New Murine Model of Bronchopneumonia Due to Cell-Bound *Haemophilus influenzae*

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This murine model of nontypeable (unencapsulated) *Haemophilus influenzae* (NTHI) bronchopneumonia used organisms bound to mouse fetal lung (MFL) cells as an inoculum. Pretreatment of the mice with 40 μL of 1% formalin 3 days before intranasal instillation of the bacteria was necessary to allow infection. The number of NTHI recovered from the lungs plus trachea on day 7 after instillation was >100 times the number originally inoculated. Later, however, the number of recovered bacteria diminished gradually, and by day 14 it was almost identical to the original inoculum size. Serum IgM also peaked on day 7 after infection, after which IgG increased while IgM decreased. Histologically, bronchoalveolar infiltration of neutrophils was observed on day 3 after inoculation and continued at least for the following 4 days. The present experiment demonstrates that MFL cells can protect bacteria that have invaded the cells from the opsonizing and killing activities of host humoral defense mechanisms.

In a rat model of nontypeable (unencapsulated) *Haemophilus influenzae* (NTHI) pulmonary infection, the inoculated bacteria are encased in agar beads to prevent their rapid removal by mucociliary clearance [1, 2]. Unfortunately, this method of inoculation requires a tracheotomy. More recently, a model of acute respiratory infection was established in which the bacteria are embedded in molten agar and nonsurgically instilled into the trachea of neutropenic weanling rats [3].

A number of bacteria, including *Shigella* species, *Legionella pneumophila*, and *Listeria monocytogenes*, are widely recognized as intracellular parasites. Although NTHI are not usually viewed as intracellular organisms, they have been shown to invade monolayer cells in vitro through a process that is inhibited by cytochalasin D and colchicine [4]. Moreover, NTHI have been found intracellularly in adenoid tissues of each of 10 clinically infection-free children [5]. Accordingly, it seems possible that oropharyngeal epithelial cells carrying cell-bound *H. influenzae* may exfoliate and enter the trachea, thus allowing the bacteria to escape normal host defense mechanisms, establish themselves in the lower respiratory tract, and eventually produce infection. In the present report, we describe a novel murine model of NTHI bronchopneumonia that uses cell-bound organisms as the inoculum.

Materials and Methods

Bacteria. NTHI strain TUM8 was isolated at our hospital. The bacteria were cultured overnight in brain-heart infusion (BHI) broth (Difco, Detroit) supplemented with NAD and hemin. A portion of this culture was inoculated, as a preculture, into a fresh sample of BHI broth at a final concentration of 5%. The new culture was then incubated for 3 h at 35°C. After centrifugation, the organisms were washed three times with PBS and suspended in minimal Eagle basal medium (MEM) at a volume similar to the original culture (mean viable organism count, 4 × 10^8 cfu/mL). This bacterial suspension was used for assay of bacterial killing by normal human serum.

Preparation of cell-bound organisms. Mouse fetal lung (MFL) cells [6] were cultured in MEM with 10% heat-inactivated fetal calf serum. The cell line was grown in flat plastic flasks (25 cm^2; Corning Glass Works, Corning, NY) at 35°C in a 5% CO_2_ incubator. The subconfluent monolayer was washed three times with PBS, inoculated with 10 μL of the bacterial suspension described above, and gently shaken for 60 min at 35°C. Nonadherent bacteria were then removed by washing three times with PBS. Cells with their adherent bacteria, hereafter referred to as cell-bound organisms (CBO), were separated from the tissue culture flask by a scraper. The separated CBO were then suspended in 6 mL of MEM. To determine the number of viable bacteria, a portion of the CBO suspension was vigorously agitated with glass beads, and serial dilutions were plated on enriched (IsoVitalex; Baltimore Biological Laboratories, Baltimore, MD) chocolate agar. The number of viable bacteria ranged from 2 to 5 × 10^6 cfu/mL and the number of MFL cells from 1 to 2 × 10^5 cells/mL.

Experimental infection in mice. Three days after their airways were impaired by intranasal instillation of 40 μL of 1% formalin, 4-week-old male Sle/ICR mice (Sankyo Labservice, Tokyo) in groups of 6 mice each, under ketamine-xylazine anesthesia, received intranasal instillations of 50 μL of the CBO suspension in MEM. The number of bacteria needed to produce respiratory infection in formalin-treated mice was determined by counting bacteria in lower respiratory organs 5 days after infection, using dosages of 3.1 × 10^5 cfu/animal and 5-fold serial dilutions of the CBO suspension.

The lower respiratory organs were removed on days 0, 1, 3, 5, 7, and 14 after infection, weighed (mean, 0.2 g), homogenized, and diluted in saline. To determine the number of viable bacteria, a
0.1-mL portion of each sample was plated in duplicate on enriched chocolate agar. Since the original tissue homogenate suspension inhibited growth of *H. influenzae*, but a 10-fold dilution of it did not, the detectable limit of bacteria in the lower respiratory organs was $5 \times 10^2$ cfu/g of tissue.

For histopathology, lungs and tracheal tissue samples were fixed in a phosphate-buffered 4% paraformaldehyde solution and processed using a conventional paraffin-embedding procedure for hematoxylin-eosin staining.

Paraffin-embedded tissue sections were also subjected to immunohistochemical analysis. Deparaffinized and rehydrated sections were treated with 0.25% trypsin–0.02% calcium chloride in 0.1 M PBS for 1 h at 35°C and then incubated overnight at 4°C with the primary antibody, rabbit hyperimmune serum against strain TUM8 organisms. The sections were then processed with a kit for rabbit primary antibody (affinity-isolated biotinylated goat IgG, Histofine SAB-P0; Nichirei, Tokyo).

Transmission electron microscopy was done according to previous reports [5].

**Serum antibody.** Organisms cultured for 3 h were washed three times with PBS, resuspended in PBS, and sonicated (Sonifier; Benson Sonic Power, CT) for 5 min in an ice-water bath. The suspension was then centrifuged under refrigeration (4°C) at 8000 g for 10 min, and the supernatant was diluted with 50 mmol/L carbonate buffer, pH 9.6, to a final concentration of 10 µg of protein/mL, which was used as an antigen. The content of antibody against infecting organisms in mouse serum was assayed by standard ELISA.

**Bactericidal effect of normal human serum and gentamicin treatment.** Sera from 3 healthy adult volunteers were processed using a conventional paraffin-embedding procedure for hematoxylin-eosin staining. Histofine SAB-PO; Nichirei, Tokyo).

Data analysis. The numbers of organisms in different groups of animals were compared by the Bonferroni multiple comparison test.

### Results

**Influence of inoculum size.** Respiratory infection occurred in all mice inoculated with $>10^3$ cfu/animal, with little animal-to-animal variation in the number of organisms recovered from the lower respiratory organs (mean, $10^3$ cfu/g of tissue). The mean viable organism count from lower respiratory organs in mice inoculated with $2.5 \times 10^3$ or $5.0 \times 10^2$ cfu/animal was also $10^3$ cfu/g of tissue, but there was greater variation in the number of recovered organisms.

**Changes in bacterial number with time.** In animals inoculated with $10^2$ cfu, the mean viable bacteria increased to $10^3$ cfu/g of tissue after 7 days (figure 1). Thereafter, however, the number of bacteria gradually decreased, falling to $3 \times 10^1$ cfu/g on day 14 after inoculation.

**Histopathologic changes.** Inoculation of MFL cells alone did not produce inflammatory changes in the lungs of formalin-treated mice (figure 2A). In mice infected by the CBO method, the bronchioles and adjacent alveoli were filled with neutrophils, macrophages, and degenerating epithelial cells. Furthermore, the lumina of the secondary and tertiary bronchi were often dilated and occasionally contained an exudate that included neutrophils and epithelial cells (figure 2B); in some cases, cells were deciliated, while in others, cuboidal to squamous cells exhibited hyperplasia.

A pneumonic focus, consisting of proliferating alveolar type II cells and fibroblasts in interalveolar septa with inflammatory cell infiltration, was detected in mice sacrificed on day 3 after inoculation (figure 2C). On occasion, the tracheas of mice sacrificed on day 7 were partially occluded by granulomatous nodules, which consisted of a mass of degenerating cells together with epithelial and fibroblastic components that had proliferated from the mucous layer. Similar observations were made in secondary bronchi of mice sacrificed on postinoculation days 3 and 7.

Immunohistologic examination revealed that these histologic lesions were associated with abundant *H. influenzae* TUM8 antigen, mostly in the cytoplasm of neutrophils and macrophages found in bronchial and alveolar exudates (figure 2D). The lungs of infected mice contained a large percentage of *H.
influenzae organisms on days 1 and 3 after infection, then the bacterial numbers in the lungs gradually decreased.

Thin-section transmission electron microscopy demonstrated that NTHI cells in formalin-treated mice multiplied on the bronchiolar surface and did not invade epithelial cells (figure 2E).

**Humoral immune response.** Serum IgM appeared on day 5 and peaked on day 7 following infection, then gradually decreased (figure 1). Serum IgG increased gradually beginning on day 7 after infection.

**Bactericidal activity of normal human serum and gentamicin.** There was essentially no change in the number of viable
bacteria following a 2-h incubation in medium without normal human serum or gentamicin (control medium). When free organisms were incubated with normal human serum, the mean number of viable bacteria were $3 \times 10^4$, $2 \times 10^5$, and lower than the detectable limit following incubation times of 0.5, 1, and 2 h, respectively. Each value represents a significant ($P < .01$) reduction compared with levels in the corresponding control.

On the other hand, the number of viable bacteria in CBO samples treated with normal human serum or gentamicin were, respectively, $8 \times 10^2$ and $2 \times 10^4$ following a 30-min incubation, $8 \times 10^3$ and $1 \times 10^5$ following a 1-h incubation, and $8 \times 10^3$ and $1 \times 10^4$ following a 2-h incubation. These values also are significantly ($P < .01$) lower than those for the corresponding controls. However, the counts following 1- and 2-h incubations were significantly greater than those for free bacteria treated with normal human serum ($P < .01$).

Discussion
The rat model of pulmonary infection by *H. influenzae* using the agar bead methodology has been reported [1–3]. We tried to apply this method in normal and 1% formalin-treated mice, but it did not enable us to develop a murine model of pulmonary infection.

Fortunately, we succeeded in developing a new murine model by inducing bronchopneumonia through intranasal instillation of *H. influenzae* bound to cells instead of to agar beads without a tracheotomy. The model of bronchopneumonia reported here is critically dependent on two features. One is that the inoculum consists of organisms bound to cells or actually located intracellularly. The other is that the mice are pretreated with formalin to impair mucociliary clearance in their airways. In addition, in the CBO model, the bacterial numbers decreased depending on the increase of humoral immune titers, but in the agar beads model, the bacterial numbers did not decrease, although titers of serum antibodies against outer membranes derived from the infecting organisms increased [1].

Since the opsonizing and bactericidal effects of normal human serum appear to be related to protection from *H. influenzae* infection, there has been interest in studying the bactericidal activity of human serum against NTHI [7–11]. Musher et al. [9] reported that incubating NTHI with normal human serum caused dose- and time-dependent killing. The present study reconfirmed that incubation with normal human serum caused a time-dependent killing of free organisms.

Of interest, there are reports that NTHI may be an intracellular parasite [4, 5]. This possibility is supported by our observation that intranasal instillation of free bacteria, even at an inoculum size of $10^7$ cfu/animal, did not induce NTHI bronchopneumonia in formalin-treated mice. Furthermore, although the number of viable organisms in our CBO preparation diminished following a 30-min incubation with normal serum, the rate of killing at later times was minimal. A similar pattern was seen when CBO were treated with gentamicin. This bactericidal antibiotic is well known for its inability to enter eukaryotic cells; thus, while it kills bacteria adherent to epithelial cells, intracellular organisms are protected from its effects [12].

On the basis of these findings, we think that one of the CBO method’s benefits may be that some NTHI actually enter MFL cells, where they are protected against host defense systems, especially the opsonizing and bactericidal activities in serum. We also think that CBO may play an important role in naturally occurring murine NTHI bronchopneumonia. We found that 0.2% of CBO escaped killing by gentamicin and therefore were presumptively intracellular. Using this value for the proportion of intracellular bacteria in a CBO suspension, we find that instillation of $1.2 \times 10^4$ or $5 \times 10^2$ cfu CBO/animal implies that the average number of intracellular colonies received by each animal is 24 or 1, respectively. This suggests that a few dozen intracellular NTHI may be needed to induce lower respiratory infection in mice with impaired airways.

In the present study, pretreatment with formalin was essential for induction of bronchopneumonia. Formalin is a classic tissue fixative and an irritant that produces local effects on mucous membranes of the respiratory tract [13]. Attachment of the pathogenic organisms to these membranes, followed by colonization of injured epithelial cells, represents a critical event in development of bronchopneumonia [10]. In fact, some investigators have used hexamethylphosphoramide solution to impair the respiratory epithelium [2]. However, this substance was found to be carcinogenic and caused severe microulcerative lesions of the rat respiratory epithelium [2].

Therefore, the present study used 40 μL of 1% formalin (0.013 mmol/L of formaldehyde) instead of hexamethylphosphoramide solution. This dose proved safe in mice weighing 19–21 g and produced little or no toxicity: Formaldehyde is hepatotoxic only at concentrations of $\geq 3$ mmol/L, and its mean value in plasma from 320- to 380-g formaldehyde-killed rats is 5.2 mmol/L [13]. Furthermore, we confirmed that administration of formaldehyde alone did not result in pulmonary inflammatory changes when assayed 3 days later; however, lung tissue may have been fixed by this formalin treatment.

The upper airway’s innate ability, through a number of non-specific mechanisms, to effectively prevent passage of any particle larger than $\sim 2-3 \mu m$ in diameter normally keeps the lower airway sterile. This implies that MFL cells ($\sim 20 \mu m$ in diameter) should be unable to reach the bronchioles. It also appears that free bacteria are easily cleared from the lower respiratory organs, even of formalin-treated mice, since it proved impossible to induce experimental NTHI bronchopneumonia using such organisms.

On the other hand, a study using organ culture of respiratory mucous membranes found that the number of *H. influenzae* increased from $10^3$ to $10^6$ cfu/mL over the 24-h period following inoculation [14, 15]. This result suggests that tracheobronchial mucosa may contain factors (X and V factors) that pro-
mote bacterial growth. Moreover, a recent study has demonstrated that NTHI resides and multiplies intracellularly in noninfected human adenoid tissue [5]. In light of these observations, we postulate that MFL cells bearing NTHI might enter the impaired trachea, where the bacteria then multiply in or around cells (or both). Later, organisms from the trachea might reach the bronchiole, multiply on the bronchiolar and alveolar epithelial cells, and thus induce bronchopneumonia. We further speculate that the process by which NTHI bronchopneumonia develops in humans may be more similar to the new model of murine bronchopneumonia described here than to the agar beads method [1–3].

References