammatory response with regulation of MMP/TIMP systems upon pneumococcal infection, but does not affect airway innate resistance. The exposure facilitated microbial colonization and increased total IgA quantity in the airway, both of which contribute to innate resistance. Furthermore, production of both IFN-γ and IL-4 increased in the exposed mice after infection, indicating the absence of a Th1/Th2 imbalance. Interestingly, the exposure increased the expression of TLR2, 4, and 9 mRNA8s in the lung, whereas the
infection neither enhanced nor downregulated the expression of
their messages in the exposed mice.

The so-called hygiene hypothesis postulates that limited
exposure to microbes during early childhood results in an
insufficient stimulation of Th1 cells, which cannot counterbal-
ance the expansion of Th2 cells (34, 63). Exposure to environ-
mental microbial factors early in life is shown to have an
educating effect on the development of immune regulatory
functions (12). However, the effect of early microbial expo-
sure on airway innate resistance is not fully understood. In
this study, weanling mice were nasally exposed to MME
prepared from several microbes commonly found in the
house and human body. MME exposure facilitated coloni-
zation of several microbes in the respiratory tract; espe-
cially, aerobic gram-positive bacteria, strict anaerobic mi-
croorganisms, and facultative anaerobic Lactobacillus in-
creased in the respiratory tract. Such enhanced colonization
of microflora is thought to not only function as colonization
resistance (58, 59) but also dramatically reduce the inci-
dence of inflammatory diseases (26).

So far, it is not ascertained whether and how the host
distinguishes between commensal and pathogenic bacteria.
With regard to this issue, TLRs on epithelial cells and mono-
nuclear cells are known to play an important role in recogniz-
ing conserved molecular patterns expressed by pathogens and
initiate primary responses to invading microbes. In epithelial
cells of the gut, TLR3, 7, 8, and 9 appear to localize within
intracellular compartments (22), whereas TLR5 localizes to
basolateral membrane surfaces (31). TLR2, similar to TLR4, is
either absent or attenuated in intestinal epithelial cells (1, 35,
39). Such different localization of TLRs may, at least in part,
account for the hyporesponsiveness of the gut towards its
commensal microflora. MME exposure apparently enhanced
the expression of mRNAs for TLR2, 4, and 9 in the lung
compared with control exposure. Unexpectedly, the expression
of these receptors in MME-exposed mice was neither enhanced
to a further extent nor downregulated by pneumococcal infec-
tion, whereas their protein levels apparently decreased. This
fact appears to indicate that TLR2 and TLR4, which have
already been upregulated by microbial extracts, are of rela-
tively low responsiveness towards invading pneumococci, al-
though these receptors are responsible for recognition of pneu-
ococcal cellular components (32, 50). In a mouse model for
endotoxin tolerance and/or sepsis, mice pretreated with suble-
thal doses of LPS are refractory to subsequent stimulation of
inflammatory cytokine production (49, 61). In addition, re-
peated stimulation with microbial antigens through TLRs can
cause tolerance to signaling through TLR4 or other TLRs (13,
64). In this study, increase in microbial colonization in the
airway of MME-exposed mice was not consequent to the
enhanced expression of TLRs. Rather, it is assumed that
enhanced colonization or repeated MME stimulation resulted
in upregulation of TLRs; consequently, the upregulated TLRs
were less sensitive or tolerant to components of invading
pneumococci. Such tolerance or low responsiveness of TLRs,
therefore, seems to represent a mechanism by which acute
inflammatory response following infection was lessened in
MME-exposed mice. Since dendritic cells are deeply involved
in TLR tolerance (45), further investigation on phenotypic
changes in the cells during MME exposure should be consid-
ered to elucidate the exact mechanism.

PMN is a major factor in acute inflammatory response
against pyogenic cocci. A significant decrease in the expres-
sion/production of KC and TNFα in the lung of MME-exposed
mice reflected the low degree of PMN-dominant inflammatory
response. Despite such reduced accumulation of PMNs, airway
resistance to a relatively low-dose (5 × 10⁴ cfu) infection with
pneumococci in the exposed mice was not affected. Airway
resistance in C57BL/6 mice intratracheally infected with a low
dose (10⁴ cfu) of S. pneumoniae is dependent on alveolar
macrophage function and does not require PMN recruitment to
prevent development of pneumonia (14, 28), unlike a high-
dose (≥10⁷ cfu) infection characterized by extensive PMN
recruitment (25, 48). Importantly, the low magnitude of PMN-
dominant response in MME-exposed mice during the initial
phase of infection appeared to be consequent on the preexpos-
sure to MME, since bacterial burden in the airway remained at
similar levels between the two groups of mice at least until 12 h
postinfection. In control mice, infiltrating cell numbers, PMN/
mononuclear cell ratio, MPO activity, and ELISA levels of
TNFα and KC were all increased after 12 h of infection; such
enhanced responses are likely due to increasing bacterial bur-
den in the airway. In the exposed mice, all these parameters
were significantly lower at 12 h postinfection compared with
control mice. Thus, the reduced PMN-dominant response in the
airway of MME-exposed mice during the initial phase of
infection (at least until 12 h postinfection) was due to the effect
of preexposure to MME, but not directly reflective of low
bacterial burden. In regard to the low bacterial burden, mono-
nuclear cells are shown to exhibit enhanced phagocytic bacte-
ricidal activity against type 3 pneumococci by adhering to
ECM (40). Thus, enhancement of airway resistance in MME-
exposed mice is possibly in part due to lung macrophages.
In connection with this, higher levels of IFN-γ production in
the lung of the exposed mice may contribute to activation of lung
macrophages. Together, we assume that lung macrophages are
involved in the low magnitude of pneumococcal growth in
lungs of the exposed mice.

For pneumococcal invasion into the bloodstream, the integ-
Rity of ECM of the basement membrane on blood vessels must
be degraded; this process may require both bacterial and host
factors in lung tissue (2, 44). MME-exposed mice became
bacteremic after day 3 of infection, at the time when levels of
MMP-9 production/release elevated to the same level as ob-
served in control mice at 12 h postinfection. In this regard,
the expression and activation of TIMPs capable of counterbalanc-
ing MPPMs (37), particularly TIMP-3, were enhanced in the
exposed mice. Thus, MME exposure seems to retard occur-
rence of bacteremia by lessening the degradation of ECM of
blood vessels. Moreover, TIMP-3 production was less affected
in the exposed mice compared with control animals because
MME exposure decreased production of TNFα capable of
inhibiting TIMP production (51). Furthermore, extensive ac-
cumulation of PMNs may also contribute to the high produc-
tion of MMP-9 in control mice, since PMNs contain the most
abundant quantity of this protease (19) and PMN elastase
activates the proenzymatic form of MMP-9 (15). In fact, excess
of activity in either MPPMs or leukocyte elastase is involved in
a number of pathological conditions in association with im-
pairment of ECM organization in the lung (5). In addition, high
magnitude of PMN infiltration into the confined area is well
demonstrated to induce tissue damage by releasing proteases
and reactive oxygen species (4, 20, 36, 55, 57), which was consistent with histological findings in the lung of infected control mice. MME exposure is therefore likely to minimize infection-associated tissue damage and also to delay development of bacteriaemia by lessening PMN recruitment and by regulating MMP/TIMP systems.

Finally, we described a beneficial effect of microbial exposure in the airway during the early life on local innate resistance to S. pneumoniae. Especially, enhanced resistance is associated with a significant reduction of PMN-dominant inflammatory response against pneumococci, which appears to be related to the low responsiveness of TLRs as well as the regulation of MMP/TIMP systems after infection. It will be of great interest to determine the effects of early microbial exposure on airway innate resistance to pneumococci in BALB/c mice with a tendency for a Th2 response. In conclusion, this study suggests that the hygiene hypothesis appears to be more important in preventing excess of PMN-dominant inflammatory response than in inducing Th1-skewed responses.

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